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AMINO ACID TRANSPORT IN THE CORNEA AND CRYSTALLINE LENS OF THE TOAD, BUFO MARINUS

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ABSTRACT

The uptake and efflux of amino acids in the cornea and the lens of the toad, Bufo marinus, was studied. Amino acids are accumulated across the aqueous side of the cornea, mainly by the epithelium. It appears that there are several distinct systems that mediate -aminoisobutyric acid (AIB) transport into and out of the cornea. AIB uptake is saturable but is not directly dependent on the presence of Na in the external bathing media. However, the exit site is very sensitive to changes in intracellular Na concentrations. Any factor, such as ouabain and metabolic inhibitors, which increases internal Na markedly stimulates AIB efflux. Under normal conditions efflux is very slow, about 15 percent of the rate of uptake. AIB uptake is inhibitable by alanine and leucine though not completely by either of these amino acids. The inhibitory effects of these compounds are additive, suggesting the presence of 2 distinct uptake sites. There are also sites for the exchange of internal for external amino acids, and this process is Na-dependent. However, Na does not move into or out of the cells with the AIB, as indicated in transmural ^{22}Na flux studies. Exchange efflux was more specific than uptake, since both alanine and leucine inhibit AIB uptake, but only alanine stimulates AIB efflux. The effect of various membrane 'probes' and hormones on these transport processes was studied. Although the largest amount of AIB accumulated by the cornea was present in the epithelium, evidence is presented that the endothelium and stromal keratocytes may also concentrate and retain amino acids.

In the mammalian lens it is considered that amino acids are only accumulated by the anterior epithelium and they then 'leak' into the rest of the organ. However, in the present experiments on the amphibian

lens, such a process was not found. Removal of the capsule and the anterior epithelium did not abolish accumulation of AIB against a concentration gradient. The probable reasons for this difference from the observations on the mammalian lens are discussed. AIB uptake in the lens is saturable and occurs by a system that is inhibited by alanine and is dependent upon the presence of Na in the external bathing media. Uptake of AIB occurs across all surfaces of the lens but is greater across the anterior epithelium. A gradient in concentration of AIB is built up, highest concentrations are in the periphery and the lowest are in the nucleus. Efflux of AIB is very slow; only 35 percent is lost in 16 hours. The rate of exodus declines exponentially over time and is not related to a decrease in the total concentration of AIB that occurs during this period. It is changed little by removal of the capsule and anterior epithelium or an excess of 'cold' AIB. Efflux can, however, be promoted by a Na-free external bathing solution. The retention of this amino acid by the lens may reflect its unique morphology. The layering of cells appears to protect the lens amino acid pool from the effects of changes in the bathing solution and the presence of toxic substances and drugs.

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TABLE OF CONTENTS

LIST OF TABLES

page

LIST OF FIGURES

page

ABBREVIATIONS

AIB - α-aminoisobutyric acid

PD - potential difference

I_{sc} - short-circuit current

DIDS - 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid

BCH - β-aminobicyclo(2,2,1)heptane carboxylic acid

PCMPS - ρ-chloromercuriphenyl sulfonic acid

T_3 - triiodothyronine

INTRODUCTION

Chapter I

Amino Acid Transport

The earliest observation that amino acids are accumulated in cells against a concentration gradient was made in 1913 by Van Slyke and Meyer. They compared changes in serum concentration with those in tissues after intravenous administration of a protein hydrolysate. Using a laborious nitrous acid technique, they showed that the tissues contained up to ten-times the concentration of amino acids found in the serum. The availability of radioactively labelled compounds in the late 1940's had an explosive effect on the field of study of amino acid transport. Many of the early investigations described the ability of specific cell types to accumulate amino acids against a concentration gradient and the saturability of this process. These observations indicated the presence of specific sites for amino acid transport. Amino acids were found to compete with each other for uptake, and this competition showed that the transport sites had specific structural preferences for certain amino acids (Christensen & Riggs, 1952; Christensen, et al., 1952; Riggs, Coyne & Christensen, 1954)

All of the above evidence indicated that amino acid transport is 'mediated', that is, there is binding of the amino acid to a specific site on a membrane and the amino acid is translocated into the cell without any modification of its structure. There have been several hypotheses offered to explain how this event might occur. The 'carrier' model involves the presence of a mobile component in the membrane which can bind the substrate reversibly at one face and then move through it

to release the molecule unchanged at the other face. This process can translocate substrates from the inner surface of the plasma membrane to the outside of the cell or vice versa. How this 'carrier' might 'move' through the membrane and how the recognition sites on the outer face may differ form those on the inner face has also given rise to considerable speculation and model building. The 'gate' model of Patlak, which he described in 1957, appears to be the most tenable of those presented, mainly because it is still broad enough to fit the experimental transport data obtained during the years since it was first suggested. It was proposed that a central transport unit in the membrane can exist in more than one conformation, and by movement of its components it can make a centrally located binding site accessible in turn from either side.

'Mediated' transport is usually considered to have the following features (Heinz, 1978):

1) substrate specificity
2) exhibits saturation which fits Michaelis-Menton kinetics
3) competitive inhibition by substrate analogues
4) non-competitive inhibition by metabolic inhibitors
5) activation by ions, cofactors and other substrates
6) feedback regulation by inducers and hormones

A notable event in the study of amino acid transport in cells was the development of non-metabolizable amino acid analogues which can be taken up specifically by natural amino acid transporting systems, as defined in competition studies. For example, a methyl group substituted for the α-hydrogen of alanine prevents the molecule from being incorporated into protein (Christensen, Aspen & Rice, 1956). This substi-

tution also prevents transamination (Cammarata & Cohen, 1950) and catabolism. The compound that is the result of this substitiution is α-amino isobutyric acid (AIB). This 'artificial' amino acid was found to be a competitive inhibitor of glycine, alanine and serine transport in mammalian tissues (Christensen, Parker & Riggs, 1958). Many other non-metabolizable amino aicds, which have similar transport properties to those of natural amino acids, have been used to study amino acid transport in a wide variety of organisms and tissues. As a result of this development the transport of amino acids in intact cells can be studied without the complications of their incorporation into proteins or their metabolism or degradation.

An important and highly controversial area in the study of the transport of amino acids arose when it was discovered that the presence of Na in the external bathing solution is necessary in some instances for their active uptake. In 1902, Reid observed that the addition of Na to the solution bathing the intestinal mucosa increased the absorption of glucose. By the early 1960's, the necessity for the presence of Na for the transport of solutes, including sugars and amino acids was established in many different tissues (Christensen & Riggs, 1952; Riggs, Walker & Christensen, 1958; Riklis & Quastel, 1958; Csaky, 1963). It was from these studies that the theory of cotransport arose. It is inferred that coupling between two solute flows may lead to a transfer of energy between the two. The 'driven' solute species, can be transported against its electrochemical gradient by the downhill, exergonic, transport of the coupled 'driver' species. It has been found that coupling between two solute flows can be parallel, cotransport, or anti-par-

allel, countertransport or anti-port. The following observations led to the development of theories of cotransport and counter-transport.

Cis-stimulation of transport was noted early in transport studies. The presence of Na stimulates the transport of amino acids in many preparations (only a sampling will be listed), indeed, active transport of some amino acids does not even take place in the absence of Na ions (Czaky, 1961; Parrish & Kipnis, 1964; Johnstone & Scholefield, 1965; Inui &Christensen, 1966; Wheeler & Christensen, 1969; Hajjar, Lamont & Curran, 1970). In a few of the early transport studies, it was also considered that antiport, or counter-transport might be the driving force of active solute accumulation. For example, Riggs, Walker and Christensen (1958) postulated that "migration of K from the cell (down an electrochemical potential difference) may stimulate the simultaneous entrance of amino acid". They also postulated that K inside the cell competes with amino acids for exodus and that the increased efflux of amino acid seen when tissues are incubated with ouabain is due to the decreased K_{in} and therefore a decrease in competition for exit sites. This theory however does not fit well with the time course of these effects, the K_{in} decreases very slowly with time and the stimulation of amino acid efflux is very rapid. The antiport theory of K exchanging for the amino acid, although plausible, has never been unequivocally verified.

The addition of the non-electrolyte substrate amino acid should also stimulate the movement of the cotransported ion across the membrane. This is difficult to accurately determine in cultures of single cells, but in intestinal epithelial preparations, across which Na movements can easily be measured, it has been shown that the addition of amino acids

to the mucosal solution increases the transmural flux of Na (Schultz, Fuisz & Curran, 1966).

If the 'driven' solute moves in the same direction as the 'driver', then a reversal of the gradient should cause a reversal in the direction of accumulation. This was demonstrated in the pigeon erythrocyte by Vidaver (1964 a,b,c). When Na gradients were reversed, but only when metabolic inhibitors were present, there was a net movement of the amino acid from the inside to the outside against a concentration gradient.

If the cotransport model is true, one would expect a fixed stoichiometry to exist between the amino acid and the driver ion. Schafer & Jacquez (1967) simultaneously measured ^{22}Na and AIB influx and found there to be a 1:1 relationship. For each molecule of AIB that enters the cell, one Na ion also enters. They compared this result to their findings with phenylalanine, the transport of which is not Na-dependent, and they could not correlate its movement with that of Na. It was inferred that 1 Na was cotransported with each AIB molecule which requires the Na for active accumulation, and that phenylalanine, which has no Na requirement, did not associate with Na, and no Na was cotransported.

If cotransport provides all of the energy required for transport then even if metabolism is completely inhibited, active transport of the driven solute should still occur as long as the gradient for the driver ion exists. Thus the lack of an immediate effect of metabolic inhibitors on amino acid transport was noted in many tissues (Christensen, et al., 1952; Schafer & Jacquez, 1967).

Although there is much evidence in support of the cotransport

theory, there are many experiments and theoretical considerations which argue against it being the only or even the major energy source for active transport of solutes, such as amino acids. Firstly, there are a large number of amino acids that do not require the presence of Na for their active accumulation (Oxender & Christensen, 1963; Cotlier & Beaty, 1967; Friedenthal & Scott, 1973; Caldwell & Lea, 1978). Secondly, reversal of the Na-K gradient without the addition of metabolic inhibitors did not inhibit the normal accumulation of AIB in the opposite direction (Schafer & Jacquez, 1968). They also calculated that the energy derived from electrochemical gradients for Na was insufficient to maintain the observed concentration gradients of AIB across the cell membrane.

If the energy derived by coupling to the downhill movement of another solute is not sufficient to maintain large concentration gradients then what is the source of energy for such an active process? There are several possibilities. Solute coupling is considered secondary active transport since the 'driver' solute gradient is maintained by an active process, in the case of Na coupled transport, Na-K-ATPase. On the other hand, primary active transport is directly linked to chemical reactions and it has been postulated that it could be directly linked to Na-K-ATPase deriving energy from the hydrolysis of ATP. Other, more recent findings, indicate that there may be a microsomal electron transport chain which is directly linked to, and provides energy for, amino acid transport (Scott & Friedenthal,1973; Christensen, 1977). Tertiary active transport implies a situation in which solutes are accumulated by linkage to solutes transported via secondary active transport. For instance,

there is evidence that some amino acids may accumulate in cells by heteroexchange with another amino acid which has previously been actively transported into the cell by cotransport with Na ions. That exchange diffusion mechanisms could energize amino acid accumulation was first suggested by Heinz & Walsh (1958), and has since been noted in many other systems (Oxender & Christensen, 1963; Caldwell & Lea, 1978). There are also Na-dependent (Winter & Christensen, 1965) and Na-independent (Caldwell & Lea, 1978) exchange systems for amino acids. Such an exchange system has been regarded as evidence for mobile carriers in membranes (Wilbrandt & Rosenberg, 1961).

Using both natural and synthetic amino acids, it is possible to distinguish several different systems which transport amino acids. The classical definitions for these transport systems come primarily from the pioneering work of Christensen and will be summarized here (Christensen, 1975; Christensen, 1977). It must be emphasized that there is a large degree of overlap among these systems, and all amino acids can be transported by more than one system. For the neutral amino acids, there are Na-dependent and Na-independent transport systems. There are two Na-dependent systems:

1) the 'A', or alanine preferring, system which has broad specificity, will accept amino acids with short polar, or linear side chains It is highly concentrative and will transport amino acids with N-methyl groups, such as α-(methylamino)isobutyric acid.

2) the 'ASC' system has a much narrower specificity, it is limited to transporting alanine, serine and cysteine (Christensen, Liang & Archer, 1967). It does not tolerate an N-methylated amino acid. Sys-

tem 'ASC' is apparently ubiquitous, it is even present in erythrocytes which lack system 'A'. From the observation that the stereospecificity of exodus is greater than the stereospecificity of entry, Christensen suggested that the highly stereospecific 'ASC' may in some situations mediate amino acid release more than uptake (Thomas & Christensen, 1971).

The two Na-independent systems are:

1) 'L', or leucine preferring, system which reacts most strongly with amino acids with an apolar mass on the side chain, even if it is branched or bicyclic. This system is most conspicuous in the red blood cell but this is most likely due to the absence of the 'A' system. The 'L' system in the Ehrlich cell is thought to be mainly an exchange system (Oxender & Christensen, 1963). The Na-independence of this system is the most important charateristic used to distinguish it from other systems (Inui & Christensen, 1966; McGivan, Bradford & Mendes-Mourao, 1977).

2) 'X' or non-saturable Na-independent system has been demonstrated in the Ehrlich cell (Oxender & Christensen, 1963; Christensen, 1966). Non-saturable components of amino acid uptake have also been demonstrated in skeletal muscle (Grinstein & Erlij, 1977) and brain (Daniel, Pratt & Wilson, 1977).

In addition to all of the above there is a specific system for basic amino acids, 'Ly', which requires an unambiguous cationic group.

The systems defined here are generalizations, they provide guidelines to follow when studying amino acid transport. They are not all present in every type of cell. There is considerable heterogeneity in the transport systems of different tissues. For example, erythrocytes do not have any 'A' system at all. That elements involved in transport

mechanisms may not be identical in all tissues would appear to follow from the fact that each cell type has different metabolic abilities and nutritional needs.

The study of amino acid transport in tissues is further complicated when regulatory feedback inhibition and stimulation, and hormonal controls are superimposed. As cells enter different phases of the mitotic cycle, growth and differentiation, amino acid transport systems fluctuate from high to low activity and may even be absent. For example, the 'A' system is very active in growing cells (Tramcere, Borghetti & Guidotti, 1977) whereas 'L' becomes more active when cells enter a quiescent phase (Goldberg & John, 1976). Mitogenic lectins such as concanavalin A, increase amino acid transport (Van den Berg & Betel, 1973) whereas non-mitogenic lectins (wheat germ agglutinin) (Li & Kornfeld, 1977) have a slightly depressant effect on transport.

The nutritional state of the cells is also a factor in controlling the rate of uptake and activity of the various systems. When cells are depleted of amino acids the activity of the 'L' system decreases significantly. However, if the cells are preloaded with 'L' system reactive amino acids, transport activity greatly increases (Oxender, Lee & Cecchini, 1977). These observations may reflect the relative importance of exchange for system 'L' in these cells. In contrast 'A' was unaffected by depletion and there was a slight decrease in uptake by cells which were preloaded with the relevant amino acid (Heaton & Gelehrter, 1977). This may be evidence of a <u>trans</u> inhibition which has been postulated to be important in limiting the intracellular concentration of amino acids. Such regulatory controls are necessary if a tissue is expected to have

a complete pool of amino acids available for metabolism and protein synthesis.

Hormones have also been demonstrated to significantly affect amino acid transport. Insulin stimulates such transport in skeletal muscle (Kipnis & Noall, 1958), fat cells (Touabi & Jeanrenaud, 1969), hepatocytes (Le Cam & Freychet, 1978) and thyroid (Debons & Pittman, 1966), to name a few. Triiodothyronine (T_3) has also been demonstrated to increase amino acid levels, not by increasing uptake, but by decreasing efflux (Goldfine, et al., 1975).

Chapter II

The Cornea

It has been shown that there are remarkable similarities in both structure and physiological functioning of the cornea and the lens in different groups of vertebrates. As will be demonstrated in the following chapters, it is not of purely comparative interest to study corneal and lens nutrition in amphibians as such studies are also of practical value for the investigation of the normal physiological functioning of these tissues in mammals, including man.

The cornea is an avascular tissue whose major physiological function is the maintenance of its transparency. This function is absolutely dependent upon the metabolism and integrity of the bounding membranes which sandwich the stroma; the anterior epithelium and posterior endothelium (see Fig. 1). The cornea is a refractive organ, light scattering is minimal and the tissue has a low refractive index due to the arrangement of its structural constituents, its smooth curvature and the presence of a tear film. Blindness due to corneal opacity is "the cause of more blindness than any single condition (Duke-Elder, 1958).

A particularly enlightening treatise on the structure of the corneal epithelium was provided 100 years ago (Stirling & Skinner, 1880). The observations then made on the rabbit cornea have been extended to the corneas of many other species. The columnar nature of the basal layer of cells and the squamous appearance of the surface cells was described. The middle layers of cells were found to interdigitate with the columnar cells and themselves. There are 4 to 6 layers of cells in the cor-

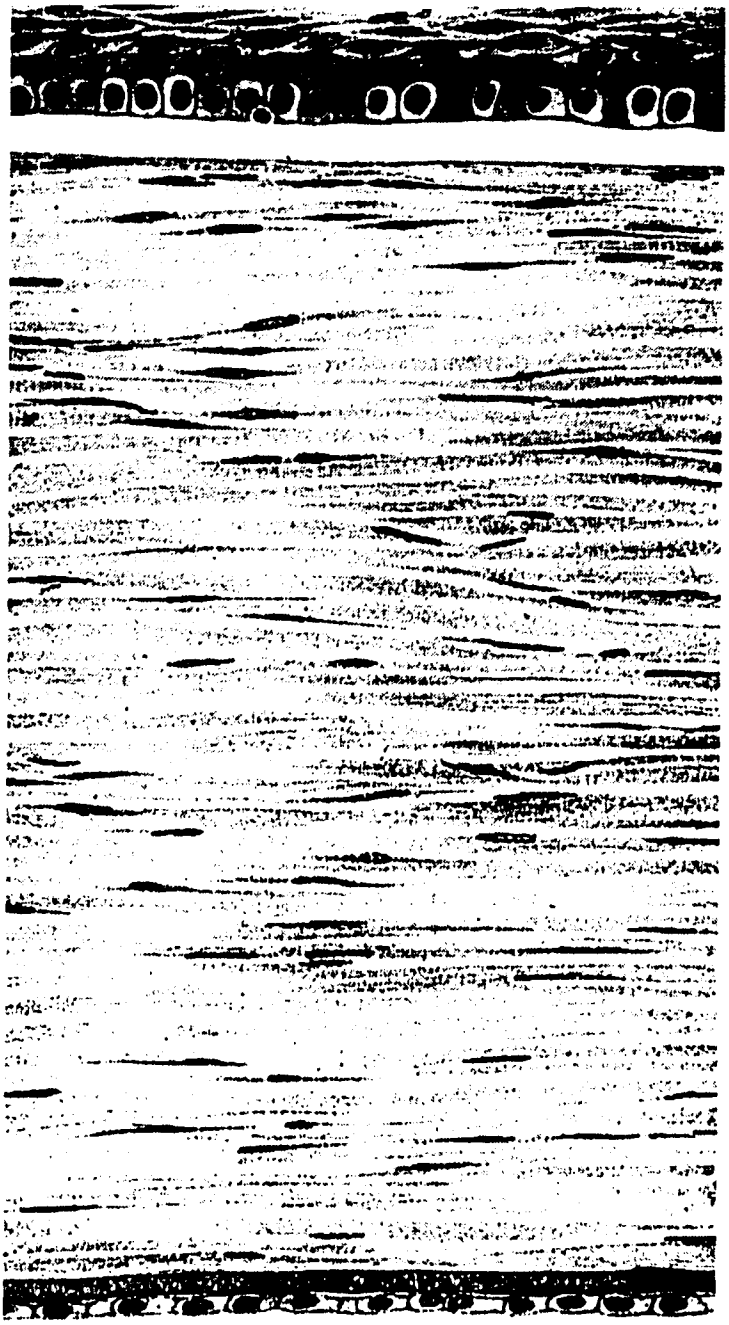


Fig. 1. A diagrammatic cross-sectional view of the cornea (Slyvester, 1977)

neal epithelium. Several investigators (Friedenwald & Bushke, 1944; Hanna & O'Brien, 1960; Hanna, Bicknell & O'Brien, 1961; Bertalanffy & Lau, 1962) have noted the high mitotic rate of the basal cells and it has been found that the epithelium completely renews itself every 3-7 days. This renewal is solely by division of the basal cells which move toward the surface and replace the outer cells which undergo desquamation. If the entire epithelium is removed, epithelial cells migrate in from the sclera to cover the surface of the cornea and they then divide to form the normal epithelium within three to five weeks (Friedenwald, 1951). Recently, Friend and Thoft (1978) demonstrated that this new epithelium is not functionally competent for at least 6 weeks. The outermost layer of cells has on its exposed surface a network of microvilli and ridge-like structures which are thought to assist in holding the tear film over the outside of the cornea (Blumcke & Morgenroth, 1967). The tear film constitutes the main refractive surface of the eye and decreases in the production of this fluid have been found to result in debilitating corneal disease (McDonald, 1969). Damage (Henkes & Waubke, 1978), disease (Tripathi & Bron, 1973; Bron & Tripathi, 1973) or removal of this epithelial layer results in distortions of vision and can lead to swelling and infection of the underlying stroma, the structure of which is integral to the transparency of the cornea.

The stroma is composed of a collagen backbone in a mucopolysaccharide matrix. The collagen backbone provides the cornea with tremendous strength and resiliency. The collagen fibers are parallel to the surface of the cornea. They are in addition, arranged in such a way, with respect to one another, that there is little distortion of light as it

passes through the cornea (Maurice, 1957). Any disruption of this distinct arrangement, such as when the cornea swells, causes the tissue to appear cloudy and disrupts normal vision. The swelling of the cornea is due to the increase in volume of the interfibrillar matrix and this occurs when either of the two bounding membranes is not functioning properly. The stroma is constantly taking up water from the aqueous humor. This imbibition is normally countered by an active pump mechanism which is thought to remove fluid from the stroma in association with the movement of ions. That this pumping requires metabolic activity is supported by the observation that cooling to 5°C causes rabbit corneas to swell and this swelling is reversible when the corneas are brought back up to 35°C (Davson, 1954). Metabolic inhibitors also cause the cornea to swell. There are ion pumps in the endothelium (Hodson & Miller, 1976) which may be responsible for this fluid pumping activity. In addition, the epithelium provides a barrier to water movement into the stroma (Mishima & Maurice, 1961). Interspersed in this collagen framework are fibroblast-like cells, keratocytes, whose metabolic function appears to be the turnover of matrix mucopolysaccharides.

The endothelium, which has the same embryonic origin as the endothelial lining of the vasculature, is a single layer of cells which lines the posterior surface of the cornea and is in contact with the aqueous humor. The maintenance of corneal hydration is an active process that is dependent on the presence of an intact and metabolizing endothelium. The presence of mitotically active endothelial cells in the adult human has not been demonstrated (Kaufman, 1976). It is postulated that any repair or replacement that may be necessary is accomplished by the spreading of

already existing cells. The structure and permeability properties of the endothelium has attracted much attention. Substrates as large as 2 to 3 nM in diameter, for example horseradish peroxidase, appear to penetrate the endothelium through intercellular spaces (Kaye, Sibley & Hoefle, 1973). In the rabbit cornea there are terminal bars which have been called zona occludens, however in the amphibian and human cornea these terminal bars do not exist (Kaye, 1962). The amphibian and human corneal endothelium are also similar as the intercellular channels follow a very tortuous route in both (Jakus, 1961).

Since the cornea is avascular, the problem of the nutrition of such a metabolically active tissue is a special one. There are three possible sources of the subtrates needed for metabolism: the tears on the epithelial surface, the capillaries at the corneo-scleral junction and the aqueous humor bathing the posterior surface. The outer, apical, epithelial surface has been shown to be relatively impermeable to glucose in the rabbit (Hale & Maurice, 1969; Riley, 1969) and to amino acids in the rabbit (Thoft & Friend, 1972; Riley, Campbell & Linz, 1973) and toad (Scott & Friedenthal, 1973). Oxygen, however, can diffuse across this surface, and indeed the O_2 supply to the cornea is partly from the aqueous and partly from the tear surface (Riley, 1969). Using an *in vivo* "anterior chamber blockade technique", Thoft, Friend and Dohlman (1971) demonstrated that the limbal blood supply contributed at most 20% of the total supply of glucose to the rabbit cornea. It was also found that the endothelium presents a permeability barrier to glucose since there was an increased flux of glucose across the cornea in the absence of this layer. Several studies have shown that inadequate supplies of

nutrients, such as glucose, may lead to epithelial degradation (Truss, Friend & Dohlman, 1970; Truss, Friend & Reim, 1971) and it has been suggested (Thoft & Friend, 1972) that some epithelial defects may be due to a decrease in protein synthesis as a result of inadequate amino acid supplies.

Information about the mechanisms by which amino acids accumulate in the cornea is clearly important. Reddy (1970) measured the distribution of free amino acids in the cornea. He found them to be present in high concentrations and showed that the corneal epithelium to aqueous concentration ratio was highest for acidic, lowest for basic, and intermediate for neutral amino acids. It was postulated that the high concentration ratios found in this study were maintained by an active transport mechanism. From the calculations of Maurice and Riley (1970), it was determined that only 2% of all amino acids flowing through the aqueous would be required to meet the synthetic needs of the corneal epithelium. Thoft and Friend (1972) found that, as with glucose, amino acid uptake by the rabbit cornea was almost eliminated by anterior chamber blockade. Removal of the endothelium in this study increased the rate of AIB movement across the cornea but they did not demonstrate that the endothelium reduces the net epithelial uptake of the amino acid. They estimated that the flux of amino acid across the corneal endothelium and stroma would supply the epithelium with ten times the amount of amino acid required for normal metabolism. In this <u>in vivo</u> study the amount of amino acid accumulated in the epithelium, 20 hours after the initial injection, reached a tissue to aqueous ratio of greater than 7, whereas in the rest of the cornea the ratio was less than 2.

It appears then, that as suggested by Maurice and Riley (1969), most of the nutrients needed by the cornea are supplied by the aqueous humor. This fluid is formed by secretions of the ciliary epithelium. Reddy, Rosenberg and Kinsey (1961) measured the levels of amino acids in the plasma, aqueous humor and vitreous body of the rabbit. They found that the levels of most amino acids in the aqueous are higher than those in the plasma, and suggested that these may be actively transported by the ciliary body. Indeed, in a later study (Reddy & Kinsey, 1962) they were able to saturate this transporting system by administering higher concentrations of amino acids. These higher levels of amino acids in the aqueous assure an adequate supply of these substrates to both the cornea and the lens.

In 1973, Riley and coworkers presented evidence that the amino acids, glycine, aspartic acid and AIB cross the endothelium both by diffusion and by a process that is saturable and can be inhibited by ouabain. This 'active pumping' was initially thought to be responsible for the accumulation of amino acids in the stroma at higher concentrations than those in the bathing media. A later study by the same investigator (Riley, 1977), however, demonstrated that the endothelium does not have such an active role, that amino acids are not pumped into the cornea but move across the deepithelialized cornea at equal rates in either the posterior to anterior or anterior to posterior direction. Amino acid accumulation in the keratocytes could account for the high stromal amino acid concentration observed in the initial experiments.

More detailed studies of the mechanism by which amino acids are accumulated by the cornea were done by Friedenthal and Scott (1973).

They measured, *in vitro*, amino acid uptake across the cornea of the toad, *Bufo marinus*. It was concluded that amino acids are taken up by the cornea almost entirely (96%) across the endothelial surface, and that 2 to 3 distinct systems exist for their uptake. These systems had some properties of the classic 'A' and 'L' amino acid transporting systems as defined by Christensen (1975). Amino acid (AIB) transport was however not tightly coupled to Na transport as removal of Na did not inhibit accumulation. This was an interesting observation, since AIB has been classically defined as an 'A', or Na-dependent, system amino acid. In a later study (Cooperstein & Scott, 1978), these investigators attempted to define the energy sources used for the transport of amino acids, since in the earlier investigations it was found that anaerobiosis and cyanide had no effect on the rate of uptake of all the amino acids studied, and iodoacetate did not affect the uptake of leucine. They postulated that ascorbate serves as an energy source for leucine uptake.

The foregoing section is a summary of our knowledge of corneal amino acid supply, uptake and distribution to the present time. In order to understand how steady-state levels of amino acids in the cornea are maintained, however, it is important to study not only the mechanisms for their uptake but also for their exodus.

The object of the present investigation was to determine how levels of amino acids are maintained by the cornea. As a necessary adjunct to this study, the permeability of the layers of tissue that make up the cornea was also determined. The non-metabolizable amino acid (AIB) has been used predominantly in this study. Included are determinations of the properties of amino acid accumulation and exodus from the cornea.

Interference with of energy supplies and ion gradients were used to help define the nature of these systems. An attempt to characterize the 'carrier', or 'carriers', responsible for these mechanisms was made by use of various enzymes and membrane probes. The possible effects of various hormones on amino acid transport was also investigated. These studies have implications with regard to the nature of the basic mechanisms of amino acid transport and also, it is hoped may ultimately contribute to knowledge of how the integrity of the different layers of corneal tissue is maintained and how various factors, such as drug administration, may contribute to their health or their disruption.

Chapter III

The Lens

The lens is separated from the cornea by the aqueous humor (Fig. 2). The physical organization of this specialized tissue is to refract light in a controlled manner. This function is the result of maintenance of the precise structural coordination of the components of the lens. However, despite exhaustive study, the structure-function relation of these elements, which result in transparency and the focusing properties of the lens has not been elucidated. This chapter will be divided into four parts: 1) an historical perspective of our knowledge of cataract; 2) the structure of the lens; 3) the physiology of the lens; 4) studies of amino acid transport and its relation to disease and cataract formation.

A cataract is an opacity in the lens that results in visual disturbances ranging from distortion of vision to complete blindness. Cataracts can form as the result of disease (Patterson & Benting, 1965; Matho, 1972; Beutler, et al., 1973), exposure to chemicals or drugs (Becker, 1964; Gehring and Buerge, 1969) and age. The variety of factors which cause cataract formation is indicative of the enormity of the problem of delineating the mechanisms by which opacities develop. It appears that any alteration of the molecular or cellular structure of the lens which results in changes in the refraction of light passing through this tissue, will form an opacity.

The following section is a summary of an historical perspective on cataract (Rucker, 1965). Cataracts were originally thought to result from the deposition of a "corrupt humor, possibly from the brain" in

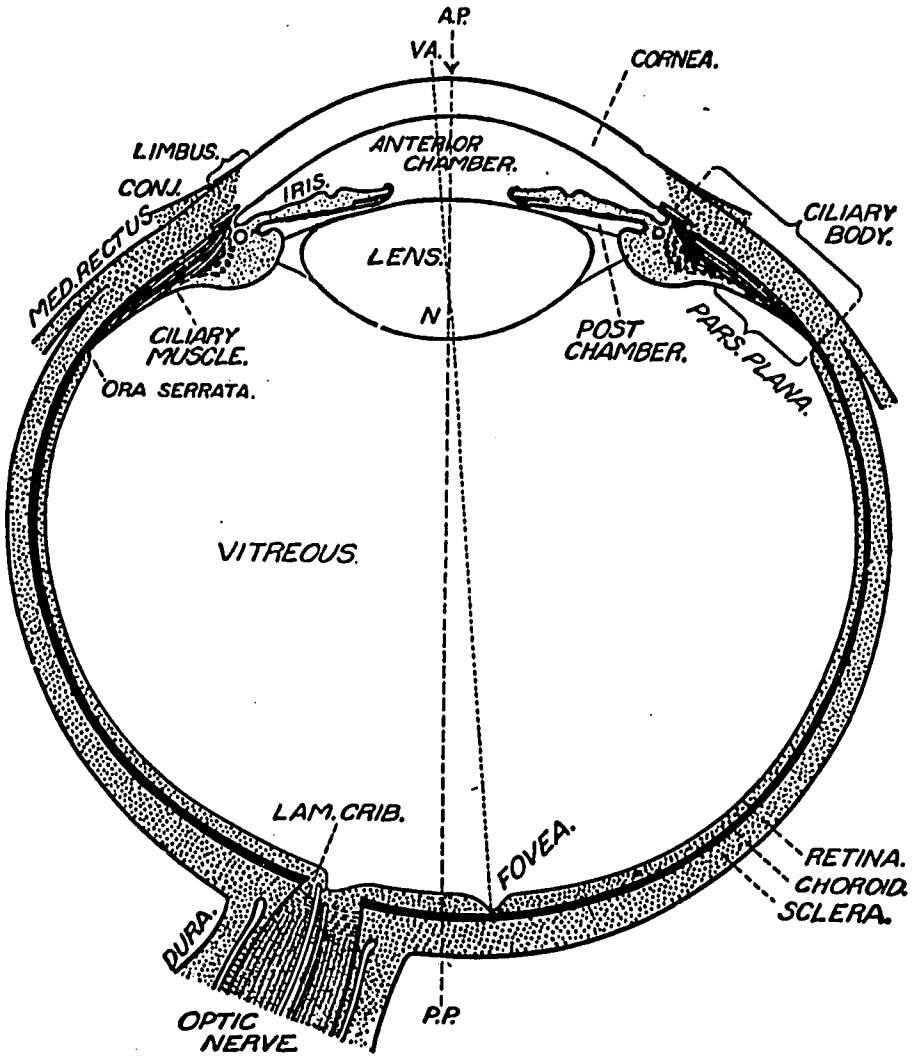


Fig. 2. A horizontal section of the eye (Sylvester, 1977)

front of the lens. The word cataracta, meaning 'waterfall' is a Latin translation of the Arabic word for 'black water' that was used centuries ago to describe cataracts. Almost 2000 years ago a description of cataract surgery was recorded by Aurelius Cornelius Celsus who practiced medicine in Rome but it was probably employed several hundred years earlier in India and Greece. This surgical method was employed until the mid-eighteenth century and involved passing a needle into the eye and pushing the cataract down below the visual axis ('couching'). It was not until 1643 that the true nature of the cataract was discovered. Francois Quarre proposed that a cataract was not a 'corrupt humor' but instead, an opaque lens. A century later a successful technique for removal of the cataract from the eye was developed by Jacques Daviel. There have been many modifications and improvements in technique, but the procedure used today is essentially the same.

The lens is completely surrounded by a 'capsule' which has been called a hyaline membrane because of its lack of visible structure. The anterior surface of the lens is covered by a single layer of epithelial cells (Fig. 3). At the periphery, these cells differentiate into fiber cells which are constantly pushed into the depth of the lens by new epithelial mitoses and differentiation. There is no sloughing off of cells as seen in the cornea as the surrounding capsule prevents this. Therefore all of the cells present from embryonic development on, remain in the lens throughout life. As the epithelial cells differentiate into fiber cells, they enlarge, sending one process below the anterior epithelium and one beneath the posterior capsule. An electron microscope study of epithelial cell differentiation in mice has shown that the fiber cell

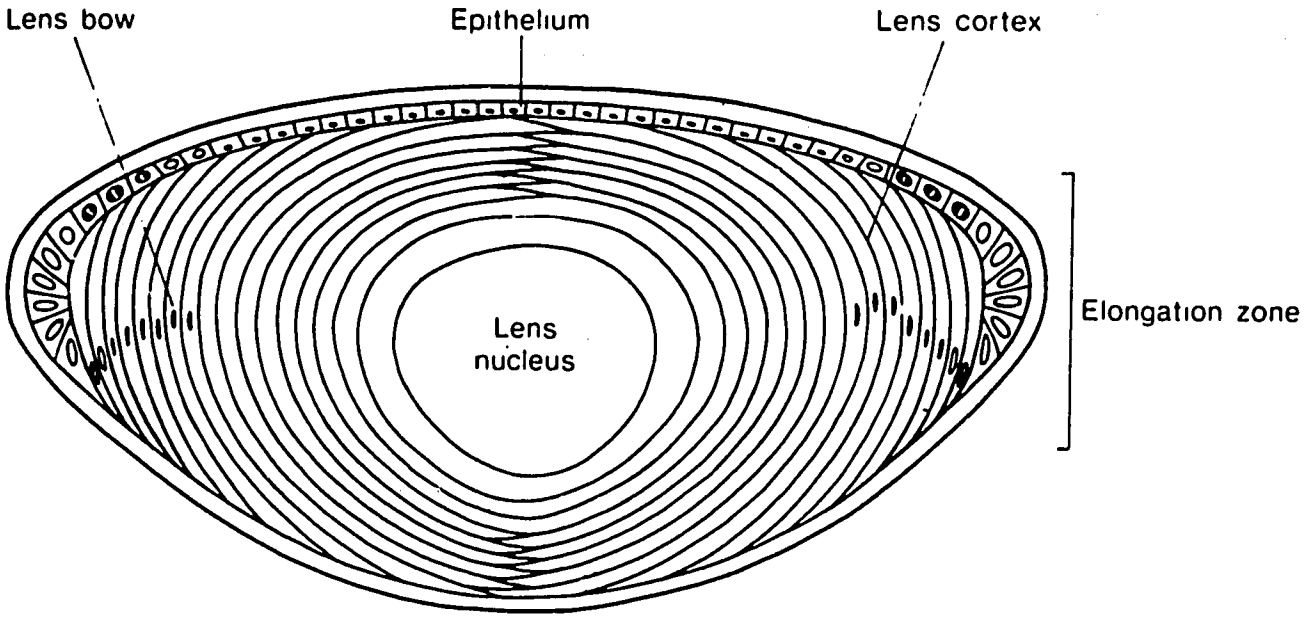
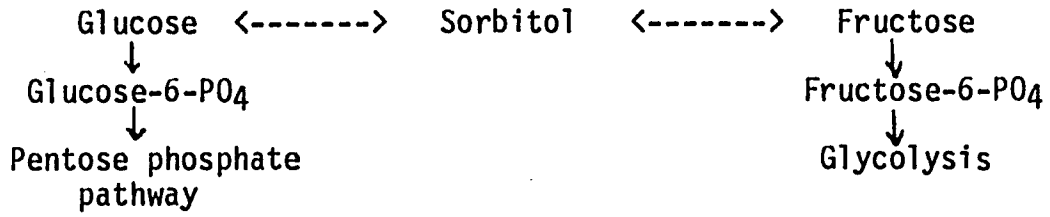


Fig. 3. A schematic drawing of a mammalian eye lens (Bloemendahl, 1977)

nucleus gradually merges with the cytoplasm (pyknosis) and disappears. The loss of the nucleus and concomitant loss of the intercellular organelles, the lack of appreciable intercellular space and absence of blood vessels and nerves may be necessary to insure transparency (Cogan, 1962; Krebs, 1972). The fiber cell membrane is, however, maintained for many years in the outer layer of fiber cells, or cortex. As the cells are continuously pushed toward the center or nucleus of the lens the cell membranes begin to break down and the nuleus has a very homogeneous nature. It is essentially packed with proteins. The lens contains the highest concentration of proteins of any other tissue in the body. 90% of these are crystallins which are considered to be the functioning units in the control of lenticular transparency (Bloemendahl & Cohen, 1976). The maintenance of the proper structure of these integral proteins is essential.

The lens, like the cornea, is an avascular tissue. It must, then, depend on the surrounding fluids for its nutrients. The aqueous humor bathes the anterior surface and the vitreous humor bathes the posterior surface of the lens. the aqueous humor contains much higher levels of nutrients than does the vitreous and so appears to be the source of most substrates. Glycolysis appears to be the major metabolic pathway utilized by the lens for production of energy (Kinoshita & Wachtl, 1958). The utilization of O_2 produces about 10-20% of the energy supply. The pentose phosphate pathway is also active in the lens. Also of great interest is the sorbitol pathway described by van Heyningen (1959a,b, 1962). Under normal conditions this pathway is postulated to regulate the activity of glycolysis.



Another notable feature of the physical arrangement of the lens is that the aqueous bathes the anterior surface of the lens which is covered by the anterior epithelium. The latter is the most active part of the lens, displaying mitotic activity and possessing several active pumping mechanisms. Na-K-ATPase is present primarily in these cells (Bonting, Caravaggio & Hawkins, 1963; Kinsey & Reddy, 1965; Palva and Palkama, 1974) and is responsible for the removal of Na which has leaked, mainly across the posterior surface, into the lens (Kinsey's 'pump-leak' hypothesis). This pump on the anterior surface mediates a net transmural movement of Na which results results in the tissue maintaining an electrical potential difference (P.D.) between its posterior and anterior surfaces (the latter positive) and it eontributes to a short-circuit current (I_{sc}) like in frog skin and toad bladder preparations.

Other active pumping mechanisms have been shown to exist in the lens epithelium and one example of this is the movement of amino acids into the lens. Amino acids were first shown to be actively transported by the lens in the early 1960's (Kern, 1962; Reddy & Kinsey, 1962; Kinsey & Reddy, 1963). The synthesis of amino acids by the lens has also been demonstrated (Kinoshita & Merola, 1961; van Heyningen, 1965). In light of the results of the early transport studies and a more recent study (Reddy, 1970b) it has been determined that amino acid transport mechanisms are more important than synthesis for the supply of amino acids to the lens.

When the capsule and epithelium were removed the amino acid concentrating ability of the rabbit lens was lost (Kinsey & Reddy, 1963). The tissue to medium ratio of amino acid present in the remaining fiber cells was 0.25 which these investigators equated with the presence of the amino acid in the extracellular space. Therefore, it would seem, that the fiber cells were not only incapable of actively accumulating amino acids, but in the absence of the capsule and epithelium were impermeable to them. In this study it was also determined (by competition experiments) that there are at least 3 distinct systems for amino acid uptake, and that 75% of energy utilized in the transport process was derived from glycolysis. A later study (Kinsey & Reddy, 1965) on the relative role of the epithelium and capsule in transport concluded that active transport of amino acids into the lens takes place exclusively across the anterior surface of epithelial cells and relegates to the capsule a passive role. They did, however notice that the concentration of amino acid in the intact lens was highest in the anterior third of the lens, lowest in the nucleus and intermediate in the posterior cortical fibers. The conclusion reached in this study was that amino acids are pumped into the lens by the anterior epithelium, and that the fiber cells are nourished by this activity. A more recent study by Kern, Ho and Ostrove (1977) disputes this "anterior pump posterior leak" theory of amino acid uptake in the rabbit lens as postulated by Kinsey and Reddy (1965). These investigators studied uptake across the anterior and posterior surfaces separately and found that the posterior fiber cells were, indeed, able to actively accumulate amino acids. Reasons for the discrepancy between these two studies were not given but may have one of two explanations. First, Kinsey and Reddy (1965) did not look at uptake across the anterior and posterior sides

separately in their investigation the capsule and epithelium were removed and no uptake was noted. In this process the lens fiber cells could have been damaged so that amino acids could not accumulate. Secondly, as will be explained below, the fiber cells lose their capacity to accumulate 'A' system amino acids during differentiation. Five separate sites for amino acid transport have been characterized (Brassil & Kern, 1968) which in analogy to the nomenclature used by Christensen (1975) were called A, L, ASC, Gly and Ly^+. In their 1977 study, Kern and coworkers measured changes in both the presence and the activity of these five systems were measured using the lens as a model for a differentiating tissue. Transport across the anterior surface represented the undifferentiated system, whereas transport across the posterior surface represented the differentiated system. They determined that the 'A' and 'L' systems regressed significantly in the posterior fiber cells but that the ASC, Gly and Ly^+ systems were maintained. Changes in the activity of various amino acid transporting systems, as noted in Chapter I, occur in many differentiating tissues (Christensen, 1975; Gazzola, et al. 1972; Franchi-Gazzola et al., 1973). Amino acid transport has been proposed to be linked to the transport of cations (Kern, 1962; Kinsey, 1965; Kinsey & Reddy, 1965; Cotlier and Beaty, 1967; Kern & Brassil, 1968). Removal of calcium from the media reduced the ability of the lens to accumulate amino acid (Kinsey, 1965; Thoft & Kinoshita, 1965).

The importance of the study of amino acid transport into the lens cannot be overemphasized. It was estimated (Merriam & Kinsey, 1950) that 5% of the lens protein turns over every day. It is obvious that the main-

tenance of normal levels of requisite proteins in the lens is dependent upon an adequate supply of amino acids and the presence of efficient mechanisms for their conservation and their transport into the lens. Many studies have indicated that there are changes in the steady-state levels of amino acids in lenses that are age and disease related (Dardenne & Kirsten, 1962; Barber, 1968; Zigler, Mauney & Sidbury, 1977). Amino acid levels have also been shown to decrease in experimentally induced cataracts (van Heyningen, 1959b; Reddy & Kinsey, 1963; Reddy, 1965). Kinoshita, Merola and Hayman (1965) demonstrated that increased hydration, which takes place in lenses with experimentally induced cataract, is primarily responsible for the loss of amino acids by the lens. Decreased incorporation of amino acids into proteins and protein synthesis is most likely due to decreases in the levels of amino acids in the lens. Cotlier (1971) found that uptake of amino acids by the lens is independent of the rate of protein synthesis but that a decrease in the rate of amino acid transport and accumulation resulted in a corresponding decrease in the levels of that amino acid incorporated into protein. This could obviously have drastic effects on the composition and structure of proteins. Amino acids have also been implicated as regulators of water and cation balance in the lens (Patterson, 1979).

In nearly all types of cataract, the concentration of glutathione is reduced (Reddy, 1971). Glutathione has an important role in the maintenance of normal cation transport and membrane permeability in the lens (Epstein & Kinoshita, 1970). Glutathione is normally present in high concentrations in the lens where its oxidation leads to decreases in Na-K-ATPase activity, a shift in Na, K, and Cl distribution and an increase

in hydration (Giblin, Chakrapani and Reddy, 1976). These processes may also influence protein synthesis since it has been shown that control of protein synthesis is associated with changes in Na and K ratios in the lens (Shinohara & Piatigorsky, 1977). Most of the glutathione present in the lens is turned over there: it is synthesized and degraded in the lens. The amino acids necessary for its synthesis msut therefore be easily accessible. That changes in protein structure and composition result in lens opacities is well known (Clark, Zigman & Lerman, 1969; Benedek, 1971; Bushell & Duncan, 1978). There are two theories of how senile cataracts develop and both involve disruption of the normal process of protein synthesis. The "Translation error-catastrophe" hypothesis of Orgel (1963) proposes that age related alterations occur in proteins during translation of messengers on ribosomes. Robinson (1974) claims that loss of amide groups from asparagine and glutamine residues after translation, serves as a "general physiological timer".

The importance of understanding the basic mechanisms of amino acid transport into the lens is obvious. Alterations in the availability of amino acids to the protein synthesis machinery can drastically alter the composition and structure of proteins, or even the basic ability of the tissue to make specific proteins. Since these proteins and their interaction with each other are presumed to be responsible for the structure which results in the transparency of the lens, their proper synthesis is vital. It was the purpose of this study to determine the basic mechanism by which amino acids enter and leave the lens. Schultz and Curran (1970) in their review of Na-solute coupled transport pointed out the difficulty, in view of its anatomical arrangement, of evaluating transport properties

of the lens. The layering of fiber cells makes measurement of unidirectional fluxes almost impossible. It was of interest to set up a model to attempt to define the interactions of the various tissue components of the lens so that the overall transport process that is seen can be better understood. Analyses of this kind are needed and to my knowledge have not been pursued. It was also the intention of this study to determine how the transport process is affected by specific drugs, membrane probes and hormones. These studies were designed to define the transport processes in the lens and to be able to compare and contrast them with other transport systems. A more precise knowledge of these mechanisms is important for an understanding of the normal functioning of the lens and the disruptions that can lead to the formation of cataracts.

Methods

Animals: Toads, Bufo marinus, were obtained from National Reagents, Bridgeport, Conn. and kept in the laboratory at 18° C.

Preparation of Tissues: Corneas and lenses were removed from the eyes of the toads by cutting through the sclera around the cornea about 3 mm from the limbus. Both tissues were placed in a modified Conway solution. The attached iris was removed and the sclera was trimmed off as completely as possible. The sclera was left on in experiments in which the corneas were mounted in Ussing-type chambers. In certain cases the epithelium was removed by scraping the anterior surface of the cornea with a scalpel blade before it was removed from the eye. The endothelium was removed by scraping the posterior surface of the cornea.

The dissection of lenses into their various layers was accomplished in the following manner. First the capsule was removed after soaking the lenses in Conway solution containing 0.5 mg/ml collagenase for 20 minutes. After this incubation, the capsule is readily pulled off, in one piece, with a fine forceps. To remove the epithelium the lens was then held, anterior surface up and the epithelium was scraped off using a scalpel blade. To expose the lens nucleus it was necessary to remove the outer cortical layer of fiber cells. The lenses were spun in glass cylinders until they were reduced to 1/3 of their initial weight (this usually took 30-90 minutes, depending on the initial size of the lens).

Measurement of potential difference (P.D.), short-circuit current (I_{sc}) and ion fluxes across the cornea and lens: Both the corneas and lenses

were mounted in Ussing-type chambers so that the transmural fluxes of ions and various compounds could be measured. The amphibian cornea and lens exhibit a transmural potential difference, anterior positive for the lens and aqueous side positive for the cornea, which in toads is 10-30 mV. This P.D. was maintained at zero, when fluxes across either the cornea or lens were measured, with an automatic voltage clamp. Transmural ^{22}Na fluxes across the cornea were measured in one set of experiments. ^{22}Na (1.0 μCi/ml) was added to either the solutions bathing the epithelial or endothelial surfaces. At 15 minute intervals 1.0 ml samples were removed from the trans solution and counted in a Beckman Biogamma II counter. The movement of ^{36}Cl (1.0 μCi/ml) across the cornea was measured in a similar manner. At 20 minute intervals, 1 ml samples were taken, 5 ml of scintillation fluid (Fisher Scintiverse) was added and the samples were counted in a liquid scintillation counter (Beckman LS-9000)

Transmural permeability studies (cornea): The permeability of each of the layers of corneal tissue was determined by mounting the corneas in Ussing-type chambers and measuring the transcorneal flux of several compounds under short-circuited conditions. Transport across intact corneas and those with the epithelium or endothelium or both these bounding membranes removed was studied. Transport of the various compounds in both directions (tear to aqueous and aqueous to tear side) was measured. The endothelial or epithelial bathing solution contained 0.1 mM and 0.2 μCi/ml of the ^{14}C-labelled compound that was to be tested. At specific time intervals 1 ml samples were counted in a scintillation counter (Beckman LS-9000) after the addition of the scintillation fluid. The corneas were

removed from the chambers, blotted, weighed and extracted in 2 ml 0.5N HCl. An aliquot of this extract was counted to determine the amount of the compound in the cornea. This method was found to be as effective as dissolving the tissues in a tissue 'solubilizer' (NCS, Amersham Searle).

Amino acid uptake: Excised paired corneas and lenses were placed in Conway solution containing the unlabelled amino acid in concentrations from 0.1 to 30 mM and 0.2 μCi/ml of the ^{14}C-labelled amino acid. At the end of the incubation the tissues were removed from the Conway solution, blotted, weighed and extracted as described above. In every case one cornea or lens of each pair was treated as the control and the contralateral cornea or lens as the experimental tissue.

Amino acid efflux: Six pairs of corneas or lenses were preloaded with 0.1 mM to 30 mM unlabelled amino acid and 1 μCi/ml of the ^{14}C-labelled amino acid for 3 hours in Conway solution. At the end of this preloading period, the tissues were removed from the bathing solution, blotted, weighed and placed in scintillation vials containing 1 ml Conway solution. They were moved serially to vials containing fresh amino acid-free Conway solution at prescribed time intervals so that the rate of exodus could be determined. At the end of the experiment the tissues were extracted as described above. As in the uptake experiments one tissues of each pair was treated as the control and the other as the experimental preparation.

Calculations: When corneas are incubated *in vitro* they swell continually during the first few hours. In order to determine the molar concentration of amino acid in the cornea it is necessary to know its water content/unit

wet weight at a particular time. Corneas were removed from toads and incubated in Conway solution at 21° C for time periods up to 6 hours, after which they were blotted, weighed and dried in the oven at 110° C for 16 hours. The water content of the corneas was calculated from the data derived from these measurements (Table 1).

Table 1. Corneal water content. Mean + S.E. of 8 experiments.

Time (hr)	ml H_2O/g dry weight	H_2O as % wet weight
0	4.4 ± 0.7	75
1	6.4 ± 1.5	84
2	7.9 ± 1.9	86
3	8.8 ± 2.3	87
4	9.0 ± 1.4	89
5	9.6 ± 2.1	90
6	10.1 ± 1.9	92

The lenses maintain their volume even overnight so it was necessary only to determine their initial volume. Using the technique described above the water content of the whole lens was determined to be 58% of the wet weight of this tissue. It was, however, also necessary to determine the water content of the inner 1/3 of the lens (or nucleus) since amino acid uptake studies were conducted on this portions of the lens. The water content of the nucleus is much less than that of the outer cortical layer, being only 44% of the lenticular wet weight.

The concentration of the amino acid in these tissues was calculated and compared to that in the external bathing fluid and expressed as a tissue to medium (T:M) ratio.

Calculation of results of amino acid efflux studies were carried out on the PROPHET Computer System* using a program specifically designed to handle the data. The program converts raw data to nM/mg wet weight and % total counts for each time period and plots these two measures on a graph. PROPHET system programs were also used to determine the rates of efflux. The programs and equations used are described more fully in the appropriate sections of the text.

* The PROPHET system is a specialized computer resource developed by the Chemical/Biological Information Handling Program of the National Institutes of Health. A detailed description of the system features appears in the Proceedings of the National Computer Conference and Exposition, May 6-10, 1974. Chicago, Ill, Vol. 43, pp. 457-483, AFIPS Press, Montvale, N.J.

Sources of compounds:

A) Radioactively labelled compounds:

New England Nuclear, Boston, MA: ^{22}Na (2.39 mCi/ml); inulin (Carboxyl-^{14}C)(2.76 mCi/g); urea (^{14}C)(47.1 mCi/mMol); α-aminoisobutyric acid (1-^{14}C AIB) (51.6 mCi/mMol); ^{125}I-iodination kit was used to iodinate the albumin used in the transmural permeability studies.

ICN, Irvine, CA: ^{36}Cl (5.59 μCi/g Cl)

B) Reagents:

All other compounds, including the scintillation fluid (Scintiverse) were, with the following exceptions, purchased from Fisher Scientific.
Pearce Chemical Co.: 4,4'-diisothiocyano-2,2'stilbenedisulphonic acid (DIDS)

Mallinckrodt Chemical Works, St. Louis, Mo.: Ca gluconate, Na_2HPO_4-$7H_2O$ Sigma Chemical Co., St. Louis, MO: ethylene glycol-bis-(β-aminoethyl ether)N-N'tetraacetic acid (EGTA), collagenase, trypsin, hyaluronidase, AIB, alanine, leucine, Tris-Cl, Na iodoacetate, ouabain, albumin, phospholipase C.

Insulin was donsated by Lilly Pharmaceuticals, Nutley, NJ

Bumetanide was donated by Hoffman-La Roche, Nutley, NJ

SOLUTIONS

Compounds (mM)	Conway	Na-free Conway	Cl-free Conway	Tris Ringer	Na-free Tris Ringer
NaCl	72.0	---	---	103.0	---
KCl	2.5	2.5	---	2.5	2.5
$MgSO_4$	1.2	1.2	1.2	---	1.2
K_2SO_4	---	---	1.2	---	---
Na_2SO_4	0.6	---	36.6	---	---
NaH_2PO_4	0.7	---	2.2	---	---
Na_2HPO_4	2.2	---	0.7	---	---
Glucose	26.0	26.0	13.0	5.2	5.2
Ca Gluconate	1.0	1.0	1.0	1.0	1.0
Sucrose	---	---	37.3	---	---
LiCl	---	---	---	---	102.8
$NaHCO_3$	25.0	---	25.0	---	---
Tris-CL pH=7.4	---	---	---	2.0	2.0
Choline Cl	---	72.0	---	---	---
Choline HCO_3	---	27.2	---	---	---
KH_2PO_4	---	2.2	---	---	---

RESULTS

1. Transmural Fluxes Across the Cornea:

The purpose of measuring the transmural fluxes was to determine how amino acids enter the toad cornea and to see if any of its layers forms a barrier to the permeation of these nutrients. Corneas were mounted in Ussing-type chambers so that the permeability characteristics of either surface and each layer of tissue could be examined. These studies were carried out under electrically short-circuited conditions. The amino acid α-aminoisobutyric acid (AIB) cannot cross the intact cornea or in preparations with the endothelium alone removed (Table 1). When the epithelium is removed, however, transmural movement of AIB takes place and the flux is equal in either direction. The additional removal of the endothelium also does not alter this rate. Thus AIB cannot enter the cornea across the outer epithelial surface, but accumulates in the cornea across the endothelial surface.

The permeability of the stroma and endothelium to other compounds was also measured in deepithelialized corneal preparations to determine the extent to which these two layers can limit the permeation of molecules of various sizes. As shown in Table 1, the transmural flux of each molecule is inversely related to molecular size. For molecules up to the size of inulin (MW=5000) the endothelium poses no measurable permeability barrier. Although albumin (MW=60,000) crosses the deepithelialized corneal preparation in the presence of the endothelium, it does so at a reduced rate as compared to preparations with both the epithelium and endothelium removed.

Table 1. Transcorneal fluxes of various solutes in vitro.

Condition of cornea	Compound	μmoles $cm^{-2}hr^{-1}$ Efflux	Influx
Epithelium+stroma +endothelium (=intact cornea)	AIB	0	0
Endothelium removed	AIB	0	0
Epithelium removed	urea	62.0±2.3 (6)	---
	AIB	32.0±1.1 (3)	33.7±1.7 (3)
	leucine	26.0±1.9 (5)	---
	inulin	4.9±0.2 (3)	---
	albumin	0.12±0.01 (6)	---
Epithelium and endothelium removed (=stroma)	AIB	32.8±2.4 (3)	31.5±2.3 (3)
	inulin	4.5±0.2 (3)	---
	albumin	0.21±0.02 (6)	---

Corneal preparations were mounted in Ussing-type chambers in the condition described above. 0.1 mM unlabelled compound and 0.2 μCi/ml ^{14}C-labelled compound, or 2 mg/ml cold albumin and 0.2 μCi/ml ^{125}I-albumin were added to either the epithelial or endothelial bathing solution. Samples were taken from the trans bathing solution at specific time intervals to determine the flux of each compound. Efflux refers to the movement of the solute from the aqueous to the tear side, influx refers to movement from tear to aqueous. The results are presented as the mean ± S.E. of the number of experiments in parentheses.

Treatment of the corneas with either hyaluronidase and/or collagenase did not alter the permeability of the cornea to AIB, leucine or urea (Table 2).

2. Amino acid uptake by the cornea.

AIB is accumulated in the cornea in concentrations up to 10 times that of the bathing media. This uptake is linear up to 9 hours of incubation (Fig. 4). Most of the accumulation seen takes place in the epithelium since removal of this layer of cells reduces the tissue to medium ratio to 1.23 (Table 3). AIB uptake is saturable at concentrations greater than 10 mM (Fig. 5). The pH dependence of this system was tested and it was found that AIB accumulation is greater at pH 8.4 than at pH 7.4 (Table 4).

The uptake of neutral amino acids, such as AIB and alanine, has been shown, in many tissues, to be dependent upon the presence of Na in the external bathing media. The cotransport of Na with the substrate amino acid is necessary for the amino acid to enter the tissue. There was no immediate effect of removal of Na on AIB accumulation in the cornea; accumulation was unaffected at 30 minutes (Table 5), after 2 hours accumulation is slightly reduced. However, even after 7 hours incubation in Na-free media, the accumulation is not completely abolished as shown by a tissue to medium ratio of 1.76.

The lack of a rapid effect of removal of external Na on AIB accumulation suggested that changes in internal Na concentration maybe involved in this process. Incubation with ouabain, which inhibits Na-K-ATPase thereby increasing the internal concentration of Na, also lacks a

Table 2. Effects of hyaluronidase and collagenase on transcorneal fluxes of various solutes

	Outflux (μmoles $cm^{-2}hr^{-1}$)		
	Leucine	AIB	Urea
Control	26.0±1.9	29.2±3.1	64.1±4.8
Hyaluronidase (4 mg/ml)	21.7±2.1	31.7±2.5	62.3±3.1
Collagenase (0.5 mg/ml)	21.9±1.9	30.6±2.8	60.7±3.2

Corneal preparations were mounted in Ussing-type chambers and leucine, AIB or urea, 0.1 mM + 0.2 μCi/ml of the labelled compound was added to the endothelial bathing solution. Samples were taken every 30 minutes from the trans bathing solution to determine the flux. After 2 hours, hyaluronidase was added to both sides and again after 2 hours collagenase was added to both sides. The values above are the average of the last 2 samples in each of the 2 hour periods and are the mean ± S.E. of 5 experiments.

Fig. 4. Corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for the times indicated. Each point is the mean ± S.E. of at least 6 experiments.

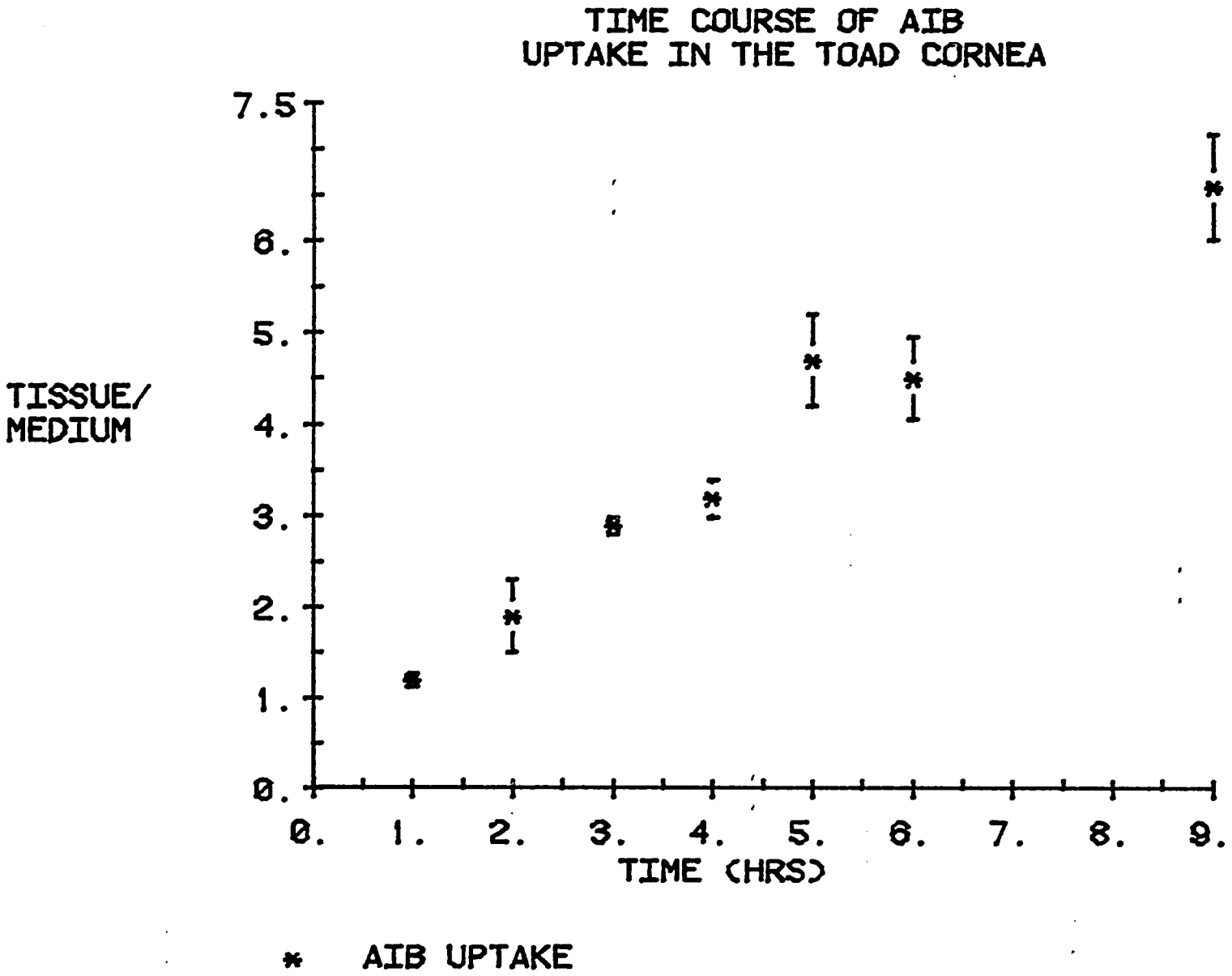

TIME COURSE OF AIB
UPTAKE IN THE TOAD CORNEA
TISSUE/
MEDIUM
7.5
6.
5.
4.
3.
2.
1.
0.
0. 1. 2. 3. 4. 5. 6. 7. 8. 9.
TIME (HRS)
* AIB UPTAKE

Table 3. Amino acid uptake in each of the corneal layers.

Condition of the cornea	Tissue:medium concentration
a. Epithelium + stroma + endothelium (=intact cornea)	3.41 ± 0.23
b. Endothelium removed	3.13 ± 0.19
c. Epithelium removed	1.23 ± 0.16
d. Epithelium and endothelium removed (=stroma)	1.07 ± 0.13

Corneas, in the condition described above, were incubated in Conway solution containing 0.1 mM AIB + 0.2 μCi/ml ^{14}C-AIB for 3 hours. The numbers represent the mean ± S.E. of 6 experiments.

	P for difference
a vs b	ns
a vs c	<0.001
c vs d	ns

Fig. 5. Corneas were incubated in Conway solution with the concentration of AIB indicated and 0.2 μCi/ml ^{14}C-AIB for 3 hours. Non-linear regression analysis was used to fit the data to the curve defined by Michaelis-Menton kinetics:

$$v = \frac{V_{max} \times [S]}{[S] + K_m}$$

This was done using a program available on the PROPHET computer system. Each value represents the mean $\pm$ S.E. of 6 experiments.

K_m = 10.13 mM

V_{max} = 10.46 umoles/mg wet weight/hr

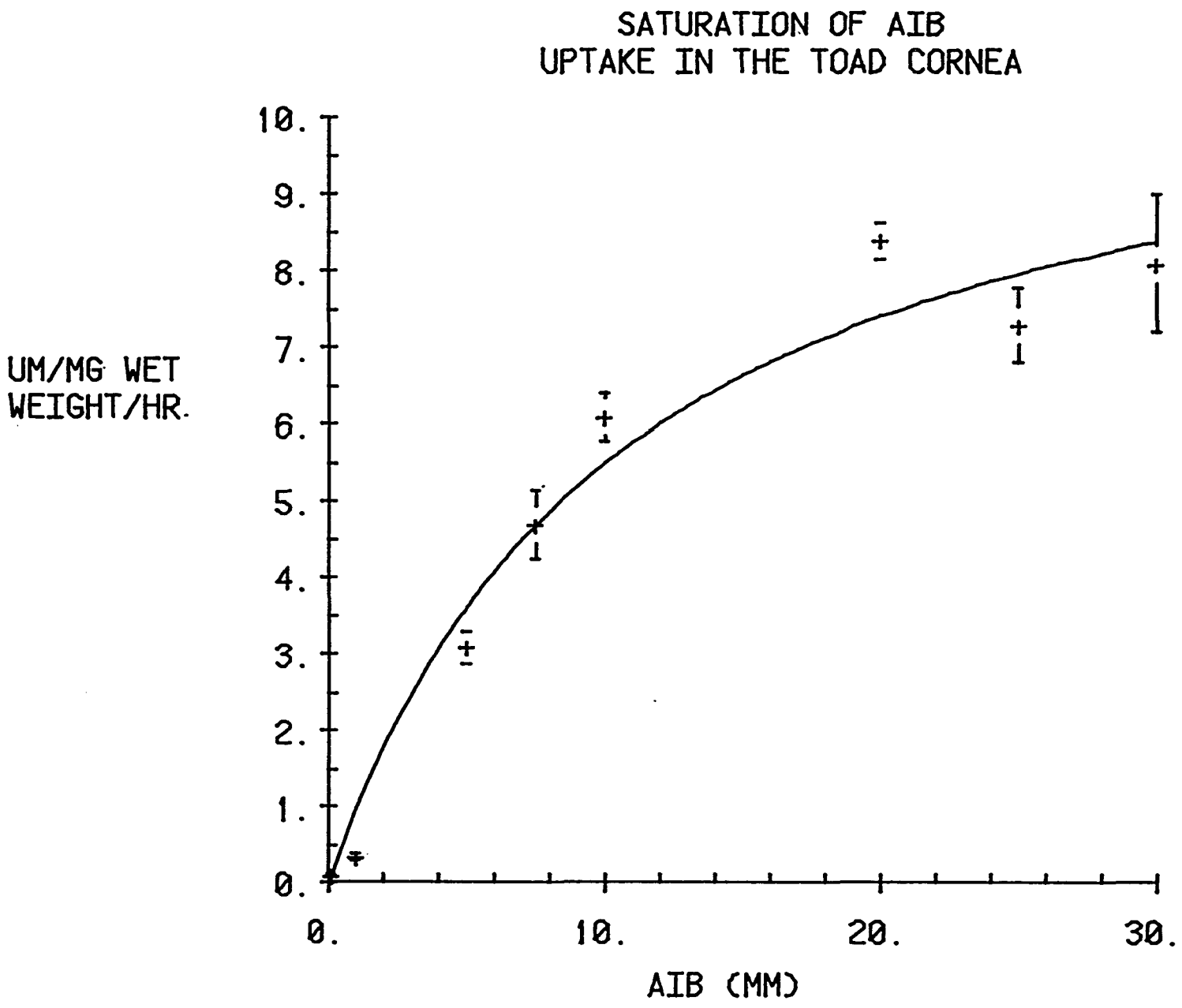

SATURATION OF AIB
UPTAKE IN THE TOAD CORNEA
UM/MG WET
WEIGHT/HR.
10.
9.
8.
7.
6.
5.
4.
3.
2.
1.
0.
0.
10.
20.
30.
AIB (MM)

Table 4. Effect of pH on AIB accumulation in the toad cornea.

	AIB (tissue:medium concentration)
pH 7.4	1.58 ± 0.03
pH 8.4	2.46 ± 0.17

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. One cornea of each pair was treated as the control and the bathing solution was bubbled with room air (pH 8.4). The other cornea was treated as the experimental one and the bathing solution was bubbled with 95% O_2 and 5% CO_2 to bring the pH to 7.4. The values above are the means ± S.E. of 6 experiments.

Table 5. Effect of incubation in Na-free media on AIB accumulation in the toad cornea.

Incubation time (hours)	Tissue:medium concentration		P for difference
	Control	Na-free media	
0.5	1.35±0.11	1.24±0.15	ns
2.0	1.91±0.09	1.45±0.06	<.01
7.0*	2.76±0.11	1.76±0.06	<.001

Corneas were paired in this experiment, one cornea of each pair was the control and the other the experimental. Experimental corneas were incubated in Na-free Conway for the times indicated. 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB were present in both solutions.

*This is the total incubation time in Na free media. AIB, both labelled and unlabelled, was added after 2 hours preincubation in Na-free solution and thus was only present for 5 hours.

rapid effect on amino acid accumulation. After 2 hours incubation in the presence of 10^{-4} M ouabain, there is no change in AIB accumulation (Table 6a). However after 7 hours, ouabain significantly reduces, but does not completely abolish, the concentration of AIB by the cornea. Absence of potassium in the external bathing media is also expected to increase internal Na concentration, and when K was removed from the external media, AIB accumulation was reduced (Table 6b). Amphotericin B is a polyene antibiotic which has been shown to increase the permeability of the cornea to Na (Candia, Bentley & Cook, 1974). When this drug was added to the preparations no change in the accumulation of AIB was observed even after 5 hours incubation (Table 6c).

The possibility that agents which increase the intracellular Na concentration cause cell swelling and, as a result, decrease amino acid concentration was considered. In other tissues, amino acids also have been shown to play a role in osmoregulation. It was important to determine if cell volume changes influence amino acid accumulation in the cornea. Corneas were therefore incubated in Conway solution in which the osmolarity was reduced by 40 mosmol. This treatment had no effect on the accumulation of AIB.

The cornea accumulates amino acids against a concentration gradient suggesting that this process may involve active transport. In order to determine if amino acid accumulation is dependent upon energy production the metabolic inhibitors iodoacetate and cyanide were added to the bathing solution. Amino acid accumulation is completely inhibited by this treatment (Table 7), but only after 7 hours incubation. Although there was a significant reduction in accumulation after 2 hours incubation the

Table 6. Effect of compounds that alter intracellular Na levels in the cornea on accumulation of AIB

	Incubation time (hr)	Tissue:medium concentration Control	Experimental	P for difference
a) Ouabain (10^{-4} M)	2.0	2.10±0.11	2.02±0.08	ns
	7.0*	3.24±0.24	1.31±0.02	<.001
b) K removed from external bathing media	5.0	3.81±0.18	1.96±0.09	<.001
c) Amphotericin B (12.5 ug/ml)	5.0	3.80±0.44	4.01±0.47	ns
d) Osmolarity reduced by 40 mosmol/l	5.0	2.60±0.06	2.42±0.12	ns

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB. One cornea of each pair was treated as the control, the other as the experimental preparation. The numbers represent the mean ± S.E. of 6 experiments.

* The incubation time here is 2 hours preincubation in the presence of ouabain after which AIB was added to the solution for the remaining 5 hours.

Table 7. Effect of metabolic inhibitors on AIB accumulation in the toad cornea

	Incubation time (hr)	Tissue:medium concentration Control	IAA & CN	P for difference
Iodoacetate (2mM) and cyanide (5mM)	0.5	1.08±0.02	0.97±0.04	ns
	2.0	2.48±0.23	1.44±0.06	<.001
	7.0*	3.20±0.14	1.05±0.03	<.001

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB. One cornea of each pair was treated as the control, the other as the experimental preparation. The values above represent the mean ± S.E. of 6 experiments.

* The incubation time here is 2 hours preincubation in the presence of the inhibitors after which AIB was added to the solution for the remaining 5 hours.

the inhibition was not complete.

Competition studies with other amino acids suggest that the uptake of AIB is a specific process since the presence of alanine significantly decreases the accumulation of AIB and, conversely, AIB has the same effect on the accumulation of alanine. Leucine inhibits the uptake of both alanine and AIB to the same extent but is not as effective as alanine or AIB are in mutually inhibiting each others uptake (Table 8). The combination of alanine and leucine completely blocks AIB accumulation. β -aminobicyclo (2,2,1) heptane carboxylic acid (BCH), which is considered a leucine analog (Christensen, et al., 1975) also inhibits AIB accumulation. However, it does not block leucine accumulation. It therefore must have its inhibitory effect on AIB acumulation via the 'A' or alanine sensitive system. As can be seen here, leucine is not accumulated in the cornea to as high a tissue to medium ratio as alanine. This agrees with the data for leucine systems in other tissues, in which the uptake mechanism for 'L' system amino acids is not highly concentrative. Any amino acid accumulation occurring in Na-free media must be occurring via Na-independent systems. AIB is still accumulated in the cornea in the absence of Na. Since leucine has been shown to inhibit amino acid uptake in the cornea and is classically defined as being taken up by a Na-independent system, it is possible that AIB is accumulated in Na-free media via the 'L' or leucine preferring system. If this is the case, then one would expect no accumulation of AIB to occur when an excess of leucine is present in a Na-free media. When leucine is added to Na-free media, accumulation of AIB is decreased as compared to Na-free alone, but not completely abolished (Table 8).

Table 8. Competitive inhibition of amino acid uptake in the toad cornea

	Tissue:medium concentration Control	Experimental	P for difference
1) AIB uptake			
+ 10 mM alanine	4.22±0.31	1.31±0.13	<.001
+ 10 mM leucine	5.13±0.36	2.22±0.07	<.001
	1.66±0.06§	1.31±0.09§	<.02
+ 10 mM BCH	4.37±0.21	2.14±0.17	<.001
+ 5 mM leucine and 5 mM alanine	5.82±0.27	1.03±0.04	<.001
2) Alanine uptake			
+ 10 mM leucine	4.16±0.20	2.21±0.07	<.001
+ 10 mM AIB	3.73±0.11	1.94±0.15	<.001
3) Leucine uptake			
+ 10 mM BCH	1.75±0.04	1.67±0.10	ns

Corneas were incubated in 0.1 mM amino acid and 0.2 μCi/ml ^{14}C-amino acid as indicated ((1) AIB and (2) alanine (3) leucine) for 5 hours. One cornea of each pair was treated as the control and 10 mM of the competing amino acid was added to solutions containing the experimental corneas. Results are expressed as the mean ± S.E. of 6 experiments. §These experiments were carried out in Na-free Conway.

Calcium is vital to the structural integrity of cellular membranes and it has been shown to influence their permeability. To determine the importance of Ca^{+2} for AIB uptake in the cornea, these tissues were incubated in Conway solution with no added Ca or Mg. These deletions had no effect on AIB accumulation. However, when Ca is left out of solution, there is still a small but significant amount of Ca (about 10^{-5}M) present in the solution. Therefore, in order to more completely eliminate Ca from the media, it was necessary to add a chelating agent. When ethylene glycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA) was added to 'Ca-free' solutions the ability of corneas to accumulate AIB was eliminated (Table 9).

In an attempt to more clearly ascertain the nature of the amino acid 'carrier(s)' in the cornea, a variety of membrane 'probes' were employed. These included a number of enzymes, and reagents that bind specifically to sulfhydryl groups, lipids, carbohydrate moieties and anion exchange proteins. Trypsin reduced AIB accumulation (Table 10), but only at high concentrations. Trypsin cleaves peptide bonds after arginine and lysine residues and therefore may disrupt the conformation of many different proteins. Phospholipase C hydrolyzes membrane phospholipids, in particular phosphatidylcholine, and has been shown to alter membrane permeability in many tissues. It did not, however, affect amino acid accumulation in the cornea (Table 10) at concentrations that elicit changes in Na permeability in the toad's lens (Yorio and Bentley, 1978). p-Chloromercuriphenylsulfonic acid (PCMPS), which binds sulphydryl (SH) groups on the outside surface of cells and is relatively impermeant, significantly decreased AIB accumulation in the cornea (Table 10). Con-

Table 9. The effect of removal of Ca and Mg on AIB accumulation in the toad cornea

	AIB (Tissue:medium concentration) Control	Experimental	P for difference
Ca and Mg-free ($Ca\ 10^{-5}M$)	3.60±0.19	3.91±0.27	ns
Ca and Mg-free + EGTA (2mM)	3.43±0.15	0.98±0.09	<.001

Corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. One cornea of each pair was treated as the control the other as the experimental. The results are expressed as the mean ± S.E. of 6 experiments.

Table 10. Effect of various 'membrane probes' on AIB accumulation in the toad cornea

	Concentration	AIB (Tissue:medium concentration) Control	Experimental	P for difference
Trypsin	10ug/ml	4.08±0.06	3.54±0.38	ns
	100ug/ml	3.08±0.12	1.48±0.36	<.001
Phospholipase C	50ug/ml	3.52±0.13	3.44±0.21	ns
PCMPS	10^{-5} M	3.32±0.25	3.18±0.07	ns
	10^{-4} M	3.15±0.18	1.68±0.12	<.001
Concanavalin A	50ug/ml	3.47±0.11	3.45±0.15	ns

The following were the specific activities of the enzymes used: 1) trypsin (14350 BAEE units*); 2) phospholipase C (6 U/mg). In all cases the corneas were incubated for 3 hours in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB. The corneas were paired and one of each pair was treated as the control, the other as the experimental The values above are the mean ± S.E. of 6 experiments.

* One BAEE unit will produce an A_{253} of 0.001 per minute at pH 7.6, at 25°C in a reaction volume of 3.2 ml (1 cm light path). Substrate α-N-benzoyl-L-arginine ethyl ester.

canavalin A (Con A) is a lectin, that is, a carbohydrate binding protein which binds specifically to α-D-glucosyl residues. This compound also had no effect on AIB accumulation (Table 10).

The toad cornea exhibits an electrical potential difference (P.D.) and short circuit-current (I_{SC}). The I_{SC} is mostly due to the net movement of Cl from the aqueous to the tears (Zadunaisky, 1966) and to a smaller extent (20%) the net movement of Na from the tears to the aqueous. It was thus of interest to determine the possible importance of Cl for amino acid transport in the cornea. When Cl in the external media was replaced by SO_4, there was no change in in the accumulation of AIB (Table 11). Bumetanide, an inhibitor of active Cl transport in the toad cornea (McGahan, Yorio and Bentley, 1977) also did not affect AIB accumulation in this tissue.

4,4'-Diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) which blocks active Cl transport across the cornea (Bentley and McGahan, 1980) (Table 12) also has a marked inhibitory effect on AIB accumulation (Table 11). These effects do not appear to be Cl-dependent for as described above, neither a Cl-free external medium nor bumetanide had any effect on AIB accumulation. It has been suggested that anion exchange (e.g. Cl^- for HCO_3^- or lactate) may be responsible for intracellular build up of Cl ions in the cornea (Bentley and Yorio, 1978). The anions subsequently leave the corneal epithelium across the more permeable apical surface, as suggested by Klyce and Wong (1977). Most likely, then, DIDS blocks anion exchange in the toad cornea like it does in erythrocytes, and the decrease in net Cl transport in the presence of DIDS is due to a reduction in intracellular Cl concentration. It is possible that a blockade of

Table 11. AIB accumulation in the toad cornea in relation to Cl transport and ion exchange

	AIB (Tissue:medium concentration) Control	Experimental	P for difference
Cl-free Conway	2.25±0.16	2.25±0.11	ns
Bumetanide (10^{-5}M)	2.41±0.30	2.28±0.20	ns
DIDS ($5x10^{-3}$M)	2.42±0.20	1.63±0.08	<.01
	4.51±0.41§	2.48±0.17§	<.02

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. Na_2SO_4 and sucrose were substituted isosmotically for NaCl in the experimental Cl-free Conway solution. The results are expressed as the mean ± S.E. of 6 experiments.

§ The uptake of alanine was measured in these experiments. The corneas were incubated with 0.1 mM alanine and 0.2 μCi/ml ^{14}C-alanine for 3 hours.

Table 12. Effects of bumetanide and DIDS on Cl fluxes and I_{sc} across the toad cornea

	Cl flux (μEq $cm^{-2}hr^{-1}$)	
	Control	Bumetanide (10^{-4} M)
efflux	0.67	0.41
influx	0.37	0.47
net flux	0.30	0.06

From McGahan, Yorio & Bentley, 1977

	Control	DIDS (10^{-3} M)
efflux	0.47±0.02	0.34±0.03
influx	0.25±0.04	0.24±0.04
net flux	0.22	0.10

Corneal preparations were mounted in Ussing-type chambers. Transepithelial potential difference (P.D.) was measured and the preparations were short-circuited with an automatic voltage clamp. The short-circuit current (I_{sc}) was recorded continuously. ^{36}Cl fluxes were measured for 2 hours before the bumetanide or DIDS was added. Both drugs were added to the endothelial side of the cornea. Efflux refers to Cl movement from the aqueous to the tear side; influx to movement from the tear to aqueous side; and net flux is the efflux minus outflux. The results are presented as the mean ± S.E. of 6 experiments.

anion exchange could also result in an increase in the intracellular concentrations of lactate and HCO_3^-. Therefore, it was possible that DIDS affected amino acid accumulation by an alteration of ion and metabolite equilibrium in the cornea (an indirect effect). However, DIDS may directly inhibit amino acid accumulation. Since this stilbene can inhibit Cl transport when it is placed on the tear or aqueous surface, and amino acids are only taken up across the latter side, it was possible to test whether the effect of DIDS was direct or indirect. Corneas were therefore mounted in Ussing type chambers and DIDS was applied to the tear side in one set of experiments and to the aqueous side in another. If DIDS placed on the tear surface inhibits AIB accumulation, then its effects maybe considered indirect. If, however, DIDS has no effect on the tear surface, but does inhibit accumulation when placed in the aqueous solution, the effect may, indeed, be direct. In fact, it was found that DIDS does not affect accumulation when it is present on the tear side, but decreases accumulation by about 50% when present on the aqueous side (Table 13).

The hormones insulin and triiodothyronine (T_3) have been shown to be anabolic in many tissues. Both hormones stimulate amino acid uptake but their effects on amino acid transport in the cornea have not, to my knowledge, been reported. Neither of these hormones affected amino acid accumulation to any significant extent under these in vitro conditions (Table 14). The corticosteroid drug, dexamethasone also had no detectable effect on AIB accumulation in the cornea.

Table 13. Effect of DIDS on the tear and aqueous surface of the cornea on AIB accumulation

	AIB(Tissue:medium concentration) Control	Experimental	P for difference
DIDS ($5x10^{-3}M$) tear side	6.21±0.28	5.86±0.40	ns
DIDS ($5x10^{-3}M$) aqueous side	4.29±0.31	2.77±0.42	<.05

Paired corneas were mounted in Ussing-type chambers. 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB were added to the Conway solution on the aqueous side. In one set of experiments, DIDS was added to the tear solution, in the other, it was added to the aqueous solution. The values are the mean ± S.E. of 6 experiments.

Table 14. Effect of hormones on AIB accumulation in the toad cornea

Hormone	Concentration (M)	Incubation time (hr)	AIB (Tissue:Medium concentration) Control	Experimental	P for difference
Dexamethasone	10^{-6}	3	3.43±0.19	3.10±0.23	ns
T_3	$5x10^{-5}$	4†	1.51±0.07	1.61±0.04	ns
Insulin	10^{-6}	4§	3.58±0.07	3.70±0.26	ns
	10^{-6}	16	9.29±0.88	9.15±0.71	ns
	10^{-7}	16	10.34±0.42	9.37±0.71	ns

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for the times indicated. One cornea of each pair was treated as the control and the other as the experimental preparation. The results are the means ± S.E. of 6 experiments.

† These corneas were preincubated in amino acid free media for 3 hours before the addition of AIB.

§ These corneas were preincubated in amino acid free media for 1 hour before the addition of AIB.

3. Amino acid efflux from the cornea.

In the previous section the emphasis of the investigation was on amino acid accumulation in the cornea and the effects of various agents on that process. However, a measure of accumulation only represents the steady-state levels of compounds. Changes in the rate of uptake may not be the only factor influencing levels of nutrients in tissues. In order to maintain the levels of nutrients necessary for normal metabolic processes there must be a balance between influx and efflux. It is therefore possible that changes in the steady-state levels of amino acids that are altered by the introduction of toxic substances or hormones are due, not only to changes in the uptake system, but also to changes in the rate of exodus of these compounds from the tissue. This section of the investigation therefore involves the study of amino acid efflux from the cornea and the effects on this process of the various compounds that were shown previously to alter the steady state levels of amino acids.

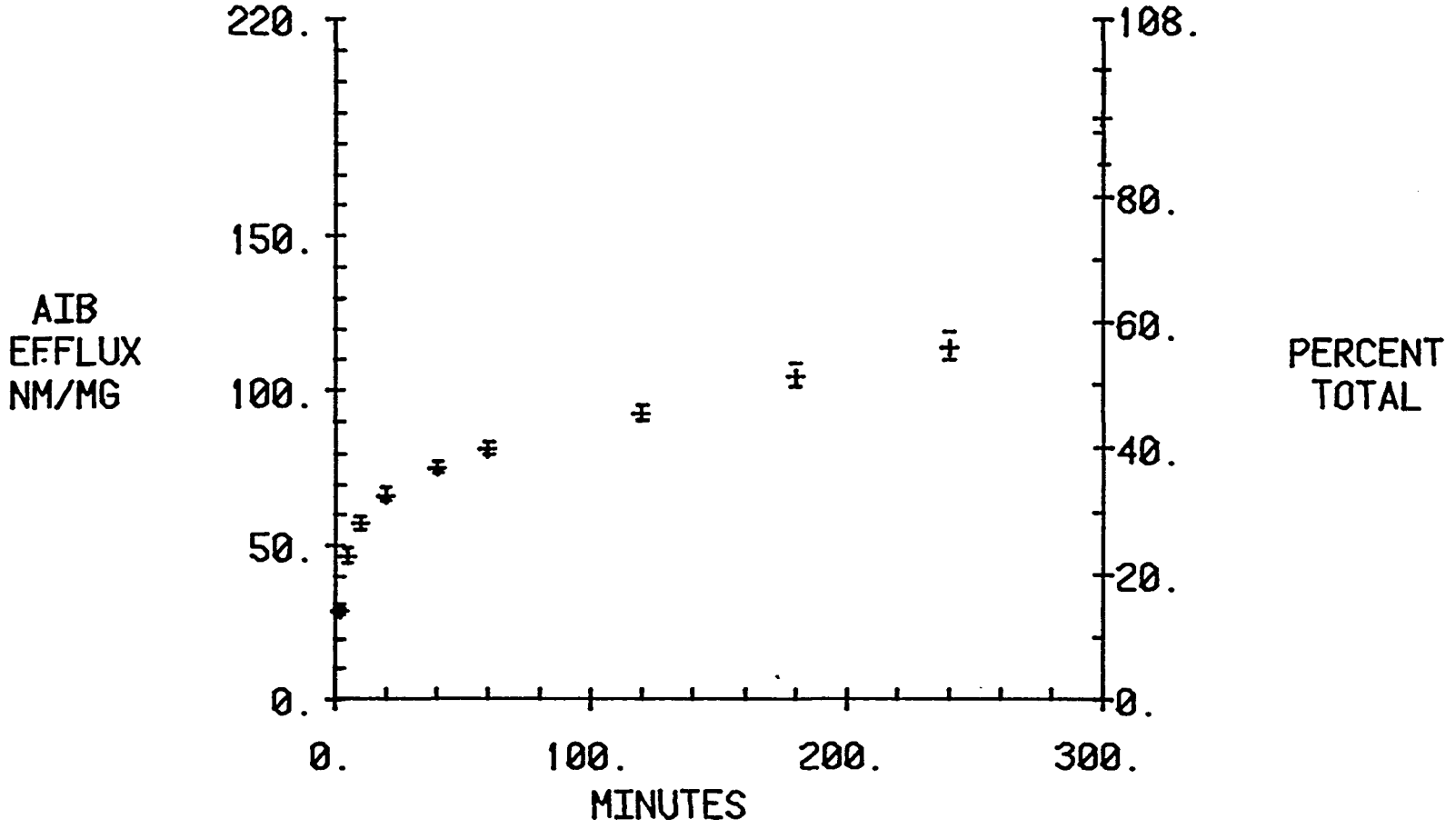
The time course of AIB exodus from corneas preloaded with this amino acid is shown in Fig. 6. It is apparent that there are two parts to this curve. An initial fast component and following that, a slower one. This is not surprising considering that the stroma, which is an extracellular space, makes up almost 90% of the volume of the cornea. Thus, it was possible to calculate the amount of amino acid present in the extra-space at equilibrium with the media, disallowing for any significant accumulation by components of the stroma. In the earlier section it was shown that the tissue:medium concentration in the stroma was not significantly different from 1.0. The amount of amino acid present was

Fig. 6. AIB efflux from the toad cornea

Corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation, corneas were placed in amino acid free Conway and moved serially to fresh Conway at the times indicated. Each point is the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

$k_1 = .0027\ min^{-1}$ $\quad T_{1/2} = 255$ min

$k_2 = .290\ min^{-1}$ $\quad T_{1/2} = 2.3$ min



\+ AIB efflux from the toad cornea

FIGURE 6

found to be about equivalent to that of the initial fast component of the efflux. The remaining amino acid, which leaves the cornea comparatively slowly, most likely corresponds to that present in the cellular compartment, predominantly the epithelium. In order to more clearly define the two components of the efflux curve, a set of equations that describe the transport of amino acids, according to the properties previously elucidated and described below were fitted to the parameters of the system using the DIFFEQ program available in the PROPHET Computer System.

A two compartment model was developed to describe the efflux of amino acids from the cornea. The model is as follows:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

1) amino acid are assumed to be present in the cornea in two compartments, a cellular compartment (A) and an extracellular one (B). After the initial preloading, the corneas are placed in an amino acid free media (C) and efflux is measured, so $k_2>0$.

2) amino acids cannot cross the epithelial surface and so must enter the bathing media from the cellular compartment through the stroma, and the stroma is not rate limiting ($k_2>k_1$).

The following equations were used to describe amino acid transport in the cornea:

$$dY_A/dt = -k_1 \times Y_A$$

$$dY_B/dt = k_1 \times Y_A - k_2 \times Y_B$$

$$dY_C/dt = k_2 \times Y_B$$

The fit of these equations to the experimental data was very good, having a P value <0.001.

The rate of exodus from the extracellular space, determined using

these equations, k_2 in Fig. 6, is equal to 0.2684 min^{-1} and therefore it is virtually empty within 10 minutes ($t_{1/2}$ = ln 2 / k). K_1 is 0.0027 min^{-1} which is much slower than k_2, and the $t_{1/2}$ for emptying this compartment is about 4 hours. This method of analysis is also useful for comparing rates of efflux under various conditions, as will be seen in the following section.

In an attempt to determine if the process of AIB efflux was saturable, corneas were preloaded with concentrations up to 20 mM. It was not possible demonstrate saturability even at intracellular concentrations estimated to be 30 mM (Fig. 7).

Removal of Na from the external bathing medium slightly increased the rate of exodus of AIB from preloaded corneas but the amount of amino acid that left either of the corneal pairs by the end of the experiment was not significantly different (Fig. 8). When corneas preloaded with AIB were exposed to ouabain (10^{-4}M) there was an immediate and dramatic rise in efflux (Fig. 9). In order to determine if this latter effect is due to an increase in intracellular Na, resulting from ouabain inhibition of Na-K-ATPase, Na was replaced by choline in the outside bathing solution. The removal of Na completely blocked the stimulation of efflux by ouabain (Fig. 10). The stimulatory effect of ouabain was not seen when the Na concentration in the external media was less than 24 mM. This Na concentration allows for the maximal response to the drug whereas lower concentrations completely blocked the stimulation of efflux. Lithium also could not replace Na in these experiments.

Metabolic inhibitors (Fig. 11), iodoacetate and cyanide, and removal of K from the external bathing media (Fig. 12) also increased the efflux

Fig. 7. Rates of AIB efflux from the cornea vs. tissue concentration

Corneas were preloaded with 0.1 to 25mM AIB in Conway solution containing 0.2 μCi/ml ^{14}C-AIB for 3 hours. The tissues were then placed in amino acid free Conway solution and moved serially to fresh solutions at the same time intervals as those indicated in Fig. 6. The rates of exodus were calculated from 60-240 minutes, so that the rates would reflect that from the cells, and not from the extracellular space. The concentration of AIB in the whole tissue was calculated from the sum of all the efflux periods and the corneal extract (see Methods). The values are the means of 6 experiments.

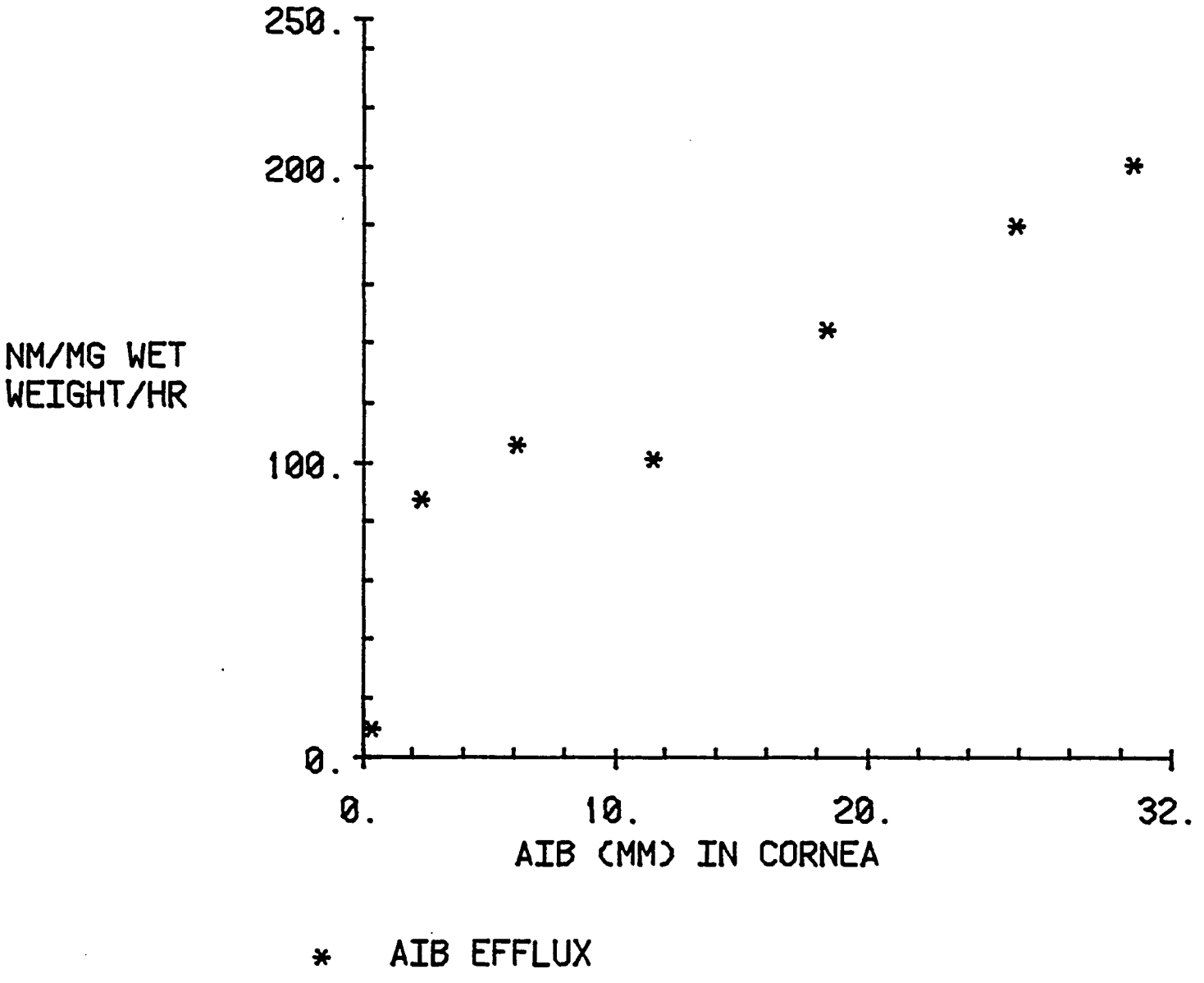

250.
200.
100.
0.
NM/MG WET
WEIGHT/HR
0.
10.
20.
32.
AIB (MM) IN CORNEA
* AIB EFFLUX

Fig. 8. Effect of removal of Na from the media on AIB efflux.

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation corneas were placed in amino acid-free Conway and moved serially at the times indicated to fresh solutions. From 60 minutes to the termination of the experiment Na was replaced by choline in the solution bathing the experimental corneas. The values are the mean ± S.E. of 6 experiments. Rate constants were derived as described in the text.

	K_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0019	363	146±8	ns
Na-free	.0028	246	180±19	

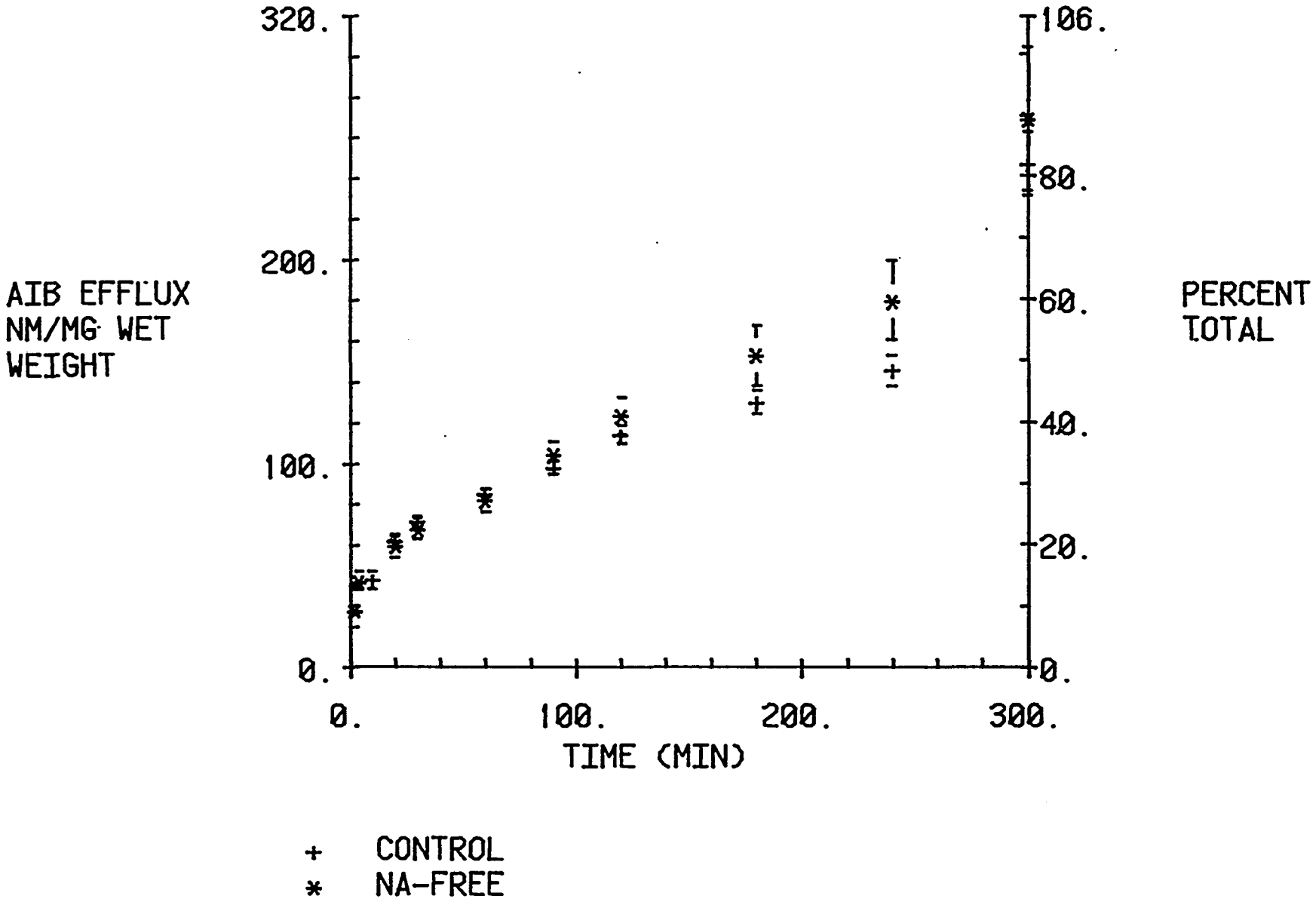
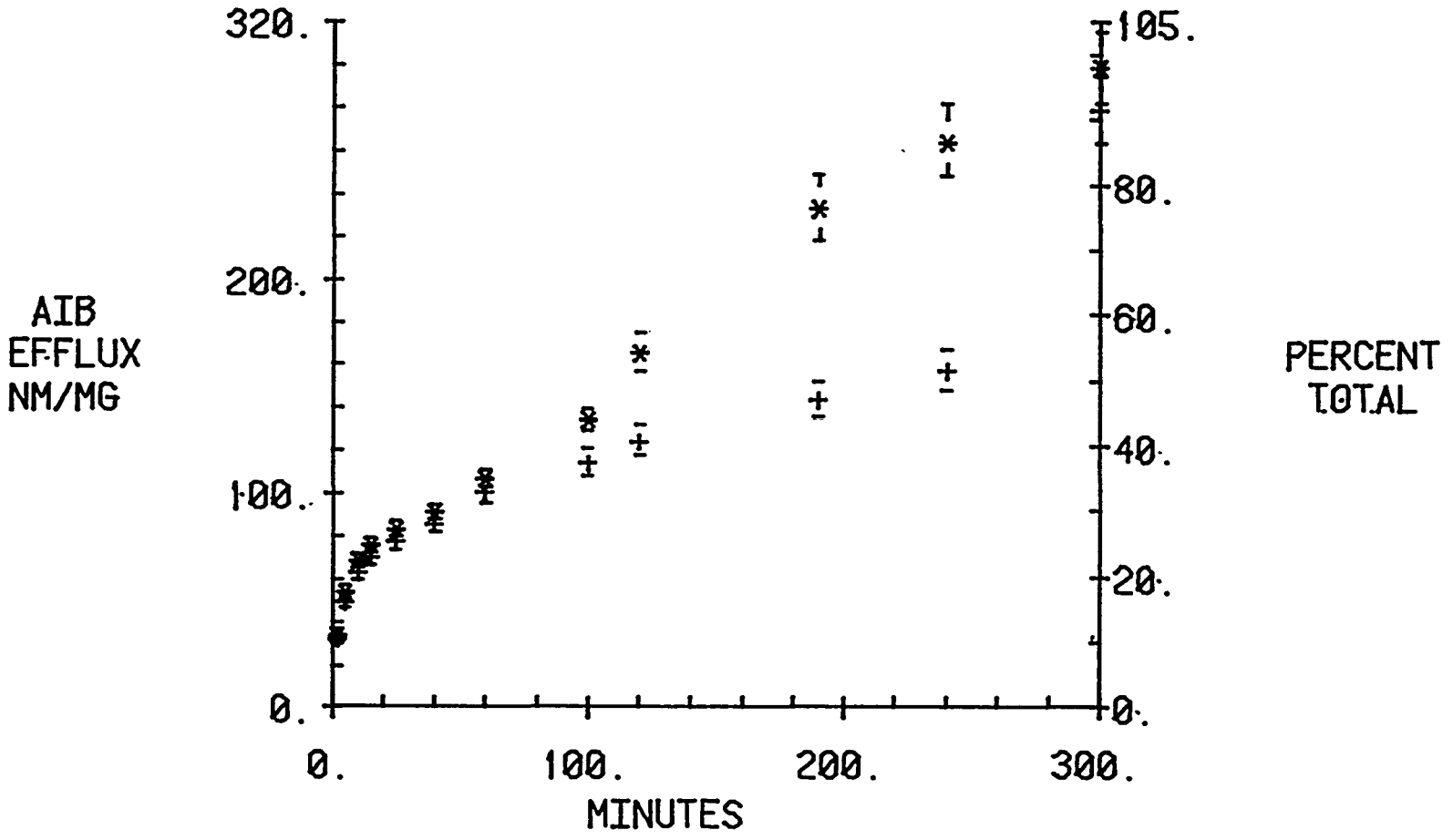

AIB EFFLUX
NM/MG WET
WEIGHT
320.
200.
100.
0.
106.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ CONTROL
* NA-FREE

Fig. 9. Effect of ouabain on AIB efflux from the cornea.

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation they were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 60 minutes to the termination of the experiment, ouabain (10^{-4} M) was present in the solution bathing the experimental corneas. Each point is the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0025	276	159$\pm$10	<.001
Ouabain	.0063	109	253$\pm$16	

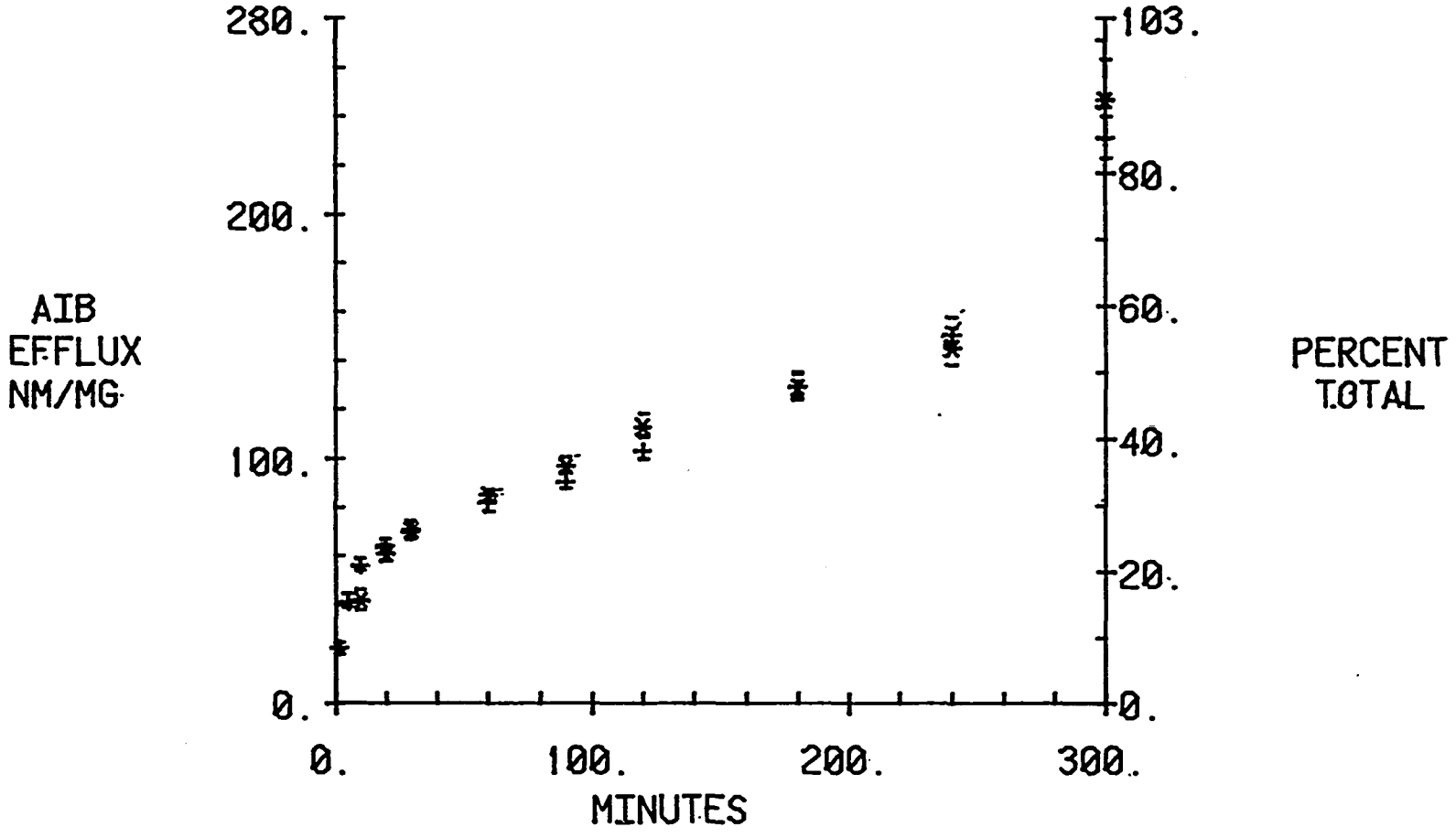


\+ Control
* Ouabain (10⁻⁴ M)

Fig. 10. Effect of ouabain in Na-free media on AIB efflux from the toad cornea

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation the corneas were placed in amino acid free Conway and moved serially at the times indicated to fresh solutions. From 60 minutes to the termination of the experiment Na was replaced by choline in the solutions bathing both control and experimental corneas and ouabain (10^{-4} M) was present in the experimental solutions. Each point represents the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Na-free	.0021	328	147$\pm$6	ns
Na-free +ouabain	.0020	345	156$\pm$7	



\+ NA-FREE

* NA-FREE + OUABAIN (10-4 M)

Fig. 11. Effect of iodoacetate and cyanide on AIB efflux from the toad cornea.

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation period, the corneas were placed in amino acid free media and moved serially at the times indicated to fresh Conway solution. From 60 minutes to the termination of the experiment, 2 mM iodoacetate and 5 mM cyanide were present in the solutions bathing the experimental corneas. Each point is the mean ± S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0013	530	126±17	<.05
Inhibitors	.0027	255	181±17	

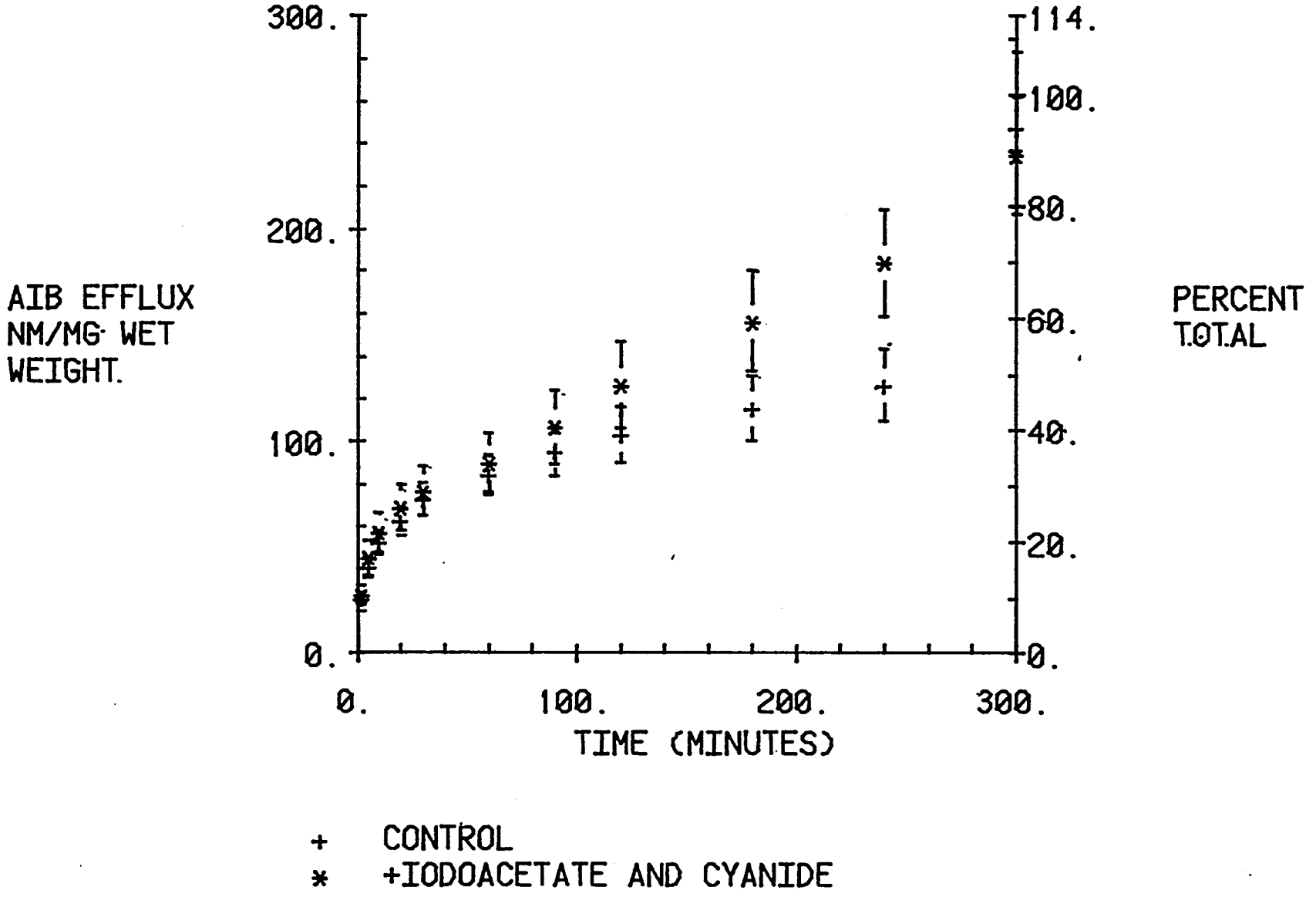
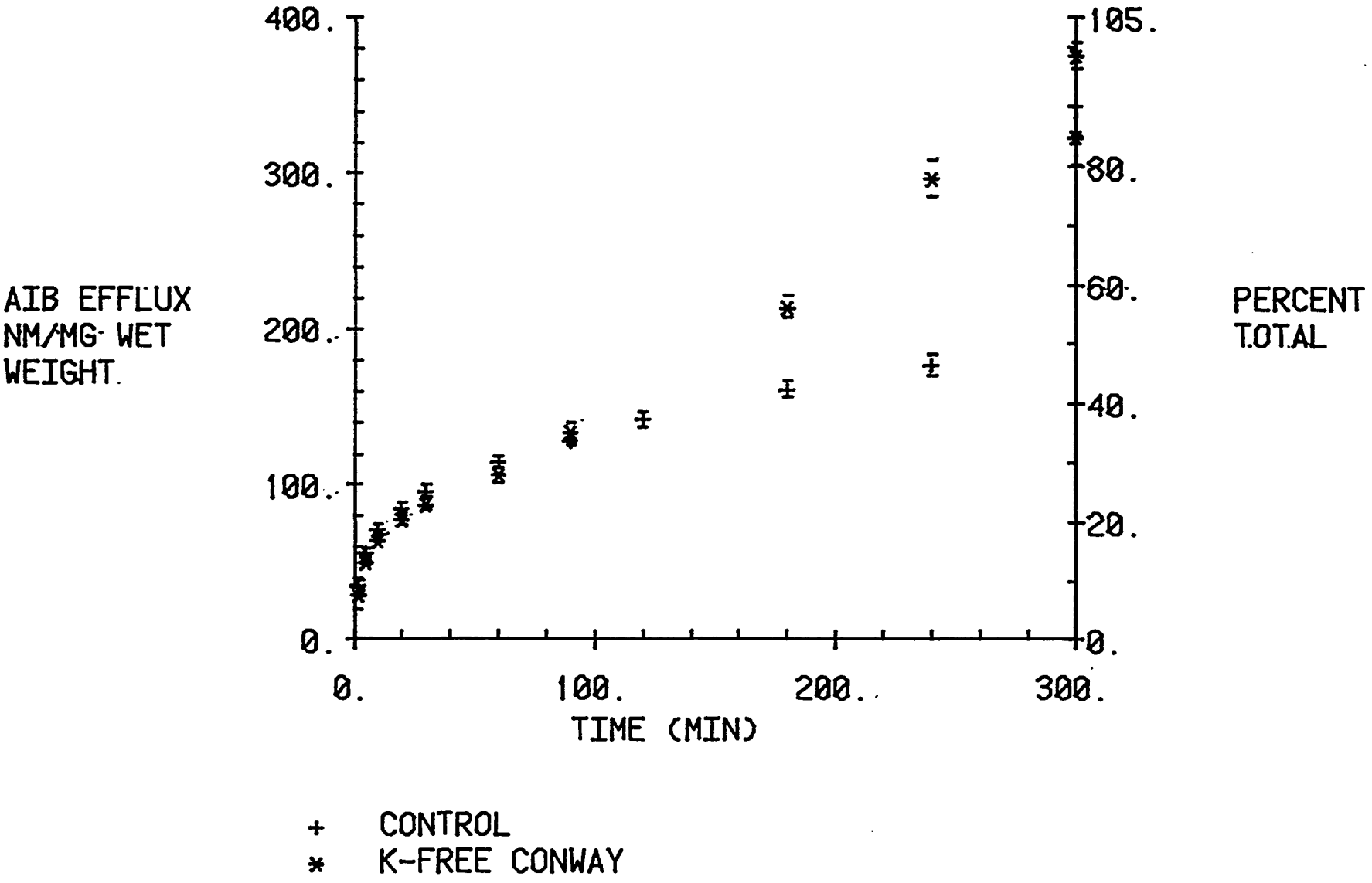

AIB EFFLUX
NM/MG WET
WEIGHT.
300.
200.
100.
0.
PERCENT
TOTAL
114.
100.
80.
60.
40.
20.
0.
0.
100.
200.
300.
TIME (MINUTES)
+ CONTROL
* +IODOACETATE AND CYANIDE

Fig. 12. Effects of removal of potassium from the external bathing solution on AIB efflux from the cornea

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation they were placed in amino acid free Conway and moved serially, at the times indicated, to fresh Conway. From 60 minutes to the termination of the experiment, the solution bathing the experimental corneas contained no potassium. Each point is the mean ± S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0036	191	177±7	<.001
K-free	.0074	93	286±11	


AIB EFFLUX
NM/MG WET
WEIGHT
400.
300.
200.
100.
0.
105.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ CONTROL
* K-FREE CONWAY

of preloaded AIB from the cornea. These responses were also abolished in the absence of external Na (Figs. 13 and 14).

Amphotericin B, which increases the permeability of the tear surface of the cornea to Na, stimulates AIB efflux (Fig. 15). However, removal of external Na does not reduce the effect of this drug (Fig. 16). The possibility that the 'pores' known to be created by the amphotericin B (Candia, Bentley & Cook, 1974) on the anterior surface of the cornea allow for passage of the preloaded AIB out of the cornea was examined. Corneas preloaded with AIB were mounted in Ussing-type chambers and amphotericin B (12.5 ug/ml) was added to the anterior surface under electrically short-circuited conditions. No efflux of AIB into the anterior bathing solution was observed.

All of the conditions just described are expected to have a common result, an increase in intracellular Na concentration. Such a change in ionic composition could result in cell swelling. In order to determine if such a change could account for the observed increase in efflux, the corneas were incubated in a solution in which the osmolarity was reduced by about 20%, by decreasing the Na concentration. Under these circumstances there was no change in the rate of AIB exodus from the cornea (Fig. 17).

Mechanisms for the exchange of internal for external amino acids have been proposed in non-ocular tissues. In order to determine if an exchange diffusion mechanism exists in the cornea, various amino acids were added to the media incubating corneas that had been preloaded with AIB. Addition of 10 mM alanine to the external bathing solution caused

Fig. 13. Effects of metabolic inhibitors in Na-free media on AIB efflux from the toad cornea

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation the corneas were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 60 minutes to the termination of the experiment the Na was replaced with choline in both the control and the experimental solutions, and 2 mM iodoacetate and 5 mM cyanide were present in the experimental solution. The values are the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Na-free	.0028	246	179±19	ns
Na-free +inhibitors	.0024	287	181±8	

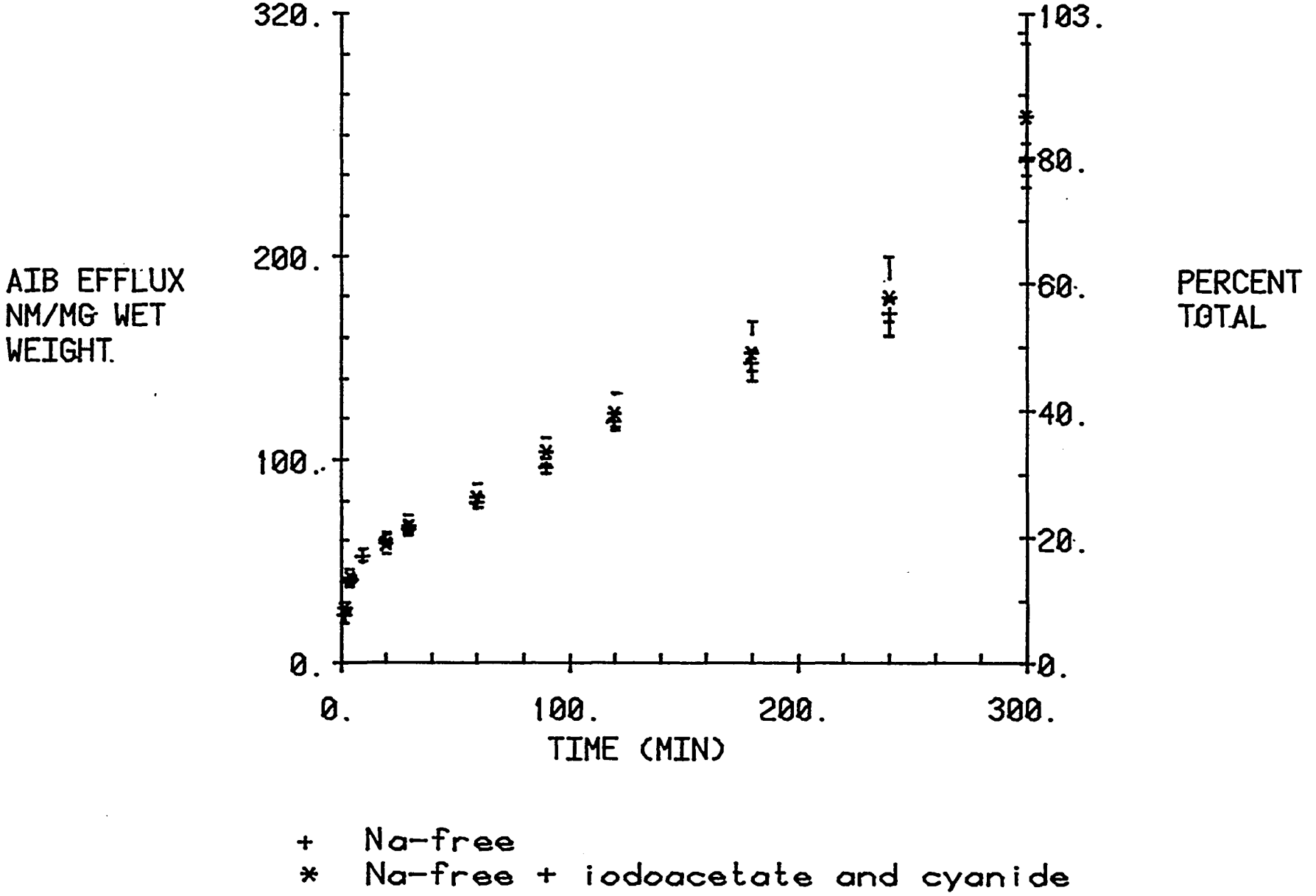

AIB EFFLUX
NM/MG WET
WEIGHT.
320.
200.
100.
0.
103.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ Na-free
* Na-free + iodoacetate and cyanide

Fig. 14. Effect of removal of Na and K on AIB efflux from the toad cornea

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation the corneas were placed in amino acid free Conway and moved serially to fresh solution at the times indicated. From 60 minutes to the termination of the experiment, K was removed from the control solutions and Na and K were removed from the experimental solutions. The values are the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Na-K-free	.0037	186	204$\pm$13	<.001
K-free	.0068	101	279$\pm$9	

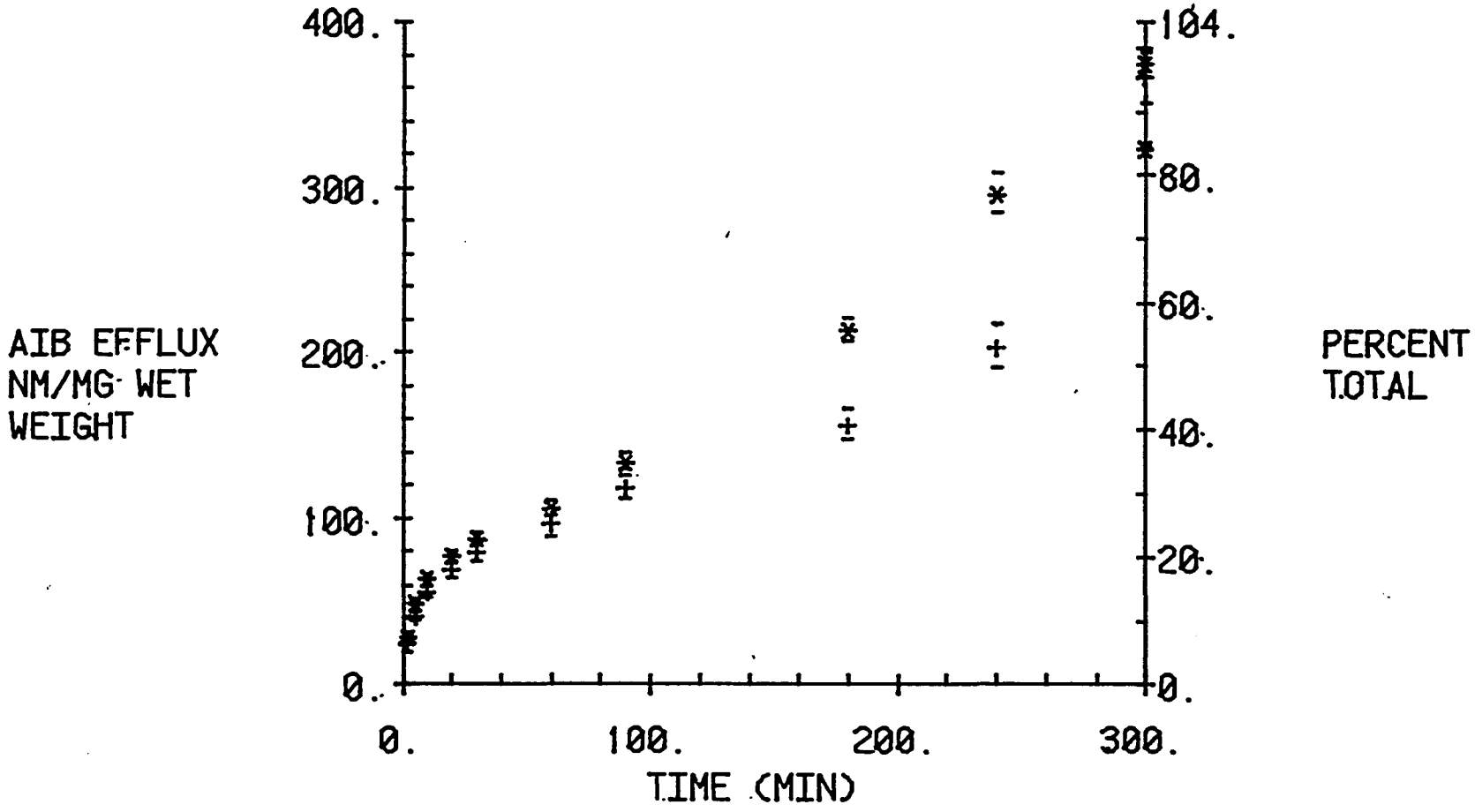
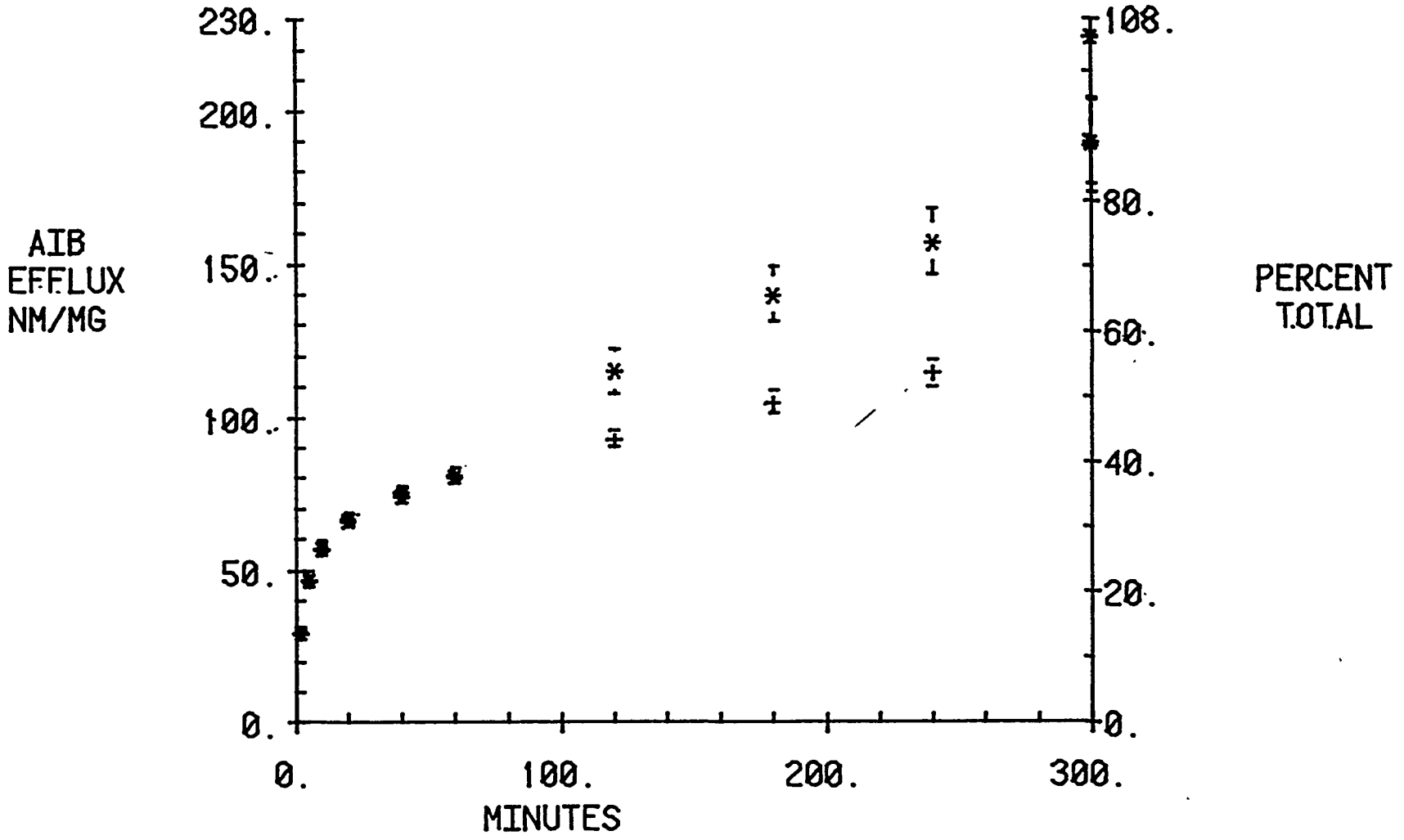

AIB EFFLUX
NM/MG WET
WEIGHT
400.
300.
200.
100.
0.
104.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ NA-K-FREE CONWAY
* K-FREE CONWAY

Fig. 15. Effect of amphotericin B on AIB efflux from the toad cornea.

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation they were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 60 minutes to the termination of the experiment amphotericin B (12.5 ug/ml) was present in the solutions bathing the experimental corneas. Each value is the mean ± S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0027	255	120±7	<.05
Amphotericin B	.0068	104	155±15	



\+ Control

* Amphotericin B (12.5 µg/ml)

Fig. 16. Effects of amphotericin B in Na-free Conway on AIB efflux from the toad cornea.

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation the corneas were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 60 minutes to the termination of the experiment, Na was replaced by choline in both the control and experimental solutions and amphotericin B (12.5 ug/ml) was present in the experimental solutions. The values are the mean ± S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Na-free	.0027	255	184±9	<.001
Na-free +amphotericin B	.0067	103	280±16	

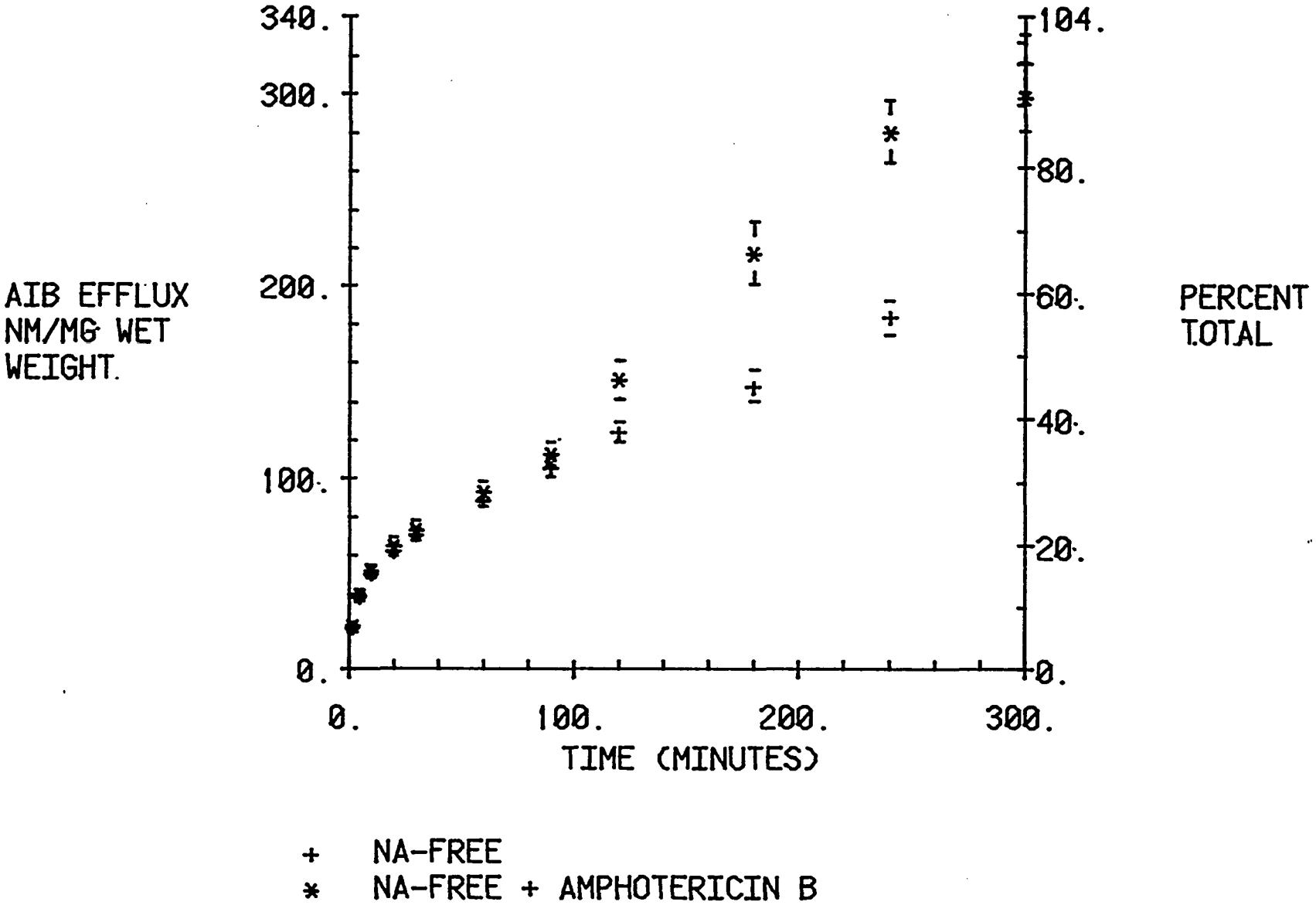
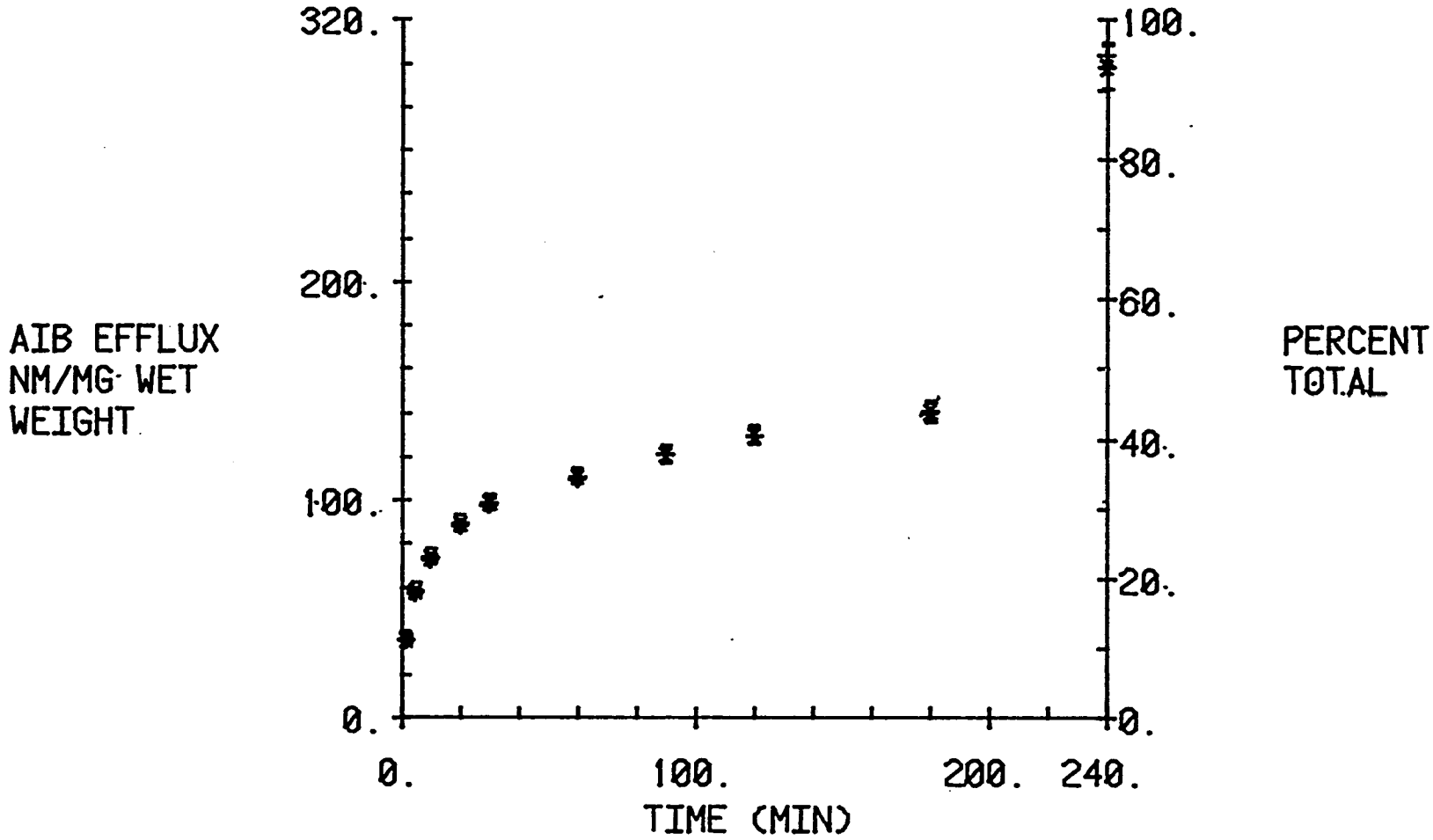

AIB EFFLUX
NM/MG WET
WEIGHT.
340.
300.
200.
100.
0.
104.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MINUTES)
+ NA-FREE
* NA-FREE + AMPHOTERICIN B

Fig. 17. Effects of reduced osmolarity on AIB efflux from the toad cornea.

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB. After this preincubation the corneas were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 60 minutes to the termination of the experiment, the osmolarity was reduced (by 40 mosmol) in the solutions bathing the experimental corneas. The values are the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 180 min nmoles/mg	P for difference
Control	.0030	230	102±3	ns
Reduced osmolarity	.0030	230	102±2	



\+ CONTROL.
* OSMOLARITY REDUCED BY 40 MOSMOL

an immediate increase in the efflux of AIB ('heteroexchange') (Fig. 18). This was not due to a change in osmolarity since an increase in efflux was not observed in control corneas to which 10 mM mannitol was added. Externally added AIB also increased the efflux ('homoexchange') (Fig.19). However, addition of leucine was without effect (Fig. 20). Replacement of Na with choline inhibited AIB stimulated efflux (Fig. 21). It also inhibited alanine stimulated efflux. However, homo- and heteroexchange still occurred when the external Na concentration was as low as 17 mM. Lithium could not substitute for Na in these experiments.

In the intestine, which exhibits a transmural transport of both amino acids and Na, the addition of amino acids to the cis-side of the membrane results in a rise in short-circuit current (I_{sc}) which reflects an increase in Na transport (Schultz & Zalusky, 1965). The movement of Na and the amino acid appeared to be coupled. The toad cornea like the bullfrog cornea (Zadunaisky, 1966) also exhibits a transmural P.D. and has a I_{sc} which is partly due to the net movement of Na from the tear to the aqueous side. Since 'homoexchange' and 'heteroexchange' are dependent on the presence of Na it was of interest to know if the Na were moving into, or out of the cells with the AIB during the exchange process. This could show up as a change in the I_{sc} or in ^{22}Na fluxes in either direction across the membrane. Addition of 10 mM AIB to the bathing solution on the aqueous side of corneas preloaded with AIB and short-circuited did not alter the I_{sc} nor the unidirectional fluxes of ^{22}Na (Table 15).

The removal of Ca and Mg from the incubation medium had no effect on AIB efflux, however, when EGTA (2 mM) was added to the Ca and Mg free

Fig. 18. Effect of addition of 10 mM alanine on AIB efflux from the toad cornea.

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. At the end of this preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway at the times indicated. From 60 minutes to the termination of the experiment, 10 mM alanine was present in the solution bathing the experimental corneas. Each point is the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0009	767	126$\pm$6	<.001
Alanine	.0034	202	206$\pm$12	

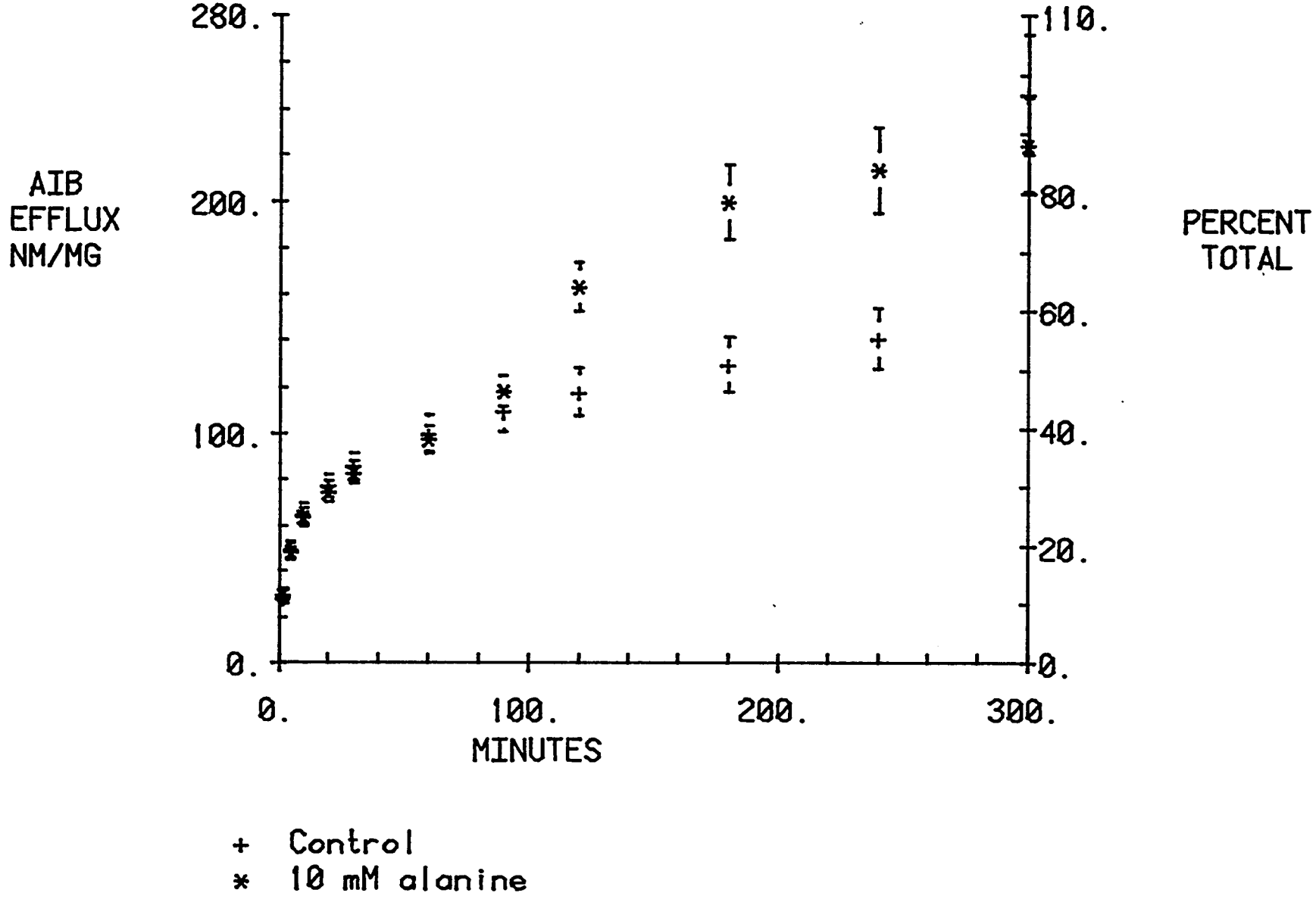

AIB
EFFLUX
NM/MG
280.
200.
100.
0.
110.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
MINUTES
+ Control
* 10 mM alanine

Fig. 19. Effect of addition of 10 mM AIB on AIB efflux from the toad cornea

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. At the end of this preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway at the times indicated. From 60 minutes to the termination of the experiment, 10 mM AIB was present in the solution bathing the experimental corneas. Each point is the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min	P for difference
Control	.0019	363	144$\pm$8	<.001
AIB	.0057	120	262$\pm$14	

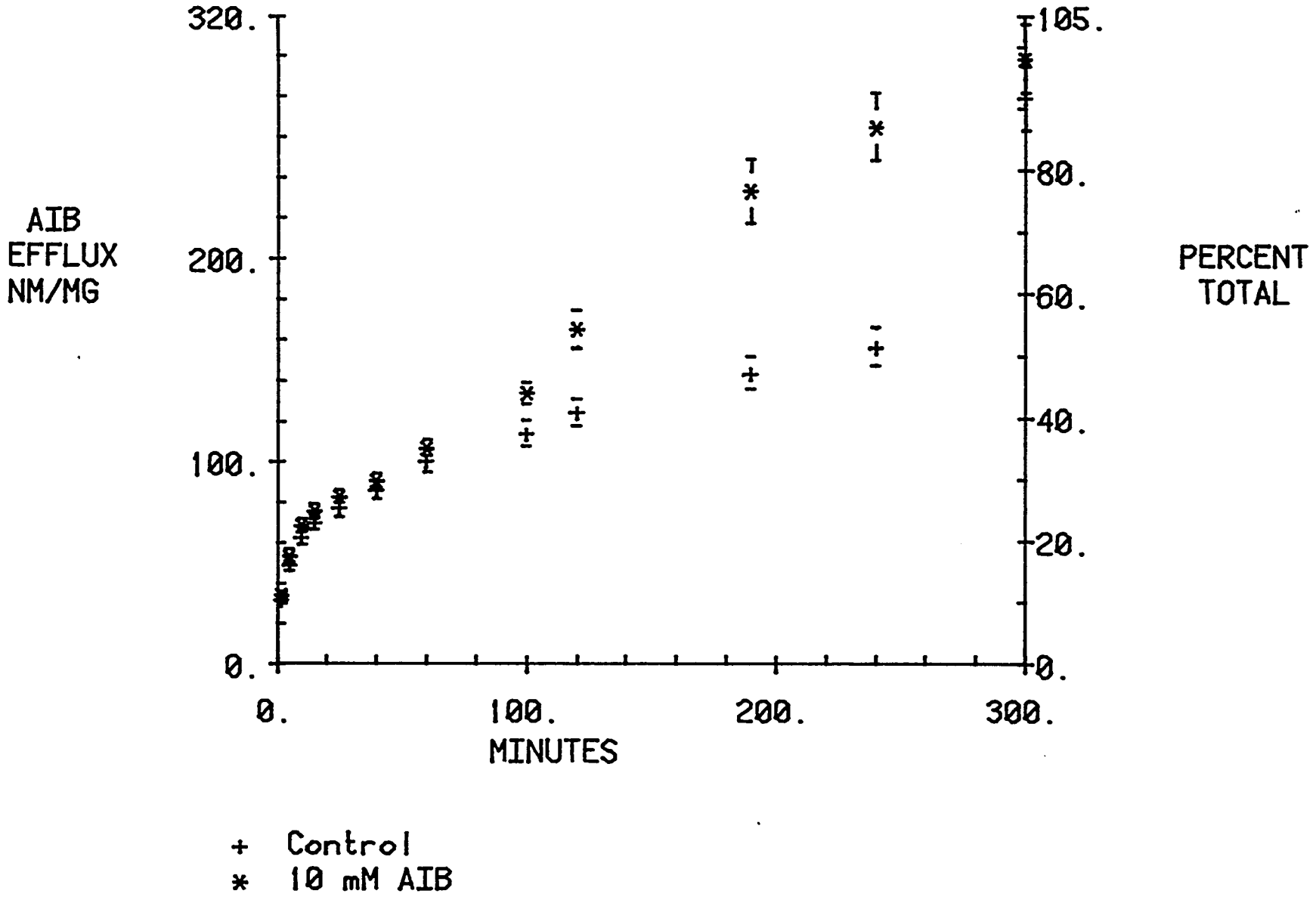

AIB
EFFLUX
NM/MG
320.
200.
100.
0.
105.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
MINUTES
+ Control
* 10 mM AIB

Fig. 20. Effect of addition of 10 mM leucine on AIB efflux from the toad cornea

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. At the end of this preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway at the times indicated. From 60 minutes to the termination of the experiment, 10 mM leucine was present in the solution bathing the experimental corneas. Each point is the mean ± S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Control	.0019	363	146±8	ns
Leucine	.0015	460	135±7	

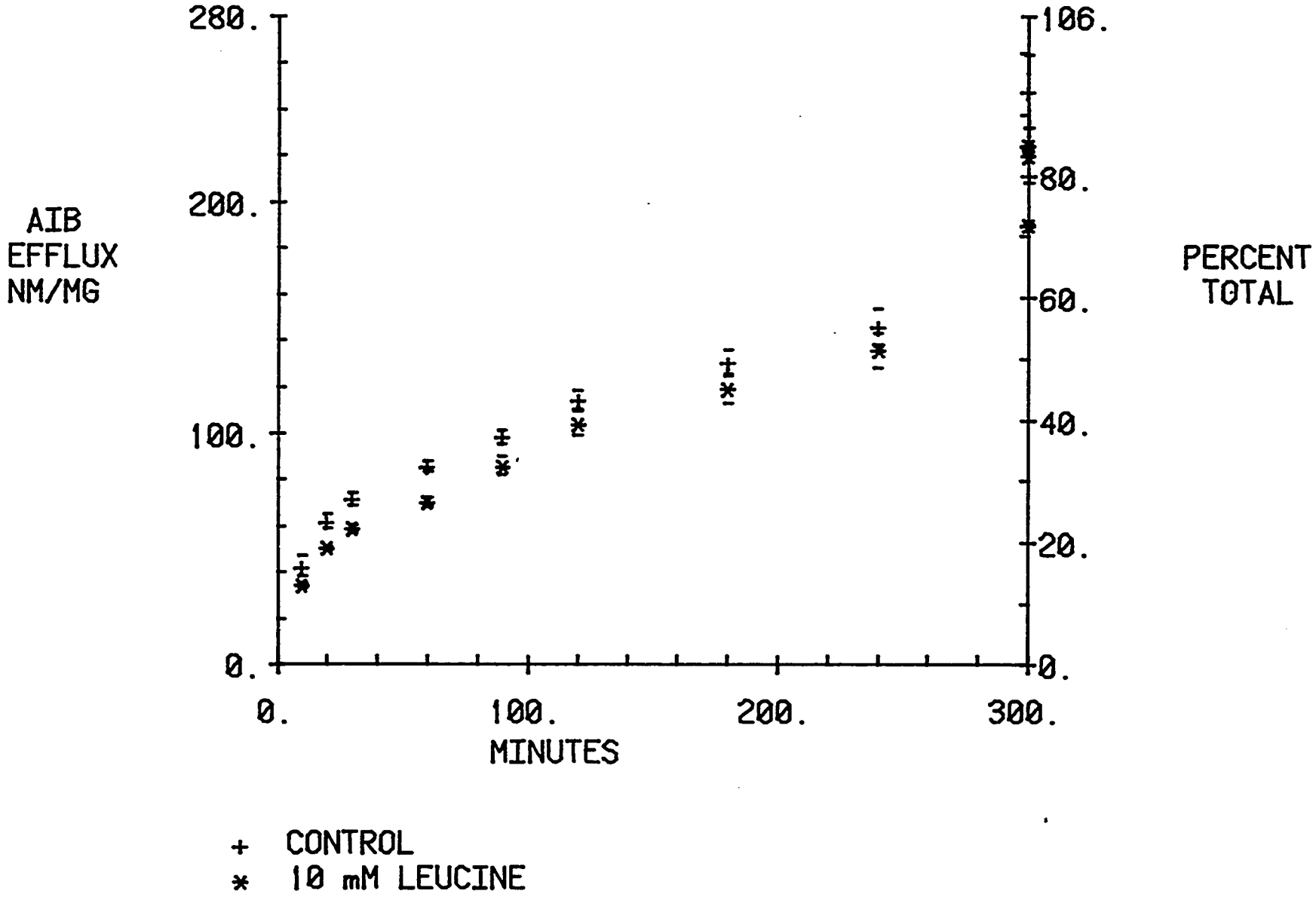

AIB
EFFLUX
NM/MG
280.
200.
100.
0.
106.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
MINUTES
+ CONTROL
* 10 mM LEUCINE

Fig. 21. Effect of addition of 10 mM AIB in Na-free Conway on AIB efflux from the toad cornea

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. At the end of this preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway at the times indicated. From 60 minutes to the termination of the experiment, Na was replaced by choline in the solutions bathing the control and the experimental corneas and 10 mM AIB was present in the solutions bathing the experimental corneas. Each point is the mean $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min nmoles/mg	P for difference
Na-free	.0021	328	161$\pm$6	ns
Na-free + AIB	.0024	287	150$\pm$8	

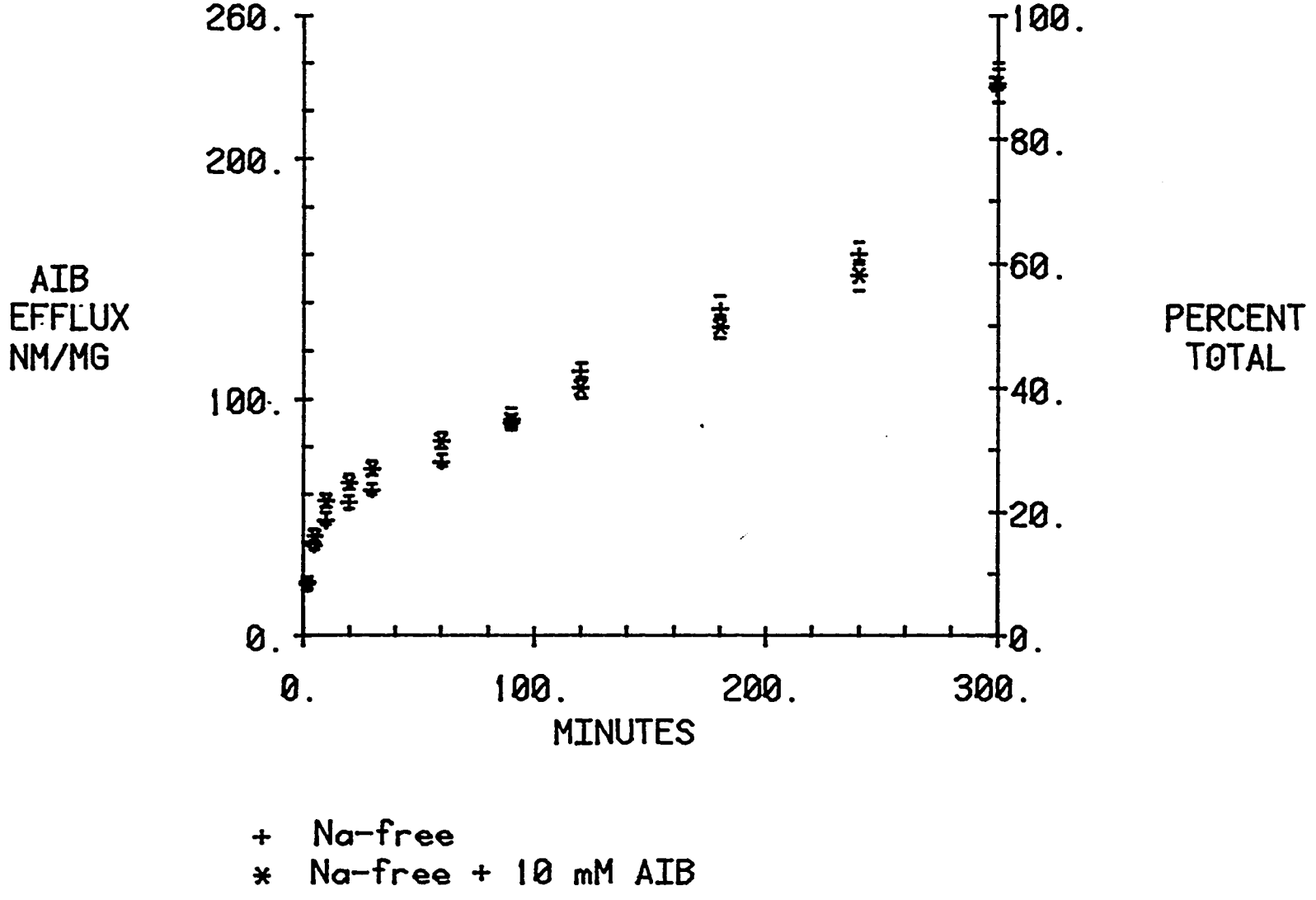

260.
200.
100.
0.
AIB
EFFLUX
NM/MG
100.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
MINUTES
+ Na-free
* Na-free + 10 mM AIB

Table 15. Effects of addition of 10 mM AIB on ^{22}Na fluxes and I_{sc} of the cornea

Time (min)	uA cm^{-2}	Influx	Efflux
60	14.6±4.2	0.44±0.05	0.35±0.02
90	14.0±4.2	0.42±0.02	0.37±0.06
120	13.6±4.1	0.45±0.04	0.37±0.03
150	13.8±4.2	0.49±0.06	0.33±0.07

Cornea preparations were mounted in Ussing-type chambers and preloaded with 0.1 mM AIB (endothelial bathing solution) for 3 hours. ^{22}Na fluxes were measured in both tear to aqueous (influx) and aqueous to tear (efflux) directions. Samples were taken from the trans bathing solution at 30 minute intervals. At 90 minutes 10 mM AIB was added to the aqueous side and 2 more samples were then taken. The results represent the mean ± S.E. of 5 experiments.

medium the rate of exodus increased (Fig. 22) and virtually all of the preloaded AIB had left the cornea by the end of 3 hours.

The pH dependence of the process of accumulation suggested that efflux and exchange mechanisms may also be altered by changes in the pH. Reducing the pH of the incubation medium to 7.4, however, had no effect on either the efflux or exchange of AIB in the cornea (Table 16).

While accumulation of amino acids in the cornea appears to occur predominantly in the epithelial cells, the possibility of a smaller uptake by the stromal keratocytes and/or endothelium was suggested from the results of the amino acid efflux experiments. Thus, when the epithelium was removed from the corneas before preloading with AIB, 80% of the accumulated amino acid leaves the cornea in the first hour, however a small but significant amount remained in the tissue and left more slowly. This portion could represent that present in the keratocytes or the endothelium. The efflux of this 'sequestered' amino acid could be stimulated upon incubation of the tissue with ouabain (Fig. 23).

It is clearly important to investigate the effect on AIB efflux of the various membrane probes that had been shown to alter its accumulation in the cornea. Trypsin greatly increased the efflux of AIB (Table 17). PCMPS had the same effect (Table 17). Since this latter compound binds to sulfhydfryl groups on the surface of the membrane, and Na-K-ATPase (a membrane enzyme) is susceptible to attack by sulfhydryl reagents it was possible that PCMPS' effect on efflux was like that of ouabain. If this was an ouabain-like effect, the elimination of

Fig. 22. AIB efflux from corneas incubated in Ca-free Conway with EGTA

Paired corneas were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway at the times indicated. The solution bathing the experimental corneas contained no calcium and had added EGTA (2 mM). The points are the means $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 180 min nmoles/mg	P for difference
Control	.0019	363	128±10	<.01
Ca-free +EGTA	.0100	69	239±23	

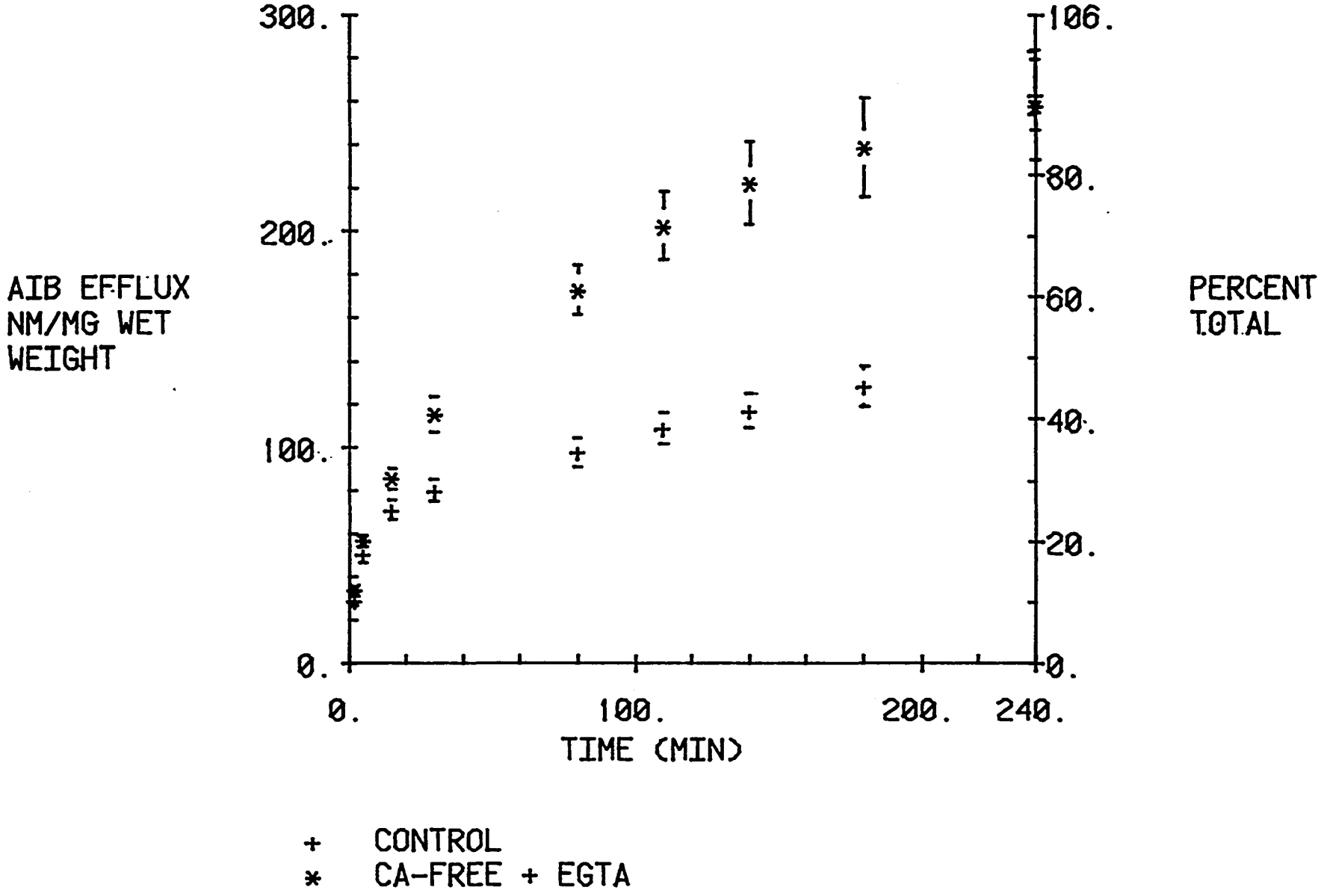

AIB EFFLUX
NM/MG WET
WEIGHT
300.
200.
100.
0.
106.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
240.
TIME (MIN)
+ CONTROL
* CA-FREE + EGTA

Table 16. Effect of pH on AIB efflux and exchange diffusion in the toad cornea

	$K_1(min^{-1})$	$T_{1/2}$(min)	Efflux at 180 min nmoles/mg	P for difference
a) Efflux				
pH 8.4	.0024	280	117±4	ns
pH 7.4	.0021	321	106±6	
b) Exchange				
pH 8.4	.0043	163	182±13	ns
pH 7.4	.0047	144	162±12	

Paired corneas were preloaded in Conway solution (pH 8.4) containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. At the end of this pre-incubation period one cornea of each pair was placed in amino acid free Conway solution (pH 8.4) and the other in Conway solution (pH 7.4) and moved serially to a fresh solution of the corresponding pH at the same times as indicated in Fig. 22. In (b) at 30 minutes 10 mM AIB was added to the external bathing media of all the corneas and was present in these solutions until the experiment was terminated. The rate constants were calculated as described in the text. The values for efflux are the mean ± S.E. of 6 experiments and represent the total amount of AIB (nM/mg wet weight) that has left the cornea by the end of the experiment.

Fig. 23. Effects of ouabain on AIB efflux from de-epithelialized corneas

Paired corneas from which the epithelium had been removed, were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway at the times indicated. From 60 minutes to the termination of the experiment, ouabain (10^{-4} M) was present in the solutions bathing the experimental corneas. The points are the means $\pm$ S.E. of 6 experiments. Rate constants were derived as described in the text.

	k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 240 min	P for difference
Control	.00022	3136	81$\pm$5	<.05
Ouabain	.0016	431	99$\pm$4	

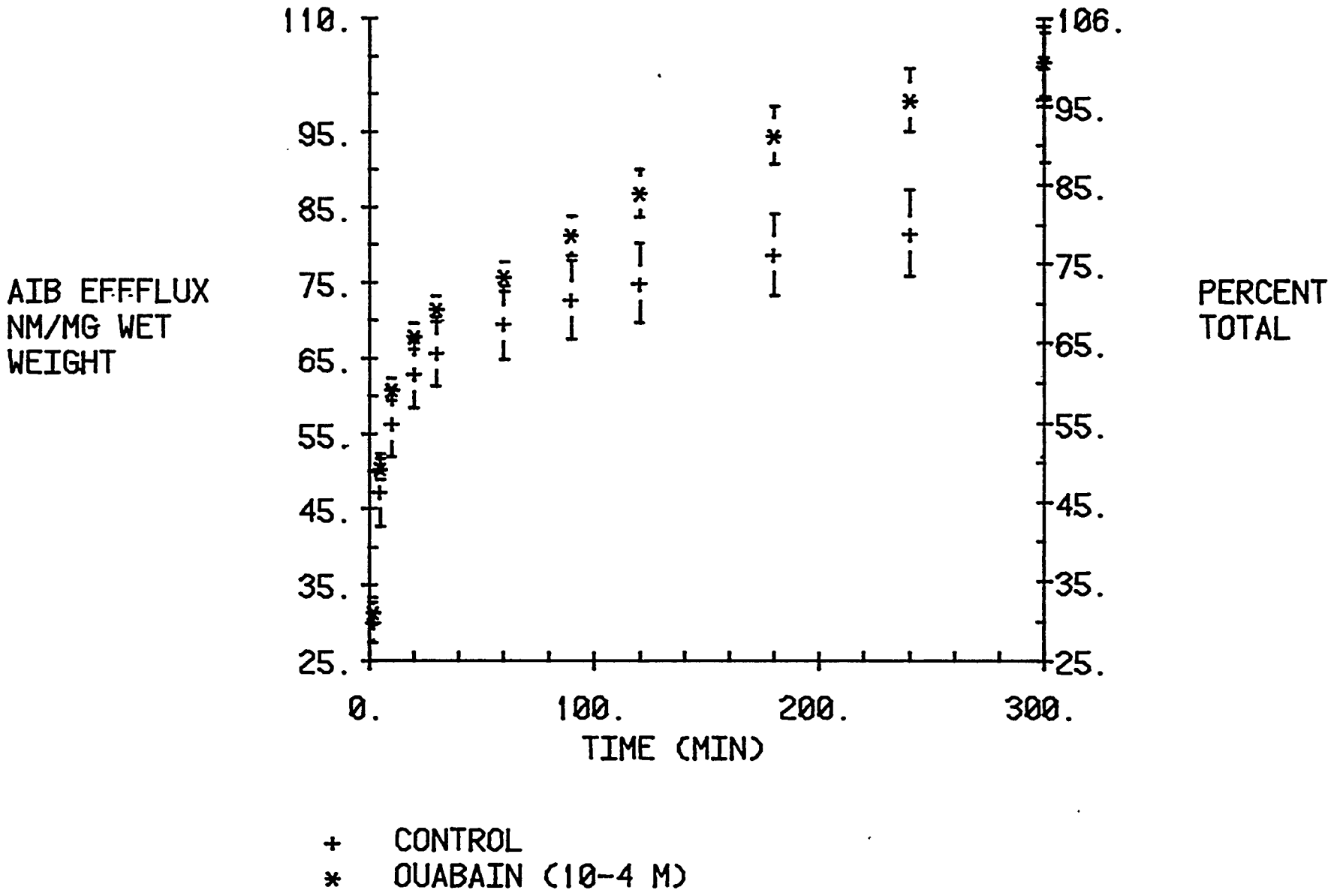

AIB EFFFLUX
NM/MG WET
WEIGHT
110.
95.
85.
75.
65.
55.
45.
35.
25.
106.
95.
85.
75.
65.
55.
45.
35.
25.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ CONTROL
* OUABAIN (10-4 M)

Table 17. Effects of membrane probes on AIB efflux from the cornea

		k_1 (min^{-1})	$T_{1/2}$ (min)	Efflux at 180 min nmoles/mg	P for difference
Trypsin (100 ug/ml)	a)	.0012	575	143±17	<.001
	b)	.0039	178	250±10	
PCMPS (10^{-4} M)	a)	.0024	280	191±4	<.001
	b)	.0051	135	249±21	
PCMPS (10^{-4} M) in Na free Conway	a)	.0027	255	178±4	ns
	b)	.0031	222	187±12	

Paired corneas were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. After this preincubation, the corneas were placed in amino acid free media and moved serially to fresh solutions at the same times as indicated in Fig. 23. Efflux from the control corneas is presented in row a. The trypsin or PCMPS was added to the experimental corneas (b) 30 minutes after efflux measurements were commenced. Rate constants were derived as described in the text. The efflux values are the mean ± S.E. of 6 experiments and represent the total amount of AIB (nM/mg wet weight) that has left the cornea by the end of the experiment.

Na from the outside bathing solution would also be expected to inhibit PCMPS' effect. This interaction was observed (Table 17).

The effect of the disulfonic stilbene, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid (DIDS) on efflux was also studied. Although DIDS had a marked effect on AIB accumulation it had no effect on either efflux of AIB or exchange diffusion (Table 18).

Table 18. Effect of DIDS on the efflux and exchange diffusion of AIB in the toad cornea

	$K_1(min^{-1})$	$T_{1/2}(min)$	Efflux at 240 min nmoles/mg	P for difference
A. Efflux				
Control	.0020	345	106±5	ns
DIDS	.0017	431	92±6	
B. Exchange				
Control	.0047	144	187±12	ns
DIDS	.0051	135	176±14	

Paired corneas were preloaded in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. One cornea of each pair was treated as the control, the other as the experimental. After the preincubation period the corneas were placed in amino acid free Conway solution and moved serially to fresh Conway solution at the same times as indicated in Fig. 23. 10 mM AIB was added to the efflux solutions at 30 minutes to all corneas (B) and was present in these solutions until the termination of the experiment. 6 pairs of corneas were used in each experiment. The rates of efflux of AIB were determined as described in the text. The concentration of DIDS in these experiments was $5x10^{-3}$ M. The efflux values are the total amount of AIB (nmoles/mg wet weight) that has left the cornea by the end of the experiment. These values are the mean ± S.E. of 6 experiments.

4. Amino acid uptake in the lens.

AIB uptake in the lens is linear up to 24 hours (Fig. 24) and is saturable at concentrations greater than 3 mM (Fig. 25). This amino acid is accumulated by the lens to concentrations up to 15-times that in the bathing media.

During the course of the studies of amino acid transport in the lens it was found that the results expressed as tissue to medium concentration ratio is dependent on the size of the organ. For example, the smaller lenses had a higher tissue:medium concentration than larger lenses when both were incubated for the same time period (Table 19). When corrected for the surface area, assuming the lens is spherical, it was found that lenses of all sizes had similar transporting activity. With this observation in mind lenses of approximately the same size were used in each experiment.

As in the study of amino acid accumulation in the cornea, AIB uptake is significantly higher at pH 8.4 than at pH 7.4 (Table 20). In order to determine if metabolic energy is necessary for amino acid accumulation in the lens, the inhibitors, iodoacetate and cyanide were added to the bathing media. The presence of these inhibitors decreased the accumulation of AIB (Table 21). Accumulation, however, was not completely inhibited after 2 hours incubation, but it was abolished after 6 hours.

The question of the abilities of the various types of cells in the lens to accumulate amino acids has not been resolved. Three techniques were employed in order to provide an answer in the amphibian lens. The first procedure was to incubate paired lenses for 6 hours in Conway

Fig. 24. Time course of AIB uptake in the toad lens.

Lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for the times indicated. Each point is the mean $\pm$ S.E. of 6 experiments.

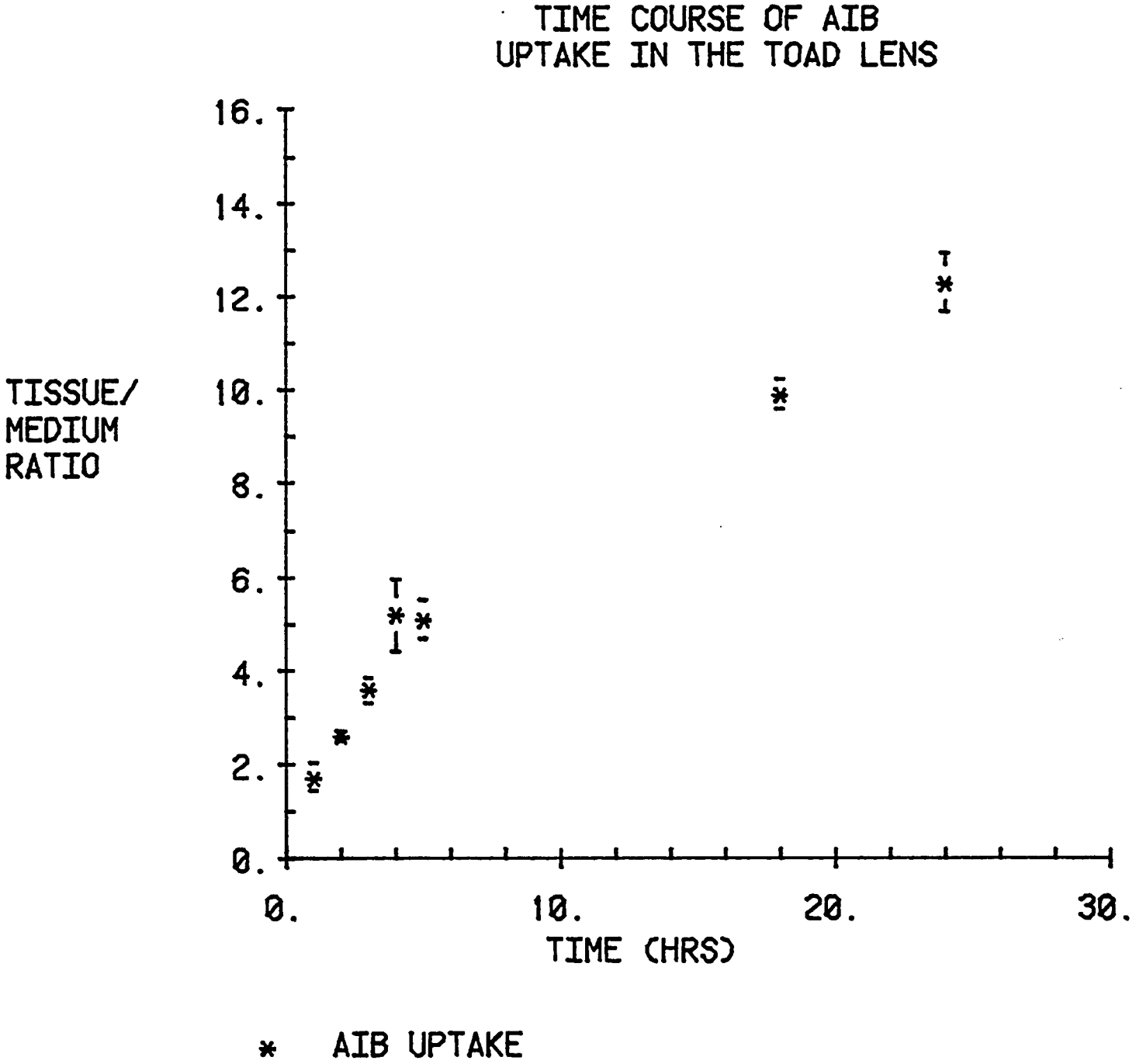

TIME COURSE OF AIB
UPTAKE IN THE TOAD LENS
TISSUE/
MEDIUM
RATIO
16.
14.
12.
10.
8.
6.
4.
2.
0.
0.
10.
20.
30.
TIME (HRS)
* AIB UPTAKE

Fig. 25. Saturation of AIB uptake in the toad lens.

Lenses were incubated in Conway solution with the concentration of AIB as indicated and 0.2 μCi/ml ^{14}C-AIB for 6 hours. Non-linear regression analysis was used to fit the data to the curve defined by Michaelis-Menton kinetics:

$$v = \frac{V_{max} \times [S]}{[S] + k_m}$$

This was done using a program available on the PROPHET computer system. Each value represents the mean $\pm$ S.E. of 6 experiments.

V_{max} = 2.32 nmoles/mg wet weight/hr

K_m = 1.24 mM

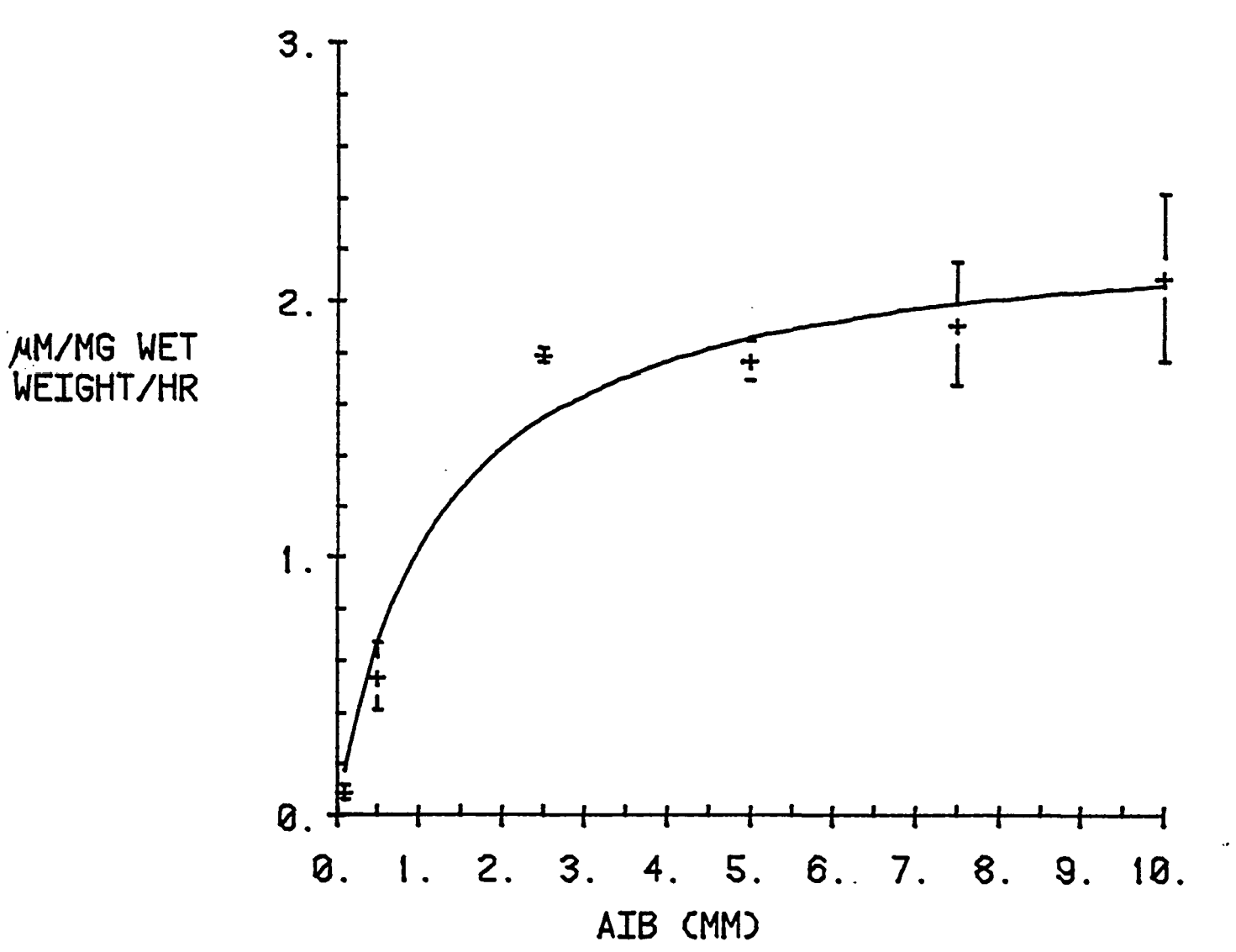

SATURATION OF
AIB UPTAKE IN THE TOAD LENS
µM/MG WET
WEIGHT/HR
3.
2.
1.
0.
0. 1. 2. 3. 4. 5. 6. 7. 8. 9. 10.
AIB (MM)

Table 19. The effect of differences in size on AIB accumulation by the lens

Lens size	Average weight	AIB (tissue:medium concentration)	umoles mm^{-2}*mg wet $weight^{-1}$
<150 mg	123.9 (4)	1.95 ± 0.31	11.1 ± 2.2
150-250 mg	193.0 (4)	1.18 ± 0.15	11.2 ± 0.7
>250 mg	281.0 (7)	0.80 ± 0.06	12.5 ± 0.6

Lenses of varying sizes were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 45 min. The values above represent the mean ± S.E. of the number in parenthesis.

*Surface area calculated on the assumption that the lens is a sphere.

Table 20. Effect of pH on AIB uptake by the lens

	AIB (tissue:medium concentration	P for difference
pH 7.4	1.53 ± 0.16	<.01
pH 8.4	2.65 ± 0.30	

Paired lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. pH 7.4 was obtained by bubbling the Conway solution with 95% O_2 and 5% CO_2 throughout the experiment. The values represent the mean ± S.E. of 6 experiments.

Table 21. The effect of metabolic inhibitors on AIB accumulation in the toad lens

	Incubation time (hrs)	AIB (tissue:medium concentration) Control	Experimental
Iodoacetate (2mM) and cyanide (5mM)	2	2.27±0.33	1.67±0.15
	6	4.71±0.23	0.54±0.04

Paired lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for the time periods indicated. One lens of each pair was treated as the control, the other as the experimental. The numbers above represent the mean ± S.E. of 6 experiments.

solution containing AIB, then remove them from the incubation solution and cut them into sections and determine the amount of amino acid in each section. The highest concentration of AIB was found in the anterior and posterior sections (Table 22). It has been postulated (Kinsey & Reddy, 1965) that the epithelium is responsible for the accumulation of amino acids in the intact lens; in order to test this hypothesis the ability of the various layers of the lens tissue to accumulate AIB was measured. Removal of the capsule and the epithelium does not abolish the ability of the lens to accumulate AIB against a concentration gradient, although the uptake is reduced. The nucleus is relatively impermeant to AIB since the AIB did not even reach a tissue:medium concentration of 1.0 in this portion of the lens (Table 23). The relative ability of the anterior and posterior surfaces to accumulate AIB was also measured. Lenses were mounted in Ussing-type chambers in order to isolate the anterior and posterior surfaces. Uptake across the anterior surface was significantly greater than uptake across the posterior surface (Table 24). It should also be noted that in these experiments the lenses were incubated for 21 hours in the chambers. This time was necessary in order to obtain significantly higher levels of amino acids in the lens than in the incubation medium. The reason for the reduced transporting activity of the lenses mounted in chambers is not known but it was a consistent observation. The lens appears to adequately maintain its metabolic acitivity over this time period, as shown by the fact that the translenticular potential difference was virtually the same at the end of the experiment (16.2$\pm$ 3.1 mV) as it was in the beginning (20.0$\pm$2.8 mV).

The importance of the presence of Na for the accumulation of AIB by

Table 22. AIB uptake in various sections of the lens

	AIB (tissue:medium concentration)
Whole lens	2.9 ± 0.6
Anterior 1.2 mm	9.5 ± 0.1
Posterior 1.2 mm	5.8 ± 0.5
Remainder	2.9 ± 0.3

Paired lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 6 hours. One lens of each pair was left intact and the other was frozen on the freezing plate of a microtome and 1.2 mm was removed from each surface. The AIB content of each layer was then determined. The values above represent the mean ± S.E. of 6 experiments.

Table 23. AIB accumulation in the whole lens, decapsulated and de-epithelialized lens and the lens nucleus

		AIB (tissue:medium concentration)	P for differences
A)	Intact lens	2.13 ± 0.21	ns
	Decapsulated lens	1.72 ± 0.22	
B)	Intact lens	3.62 ± 0.31	
	Decapsulated and epithelium removed	3.31 ± 0.22	ns
C)	Intact lens	2.02 ± 0.14	<.001
	Nucleus*	0.50 ± 0.11	

Paired lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. One lens of each pair was treated as the control, the other as the experimental which was prepared as described. Lenses were decapsulated by treatment with 0.5 mg/ml collagenase. The epithelium was then removed by scraping the anterior surface. The outer 2/3 of the lens (the cortical fibers) were removed by agitating the lenses for 1.5 hours. The values above are the mean ± S.E. of 6 experiments.

* The nucleus contains less water than the surrounding fiber cells, as discussed in the Methods section, this was taken into account in calculations of this experiment.

Table 24. AIB accumulation in the lens: anterior vs. posterior surface

AIB present on:	Incubation time (hr)	AIB (tissue:medium concentration)
Anterior side	21	2.59 ± 0.71
Posterior side	21	1.07 ± 0.18

Lenses were mounted in Ussing-type chambers and bathed on both sides with Conway solution. The potential difference was measured at the beginning and end of each experiment and the lenses were continuously short-circuited. 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB was added to either the anterior or posterior bathing solutions. Samples were taken of each bathing solution at the beginning and end of the experiment. The values above are the mean ± S.E. of 6 experiments.

the lens was studied. Unlike the cornea, the lens is completely dependent on the presence of Na for (Table 25) and the decrease is seen immediately when this ion is excluded from the external media. The activity of the Na-K pump has been shown to be important for many tissues' ability to accumulate amino acids. The effect of ouabain, an inhibitor of Na-K-ATP-ase, on AIB uptake in the lens was studied, this cardiac glycoside (Table 25) significantly reduced the concentration of AIB in the lens after 2 hours incubation, and uptake against a gradient was even further reduced after 6 hours.

Amino acids may compete with each other for uptake by tissues, indicating that specific systems exist for their transport. The ability of both alanine and leucine to compete with AIB for uptake by the lens, as shown by a reduction in accumulation of AIB in the presence of an excess of either of these amino acids (Table 26), indicates that specific transport systems for AIB are present in this tissue. Alanine was especially effective and there was no accumulation of AIB in its presence.

The hormones insulin and triiodothyronine (T_3) were incubated with the lenses to determine if they have any effect on the accumulation of AIB by the lens (Table 27). Neither hormone had a significant effect on AIB accumulation. Dexamethasone, which can be cataractogenic was also tested in this <u>in vitro</u> system. Incubation for up to 16 hours revealed no significant effect on amino acid accumulation (Table 27).

The effects of DIDS on AIB uptake in the lens was studied and the responses were found to be the same as in the cornea (Table 28). AIB accumulation was significantly decreased.

Table 25. Effect of incubation in Na-free media and the presence of ouabain on AIB accumulation in the toad lens

	Time (hr)	AIB (tissue:medium concentration) Control	Experimental	P for difference
A) Ouabain (10^{-4} M)	2	2.62 ± 0.32	1.79 ± 0.19	<.05
	6	4.34 ± 0.26	1.20 ± 0.09	<.001
B) Na-free Conway	2	2.45 ± 0.62	0.87 ± 0.13	<.001
	6	3.92 ± 0.41	0.73 ± 0.22	<.001

Paired lenses were incubated for the times indicated in solutions containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for the times indicated. One lens of each pair was treated as the control, the other as the experimental. In B, the Na in the experimental solution was replaced by choline. The values above are the mean ± S.E. of 6 experiments.

Table 26. Competitive inhibition of AIB uptake in the toad lens

	AIB (tissue:medium concentration) Control	+ Competing amino acid	P for difference
A) 10 mM alanine	2.72±0.34	0.40±0.05	<.001
B) 10 mM leucine	5.56±0.17	2.76±0.40	<.001

Paired lenses were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 3 hours. One lens of each pair was treated as the control and the other as the experimental. The values above are the mean ± S.E. of 6 experiments.

Table 27. Effect of hormones on AIB accumulation by the toad lens

	Concentration	Incubation time (hr)	AIB (tissue:medium concentration) Control	Experimental	P for difference
Insulin	10^{-6} M	4	2.76±0.16	2.88±0.79	ns
		24	12.45±0.94	14.67±0.71	ns
	10^{-7} M	18	5.54±0.53	5.48±0.60	ns
	10^{-8} M	18	9.51±0.92	11.03±0.58	ns
Dexamethasone	10^{-6} M	18	10.2±1.6	9.8±2.1	ns

Paired lenses were incubated for the times indicated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB. The hormones were added, in the concentrations indicated, to the experimental lenses. The values are the means ± S.E. of 6 experiments.

Table 28. Effect of DIDS on AIB accumulation in the toad lens

	AIB (tissue:medium concentration) control	AIB (tissue:medium concentration) experimental	P for difference
DIDS (5×10^{-3} M)	2.42±0.20	0.82±0.21	<.01

Paired lenses were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 5 hours. DIDS was present in the solution bathing the experimental lenses. The values are the mean ± S.E. of 6 experiments.

5. Amino Acid Efflux from the Lens.

The time course of AIB efflux from the lens is shown in Fig. 26. There is an initial fast component to AIB efflux (see inset Fig. 26) which is followed by a much slower one. The efflux from this tissue is extremely slow; only 35% of the preloaded AIB leaves the lens during 16 hours incubation. When the amount of AIB present in the lens is increased by extending the preloading period, the rate of exodus also increases, presumably reflecting a concentration dependence of this process (Table 29) When the efflux was measured over a period of 4 hours, the rate decreased exponentially (Fig. 27). However, this decline could not be accounted for by a decrease in the AIB concentration in the lens which only declined slightly in this time. The extracellular space of the toad lens, measured with inulin (Yorio & Bentley, 1976), is 6% of the wet weight of the tissue The fast component probably contains the amino acid present in this extracellular space. However, the amount of AIB calculated to be in the extracellular space can only account for about 10% of the total amino acid which leaves the lens in the first two minutes. Removal of the capsule and epithelium, although reducing the total amount of AIB in the tissue, did not measurably affect the rate of loss of AIB (Table 30).

The importance of Na for the amino acid accumulating mechanism in the lens, and the observations of its effects on the efflux of amino acids from the cornea prompted me to examine the response to changes in the concentration of this ion on the efflux system in the lens. Removal of Na from the media substantially increased the rate at which the amino acid left the lens (Fig. 28) so that by the end of 4 hours, 75% of the preloaded AIB had left the tissue. In the control lenses only

Fig. 26. AIB efflux from the toad lens

Lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 1 hour. After this preincubation, the lenses were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. Each value is the mean ± S.E. of 6 experiments.

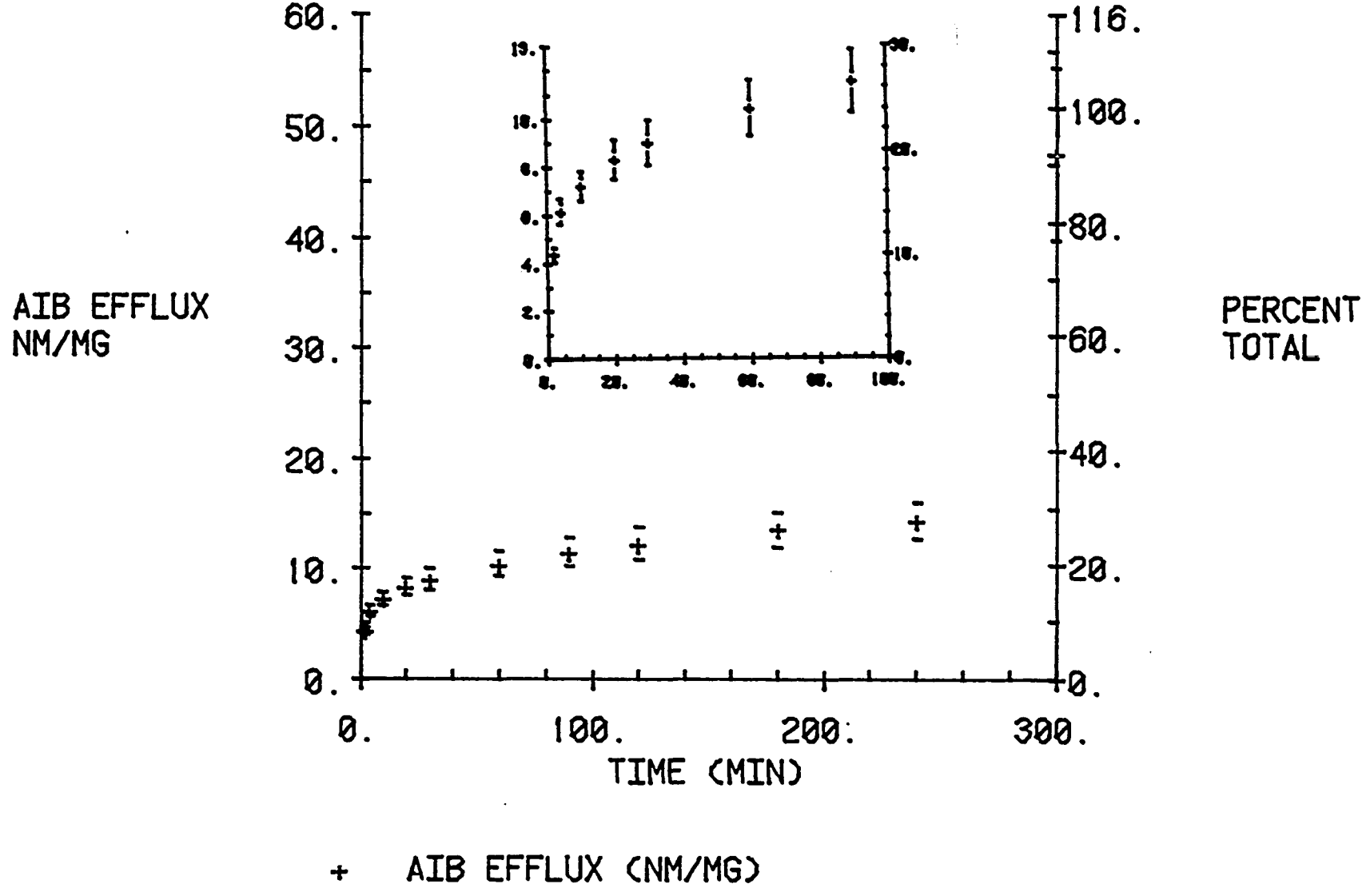

AIB EFFLUX FROM THE TOAD LENS
AIB EFFLUX
NM/MG
PERCENT
TOTAL
60.
50.
40.
30.
20.
10.
0.
116.
100.
80.
60.
40.
20.
0.
0.
100.
200.
300.
TIME (MIN)
+ AIB EFFLUX (NM/MG)

Table 29. Effect of varying the preincubation time on rate of AIB exodus from the lens

Preincubation time (hr)	Initial total AIB in lens (nM mg^{-1})	Rate of exodus (nM $mg^{-1}hr^{-1}$)
0.25	5.2 ± 0.6	0.33
	8.8 ± 0.5	0.47
0.75	33.4 ± 5.4	1.17
	67.5 ± 9.9	1.77
3	205.0 ± 27.0	4.8*
20	603.0 ± 99.0	12.8

Lenses were preincubated in Conway solutiohn with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for the times indicated. After this period, the lenses were placed in amino-acid free media and moved serially to fresh amino acid free solution at specific intervals for 4 hours (*16 hours). The rate of exodus was calculated from the last 3 hours (*15 hours) of the experiment. These values represent the mean ± S.E. of 6 experiments.

Fig. 27. The relationship between internal AIB concentrations and the rate of AIB efflux from the lens

Lenses were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 45 min. After this preincubation, the lenses were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. For each time interval, the amount of AIB left in each lens was determined and the rate of exodus at that time was calculated. Both of these values are plotted versus time in this figure.

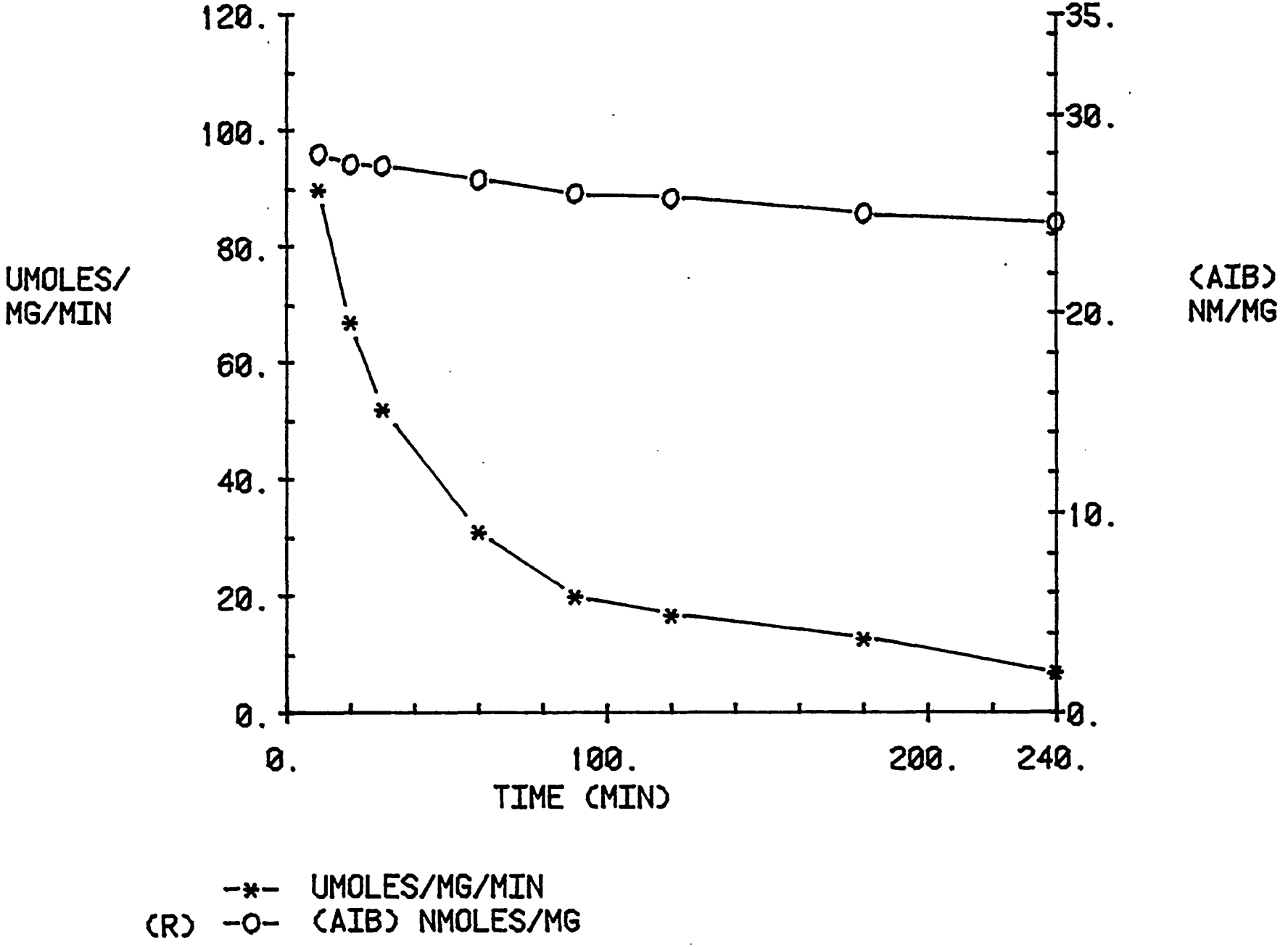

120.
100.
80.
60.
40.
20.
0.
UMOLES/
MG/MIN
35.
30.
20.
10.
0.
(AIB)
NM/MG
0.
100.
200.
240.
TIME (MIN)
-*- UMOLES/MG/MIN
(R) -O- (AIB) NMOLES/MG

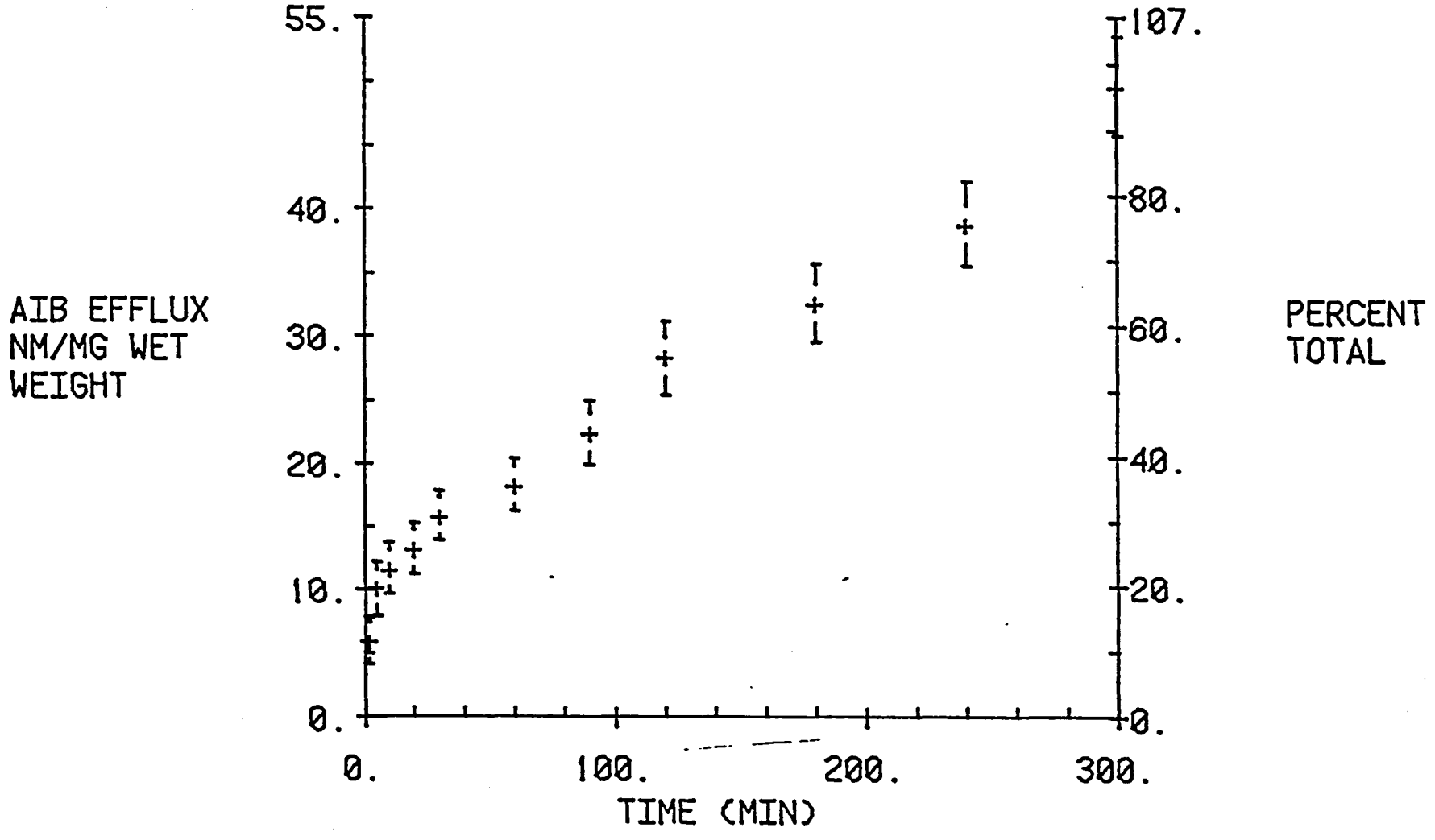
Table 30. Effect of removal of the capsule and epithelium on efflux of AIB from the lens

	Initial (total AIB in lens) nM/mg wet weight	AIB efflux nmoles $mg^{-1}hr^{-1}$
Intact	27.6 ± 1.9	1.26
Epithelium and capsule removed	21.4 ± 3.2	1.37

Paired lenses were incubated in Conway solution with 0.1 mM AIB and ^{14}C-AIB (0.2 μCi/ml) for 45 min. One lens of each pair was the control the other lens had its capsule and epithelium removed as described in the Methods section. After this incubation period the lenses were placed in amino acid-free media and moved serially to fresh bathing media at specific thime intervals up to 4 hours to determine the rate of AIB exodus. The values represent the mean ± S.E. of 6 experiments.

Fig. 28. Effect of incubation in Na-free media on AIB efflux from the toad lens

Lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 45 minutes. After this preincubation, the lenses were placed in Na-free, amino acid free media and moved serially to fresh solutions at the times indicated. Each value is the mean ± S.E. of 6 experiments.


AIB EFFLUX
NM/MG WET
WEIGHT
55.
40.
30.
20.
10.
0.
107.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ AIB EFFLUX NM/MG

30% was lost in this time. A surprising result, in view of the large effects of ouabain on AIB efflux from the cornea, was the small effect of this drug on the efflux from the lens (Fig. 29). Amphotericin B is known to alter the permeability of the lens to Na and it also increases AIB efflux from the cornea. Again the efflux of AIB from the lens was relatively insensitive to this drug (Fig. 30).

The presence of an exchange diffusion mechanism for amino acids was also sought in the lens. The addition of 10mM AIB to the solution bathing preloaded lenses stimulated the efflux of AIB (Fig. 31), but only very slightly compared to the increase seen in the cornea under similar conditions. It was not possible to determine if any of these effects were dependent on the presence of Na since removal of Na from the external bathing media had a much larger effect on efflux than any of the above treatments.

Addition of DIDS to the bathing solution had no effect on efflux of AIB from the lens or on exchange diffusion in this tissue, this parallels the effects of DIDS on the cornea.

Fig. 29. Effect of ouabain on AIB efflux from the toad lens

Paired lenses were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 45 min. At the end of this preincubation, the lenses were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 30 minutes to the end of the experiment, the solutions bathing the experimental lenses contained ouabain (10^{-4} M). The values are the mean $\pm$ S.E. of 6 experiments.

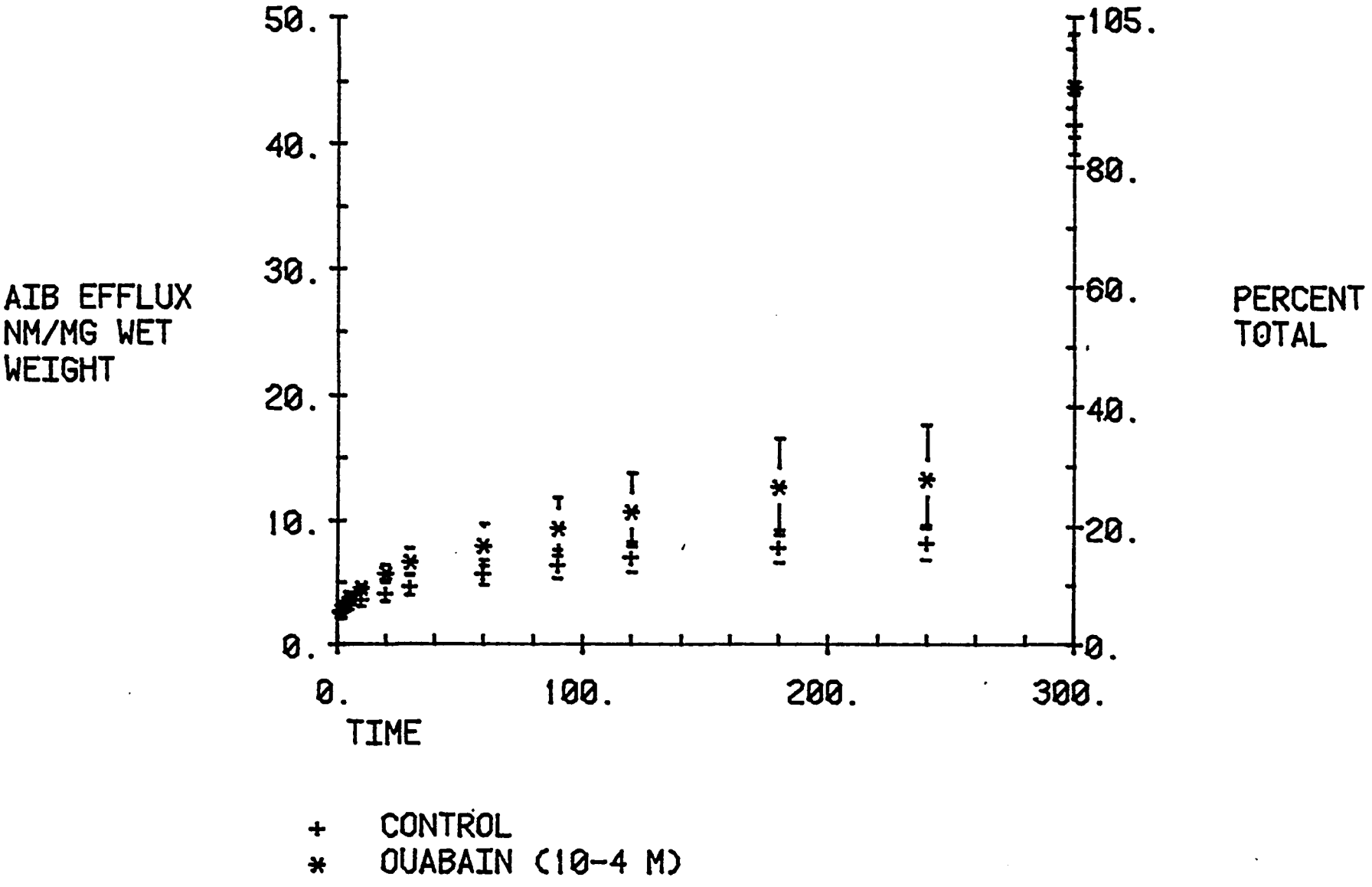

AIB EFFLUX
NM/MG WET
WEIGHT
50.
40.
30.
20.
10.
0.
105.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME
+ CONTROL
* OUABAIN (10-4 M)

Fig. 30. Effect of amphotericin B on AIB efflux from the toad lens

Paired lenses were incubated in Conway solution containing 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 45 minutes. At the end of this preincubation, the lenses were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 30 minutes to the termination of the experiment, the solutions bathing the experimental lenses contained amphotericin B (12.5 ug/ml). The values are the mean ± S.E. of 6 experiments.

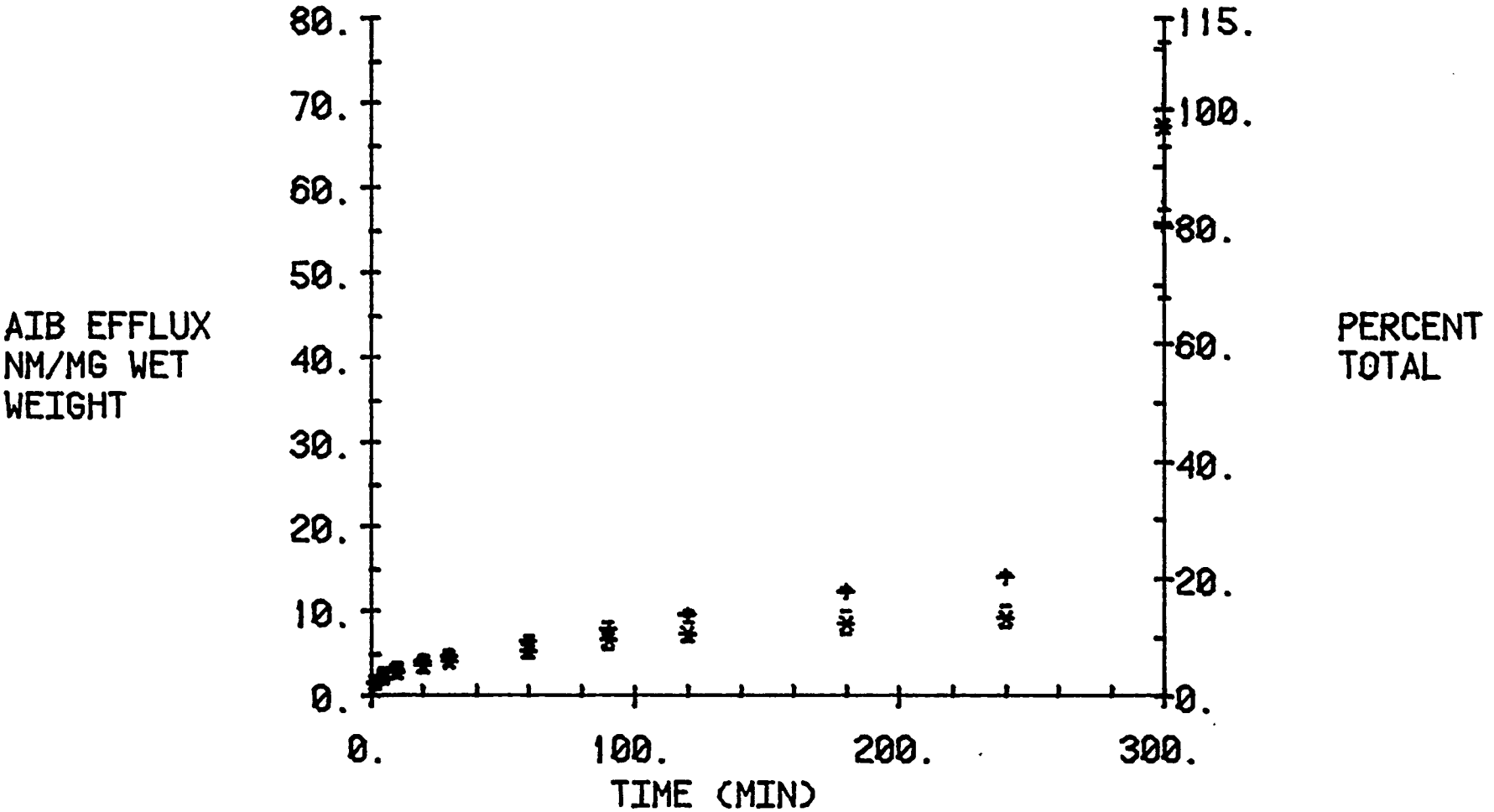
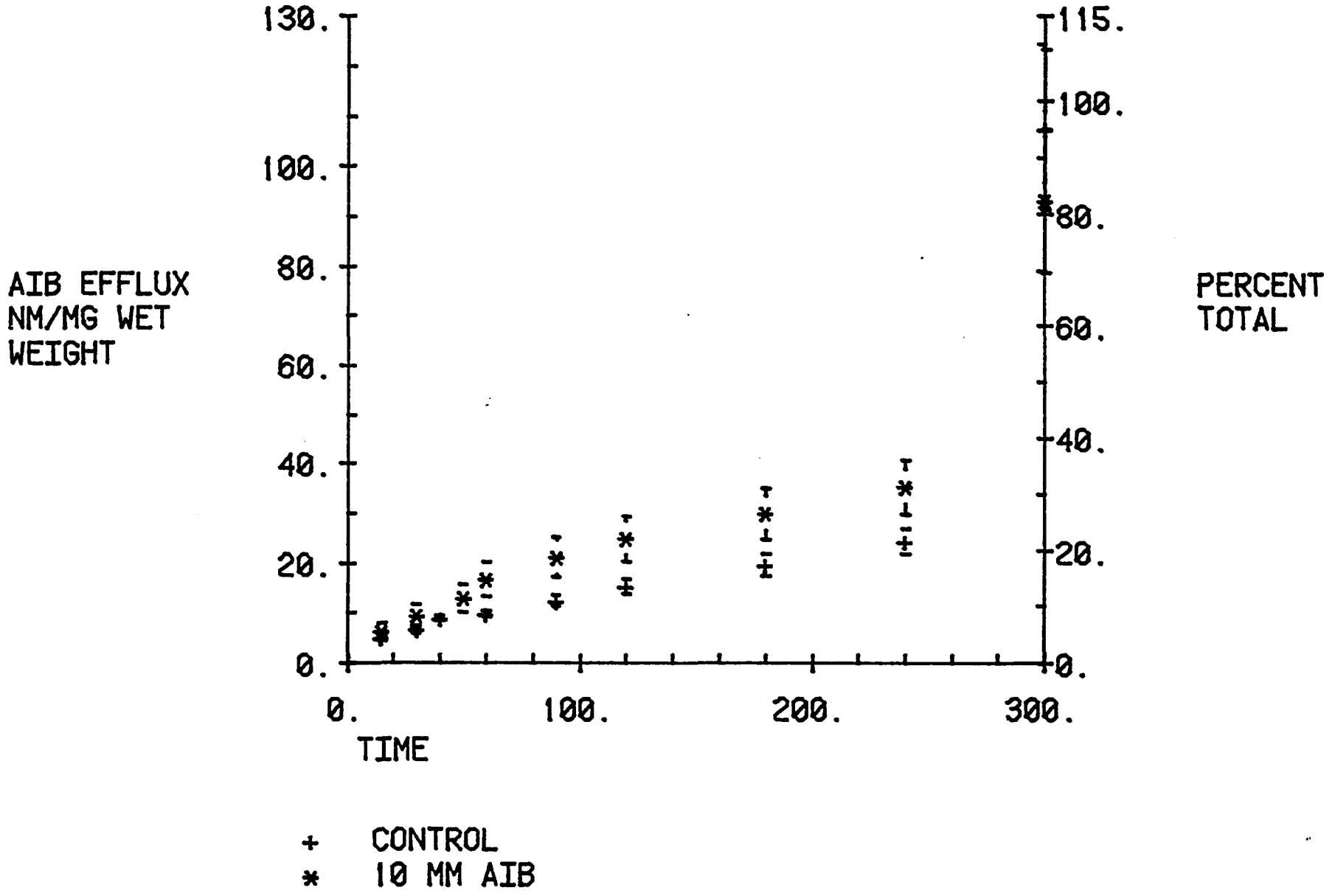

AIB EFFLUX
NM/MG WET
WEIGHT
80.
70.
60.
50.
40.
30.
20.
10.
0.
115.
100.
80.
60.
40.
20.
0.
PERCENT
TOTAL
0.
100.
200.
300.
TIME (MIN)
+ CONTROL
* AMPHOTERICIN B

Fig. 31. Effect of addition of 10 mM AIB to the external media on AIB efflux from the lens

Paired lenses were incubated in Conway solution with 0.1 mM AIB and 0.2 μCi/ml ^{14}C-AIB for 45 minutes. At the end of this preincubation, the lenses were placed in amino acid free Conway and moved serially to fresh solutions at the times indicated. From 30 minutes to the termination of the experiment, the experimental lenses were placed in Conway solution with 10 mM AIB. The values are the mean ± S.E. of 6 experiments.


AIB EFFLUX
NM/MG WET
WEIGHT
PERCENT
TOTAL
130.
100.
80.
60.
40.
20.
0.
115.
100.
80.
60.
40.
20.
0.
0.
100.
200.
300.
TIME
+ CONTROL
* 10 MM AIB

DISCUSSION

1. Cornea: Transmural Permeability

The observation that amino acids cannot cross the toad cornea or enter it from the outer tear surface is in agreement with the work of Riley, Sibley, and Hoefle (1973) on the rabbit cornea and confirms that of Friedenthal and Scott (1973). As this property is lost when the epithelium is removed, the outer side of these cells appears to prevent the loss of nutrients by leakage to the tear film. In the present studies of transmural permeability the endothelium appears to be an obsequeous entity, providing neither a facilitatory nor a restrictive barrier for the transport of nutrients such as amino acids. However, the endothelium does limit the movement of molecules the size of albumin (MW=60,000; diameter=37A), as the flux of this protein across the deepithelialized cornea doubles in its absence. In other studies of endothelial permeability in rabbits (Kaye, Sibley & Hoefle, 1973) molecules of approximately the same size as albumin (for example, horseradish peroxidase) were found to cross the endothelial surface through intercellular spaces.

In addition, it was found that removal of the endothelium does not influence the accumulation of AIB in the cornea, nor does it affect the rate of its efflux. These observations thus also suggest that the endothelium has a passive role in corneal amino acid transport. The permeability characteristics of the endothelium of the toad cornea appear to be similar to those of the endothelial lining of the vasculature, since the capillary membrane behaves as if it has channels with a diameter of about 35A; or just small enough to prevent the passage of most of the

albumin. It is interesting to note that the corneal and vascular endothelium have the same embryonic origin.

In the case of the smaller solutes, including amino acids, the endothelium does not seem to present a barrier, the stroma presumably being the main restriction to their movement. The flux of these solutes across the corneal membranes appears to be directly related to their size. The basic structure of the stroma consists of collagen fibers embedded in a mucopolysaccharide matrix. In an attempt to determine the role of each of these structural constituents in influencing the permeability to amino acids, the denuded stroma was exposed to collagenase and hyaluronidase and the transmural flux of AIB was measured. Hyaluronidase has been shown to leech out the mucopolysaccharide matrix of the mammalian cornea (Mishima & Kudo, 1961). Neither of these agents, alone or in combination, or by direct injection into the stroma changed the permeability characteristics of this structure to AIB, leucine or urea. This maybe due to either lack of substantial effect of these enzymes on the toad cornea, or it could reflect the absence of a critical role of these basic stromal structures in controlling its permeability to such molecules.

2. Cornea: Amino Acid Uptake and Efflux

Amino acids only enter the cornea across the endothelial surface; an observation which is consistent with that of others (Maurice, 1969; Thoft & Friend, 1974) who suggested that most of the nutrients needed for corneal metabolism are obtained form the aqueous humor and, to a smaller extent, from the capillaries at the corneo-scleral junction.

Most of the amino acids accumulated by the cornea appear to be present in the epithelial cells since removal of this layer drastically reduces the accumulation of the neutral amino acid analogue, AIB, used in these studies. The epithelium must, therefore, have a tremendous capacity to concentrate amino acids as a tissue:medium ratio of up to 10 is found in the whole tissue after 18 hours incubation. Since the epithelium represents only 10% of the wet weight of the cornea it must have the capacity to accumulate AIB up to 100 times the concentration in the bathing media. Removal of the epithelium reduces the amount of AIB in the cornea to a T:M of close to 1.0. This observation, however, does not exclude the possibility that the endothelial cells or stromal keratocytes can also actively accumulate amino acids. Since the endothelium and keratocytes make up, at most, about 8% of the wet weight of the cornea, a modest accumulation of AIB in these cells would most likely be difficult to detect. However, these cells clearly do not have the large concentrative ability of the epithelium for AIB. The variations in this capacity may reflect differences in the protein synthetic requirements of the various types of cells. The epithelium is constantly dividing and renewing itself, whereas in the adult, the endothelium and the stromal keratocytes appear to have lost the ability to divide. The epithelium, then would appear to have a greater need for a constant supply of amino acids and it possesses an efficient mechanism for their concentration.

In the studies of the efflux of AIB, removal of the corneal epithelium, and even the endothelium also, indicated that there were still two components to efflux, just as seen in the intact cornea. However, the amount of AIB involved in the slow component of efflux in these preparations was much less than in intact corneas. The AIB which is appar-

ently sequestered in these scraped corneal preparations exhibits the same facilitation of its efflux as the intact cornea does to the presence of externally added amino acids (both 'homoexchange' and 'heteroexchange') and ouabain.

AIB appears to be a good analogue for alanine in the amphibian cornea since the two amino acids are mutually competitive for uptake and both AIB and alanine can exchange for AIB. A detailed account of amino acid accumulation in the toad cornea has been provided by Friedenthal and Scott (1973), and the present results are mainly in agreement with it. There is a further parallel between these transport systems as leucine inhibits both AIB and alanine accumulation to the same extent. AIB uptake is blocked by both alanine and leucine, although neither alone completely abolishes accumulation, their effects are additive. Concentration against a gradient is eliminated under these conditions. It can be assumed then then, that AIB is taken up by both an alanine and a leucine specific transport system. In the classical studies of amino acid transport in Ehrlich cells and avian erythrocytes the 'A', or alanine-preferring system, has been defined as Na-dependent and it relies on metabolic energy to drive transport against a concentraton gradient (Christensen, 1975). In the amphibian cornea the alanine transporting system for amino acid accumulation is, however, not dependent on the presence of Na and, as will be discussed later, it may only be indirectly affected by inhibition of metabolism. This Na-independence of AIB accumulation has been noted previously in the toad cornea (Scott and Friedenthal, 1973). The considerable time lag for the inhibition of accumulation of AIB following removal of Na or addition of metabolic inhibitors suggests that these

conditions do not directly affect the uptake process. However, it was possible that AIB accumulation in Na-free media took place via increased 'L' system activity. By classical definition, removal of Na from the incubation media should completely abolish uptake by the Na-dependent 'A'-system. If the 'L' system did compensate for decreased 'A' system activity in the cornea, then addition of high concentrations of leucine in Na-free media would completely block AIB accumulation. Uptake was decreased but concentration against a gradient was not completely eliminated. This, together with the fact that AIB accumulation does not occur when alanine and leucine are added together to the incubation media indicates that AIB can be accumulated by both leucine and alanine preferring systems in the absence of Na. It is thus appears that there is a Na-independent system in the cornea that has no affinity for leucine.

The removal of Na is also associated with a small increase in the rate of AIB exodus from the cornea. This may result in a decrease in the retentive capacity of the cells for actively accumulated AIB and may contribute to the apparently indirect effect of the removal of Na on the steady-state levels of AIB in the cornea. It is also possible that the internal AIB moves with Na down its concentration gradient out of the cells.

The 'L', or leucine-preferring system, is classically defined as being Na-independent (Christensen, 1975) and not highly concentrative. It has also been suggested that the 'L' system functions mainly for exchange. Oxender and Christensen (1963) proposed that every natural amino acid has a degree of affinity for both the 'A' and the 'L' uptake systems. The stereospecificity of amino acid exodus from the cornea is

greater than that of amino acid entry since leucine cannot exchange for AIB but can competitively block its uptake. This differential specificity of the processes of accumulation and efflux of amino acids has also been noted in the Ehrlich cell (Christensen, 1976).

It has been shown that amino acid uptake systems work strongly in an uphill direction and this may serve to limit the losses of nutrients which are usually gained at metabolic expense. The normal rate of loss of AIB from the cornea is only 7% the rate of their uptake. The basis for this very strong retention mechanism is not known. It is possible that there are fewer sites for the exit of amino acids or that the sites turn over more slowly. One would expect that if efflux occurred by such a mediated system and not just via simple diffusion, that it would be possible to saturate the system, as it was possible to saturate the influx influx system. However, when the corneas were preloaded with concentrations of AIB of more than 30mM, it was found that efflux was not saturated. It is possible that there are intracellular sites or 'pools' in which amino acids are 'bound' or 'sequestered' and therefore only a very small amount of the amino acid is freely available to leave the cell. Although rates of exodus can be determined rather precisely, the ability to determine the absolute intracellular concentration of the solute under study with reasonable certainty makes an accurate determination of the kinetics of efflux very difficult. This problem has been noted by others (Schultz & Curran, 1970).

If, indeed, amino acids are stored in such a way that they are not readily available for exodus, this 'storage system' may be disrupted in a number of ways. Thus, ouabain which inhibits Na-K-ATPase and would

therefore be expected to increase the intracellular concentration of Na, causes an increase in the rate of AIB efflux. This response was dependent on the presence of at least 24 mM Na in the external media. A blockade of the Na-K 'pump' would be expected to increase the internal Na to concentrations approaching the external concentration. Since only 24 mM Na in the external media is needed for ouabain to exert its effect it may be hypothesized that an increase in internal Na concentration to about 24 mM is all that is needed to release the internal stores of AIB. Neither lithium nor choline could substitute for Na in these experiments. A variety of Na-dependent processes involved in transepithelial transport (Field, Schultz & Curran, 1967) and accumulation of solutes within epithelial (Thier, 1968; Boge, Rigal & Peres, 1979) and non-epithelial cells (Charalampous, 1968; Caldwell and Lea, 1978) are affected by ouabain. However, it has also been shown that ouabain, although it inhibits net transmural transport, it has no effect on Na-dependent influx of amino acids across the mucosal brush border of the isolated rabbit ileum (Chez, et al., 1967). Ouabain increases the movement of amino acid from the intestinal epithelial cell back into the mucosal solution (Danisi, Tai & Curran, 1976) and this maybe the mechanism by which it blocks net transmural movement from the mucosa to the serosa.

Removal of K from the external media would be expected to have a similar effect to that of ouabain in increasing intracellular Na. If there is no external K to activate the internal 'Na pump' it will not function. Removal of K, like ouabain, increased AIB efflux and this effect was also Na-dependent. In the mouse ascites tumor cell removal of K from the external media caused an increase in glycine efflux which

was related to an increase in intracellular Na (Eddy, Mulcahy & Thompson, 1967). The addition of metabolic inhibitors may also increase internal Na by decreasing the energy supply to the Na pump. Such treatment increases AIB efflux, and this effect is also Na-dependent. One can only speculate as to the mechanism by which increases in internal Na increase amino acid efflux. It may be that the amino acid has low affinity for the efflux 'carrier' in the presence of low (or normal) levels of Na inside the cell. However, when internal Na levels are raised, efflux of the amino acid could be increased, either by increasing the affinity of the amino acid for the carrier or by increasing the rate of translocation. Such allosteric effects have been described by others (Jardetzky, 1966; Alvarado, 1976). Changes in cell volume do not appear to be involved as exposure to hypoosmotic media failed to increase amino acid efflux.

The total amount of amino acid accumulated in any tissue is the sum of their uptake and efflux. Both the addition of ouabain and the absence of K in the external media also decreased the ability of the cornea to accumulate AIB. This could, at least in part, be due to the observed increase in efflux and may so reflect an inability of the tissue to hold on to accumulated amino acids. However, if an increase in efflux is responsible for the decrease in ability of cells to accumulate amino acids, it is expected from the kinetic analysis that the cellular compartment would be virtually empty within 6 hours incubation time with ouabain or in the absence of external K. This is not the case. In addition, despite the increased efflux in the presence of amphotericin B, the cornea still retained its ability to concentrate AIB. These results suggest that uptake and efflux are separate systems and that AIB uptake can be a very

efficient process. It is also possible that the loss of AIB may trigger an increase in the activity of the uptake system, or that the loss may be associated with an exchange of internal for external AIB.

Initially it was thought that amphotericin B may also be having a ouabain-like effect, as it is known to increase permeability to Na in the cornea, and the internal concentration of this ion. However, in contrast to ouabain, removal of Na from the external media had no effect on the stimulation of efflux by amphotericin B.

The Na-dependence of exchange diffusion is also of interest with respect to the possible mechanism of amino acid efflux. Early studies of amino acid and sugar transport in the intestine (Schultz & Zalusky, 1964, 1965) suggested that a Na-gradient is necessary for the substrate to be transported and that the Na and substrate are cotransported into the tissue. Addition of the amino acid to the mucosal solution bathing the intestine mounted in Ussing-type chambers, and short-circuited, resulted in an abrupt rise in the short-circuit current (I_{sc}). This increase in I_{sc} was shown to be due to a stimulation of the transport of Na. More recently (Laris, et al., 1978), it has been shown that hyperpolarization of Ehrlich cells occurs when these cells are preloaded with high concentrations of amino acids and that this hyperpolarization results from the electrogenic coefflux of Na and the amino acid. Cysteine has been shown to stimulate the entry of Na into human red blood cells (Young, et al., 1979). In the present studies of amino acid exchange diffusion in the cornea, extracellular Na was essential for the exchange to occur but an external concentration of 17 mM Na was all that was necessary. A large Na gradient is apparently not important for this

effect. As suggested by the ouabain studies, it appears that a very small increase in internal Na concentration can effect a large change in efflux. It is possible that when 10 mM AIB or alanine is placed on the outside of the cells, Na may be transported with the amino acid into the cells and this rise in Na maybe responsible for the increase in AIB efflux. If Na does move into or out of the cornea with the amino acid a change in transcorneal I_{sc} or ^{22}Na fluxes might be expected. In the toad cornea the I_{sc} is only partly (20%) due to net Na movement and it occurs in the tear to aqueous direction. If internal Na increases due to cotransport with the amino acid an increase in Na efflux across the cornea, due to passive ion movement, or an increase in Na transport from the tear to aqueous side, due to increased Na pumping, may be expected. Exchange diffusion increases the efflux of AIB to 1.1 μEq cm^{-2} hr^{-1}. The normal net flux of Na is only equal to 0.10 μEq $cm^{-2}hr^{-1}$, so that if Na moved with the amino acid some change in the Na flux in either direction should be expected. Neither a change in I_{sc} nor Na fluxes was observed, so it is concluded that the presence of Na, rather than its cotransport, at some critical concentration, presumably on the inside of the membrane, is the factor necessary for exchange to take place. Alvarado (1979) suggests that even in Na-free media, a microenvironment rich in Na immediately adjacent to the outer face of the cell membrane may exist. If this is true in the corneal epithelium, and Na exchanges across the membrane only within this microenvironment, without entering the cellular Na pool, then changes in transcorneal ^{22}Na fluxes might not be seen.

The foregoing observations suggest that there are at least four systems present in the cornea which influence the steady-state levels

of AIB in the cornea, they are illustrated diagrammatically in Fig. 32.

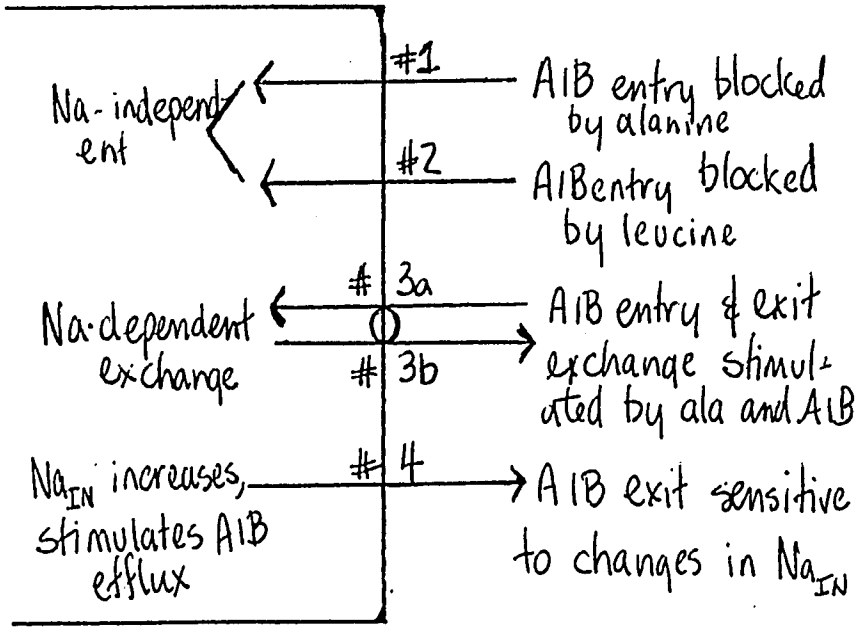


Fig. 32

1) AIB can enter via site #1, this site can be blocked by alanine. AIB entering by site #1, does not require the presence of Na.
2) AIB can enter via site #2, this site can be blocked by leucine. AIB entering by site #2 also does not require the presence of Na. It appears that, although some alanine may be taken up by #2 and leucine by #1, there are two distinct systems for AIB uptake that are blocked by alanine and leucine, since neither amino acid alone completely blocks uptake, but when added together, no AIB is accumulated by the cornea. However, it is possible that there is only one site, and that this site has a different affinity for alanine and leucine.
3) AIB can enter via site #3, and can also leave via #3, its exit is stimulated by the presence of alanine or AIB (but not leucine) in the outside bathing solution. There is also an absolute requirement for Na

in the external bathing solution in order for exchange to occur. As discussed above it has not been determined which sites, 3a and/or 3b, require Na. It would be important to determine if changes in external levels, the gradient, or internal levels of Na inhibit this exchange process. It is also possible that #1 and #3a are the same sites since both sites are stereospecific for AIB and alanine. This system appears to be similar to that described by Heinz and Walsh (1958) in which exchange occurs as a one-way migration driving a counter-flow. Such a system, by utilizing an intracellular amino acid pool derived from excessive uptake, amino acid synthesis or protein and peptide degradation could provide the cornea with a means for accumulation of similar amino acids against a concentration gradient in the absence of a direct energy source.

4) AIB efflux occurs via site #4, this site may be associated with site #1 and #2, and/or be synonomous with #3b or be a completely different site. The affinity of AIB for this site appears to be much less than its affinity for any of the uptake sites since the rate of exodus is so much less than that of uptake. Site #4 is very sensitive to changes in internal Na concentrations. Any factor that increases internal Na levels increase the rate of AIB exodus.

The hypothesis that there are separate sites for uptake and efflux is supported by the results of both the amphotericin and the DIDS experiments. Amphotericin B increases efflux, but does not affect accumulation and DIDS decreases uptake without affecting efflux or exchange. However, this does not preclude the ability of these sites to be bi-directional. The possibility that factors which increase efflux or reduce uptake may be operating at the same site causing allosteric

changes which facilitate or inhibit one activity of a particular site, for instance uptake, and not affecting another, efflux, is certainly plausible.

Having delineated these transport systems for AIB, it was of interest to try to define their physical properties. In order to attempt this a variety of membrane 'probes' were employed. The inability of concanavalin A or phospholipase C to influence transport suggests that neither α-D-glucosyl nor phosphatidylcholine residues are important for AIB transport. However, more general probes such as PCMPS and trypsin do affect transport. Since only high concentrations of trypsin could influence AIB accumulation it is likely that its effects are non-specific ones on the membrane that may influence not only uptake, but also the ability of the cornea to retain any accumulated AIB. It does, however, indicate that the integrity of membrane proteins is essential to assure the cornea's ability to accumulate AIB. PCMPS, which binds to membrane sulfhydryl groups, primarily on the exterior of the cell membrane, also decreased accumulation and increased efflux of AIB. It is interesting to note that PCMPS may bind to Na-K-ATPase thereby inactivating it; an effect which could increase internal Na like the effect of ouabain. Removal of Na abolished PCMPS' stimulation of AIB efflux.

It appears that anion exchange may have an important role in normal corneal metabolism and physiological functioning (Yorio & Bentley, 1978). It was of interest, then to determine if these exchanges could influence AIB accumulation. DIDS has been shown to block anion exchanges ($Cl^-$for HCO_3^-) in red blood cells (Cabantchik and Rothstein, 1974). It has also been shown to inhibit 'active' Cl transport across the cornea, probably

by inhibiting a similar anion exchange pump (Bentley & McGahan, 1980). In addition, DIDS decreased the accumulation of both alanine and AIB in this tissue. DIDS does not elicit its effect by increasing AIB efflux and it does not affect the amino acid exchange process. The decrease in amino acid accumulation seen here does not appear to be directly related to its effect on Cl transport for the following reasons: 1) bumetanide, which inhibits Cl transport in the cornea does not affect AIB accumulation; 2) removal of Cl from the bathing media also does not inhibit accumulation. The effect of DIDS on AIB accumulation may be more direct as indicated by the fact that when it is placed on the tear side of the cornea, DIDS does not reduce AIB accumulation, although DIDS does inhibit Cl transport at this surface. Since DIDS does decrease AIB accumulation when it is placed on the endothelial side of the cornea, it appears to be having a direct effect on this process. DIDS maybe binding to the transport site for AIB, or it may, by binding near the transport site be changing the conformation of that site and so inhibit AIB uptake.

The lack of effect of hormones on AIB uptake in the cornea does not rule out the possibility that other amino acid transport systems in this tissue may be affected by their presence and it would be worthwhile to investigate this possibility. Since the fluid pump in the corneal endothelium has been shown to be affected by insulin (Fischbarg, 1978) and insulin is present in significant quantities in the aqueous humor (Daniel, Pratt & Wilson, 1967; Greco, et al., 1973) it is possible that this hormone may affect other amino acid transport systems not investigated here.

3. Lens: Amino Acid Accumulation

The lens of the toad, Bufo marinus actively accumulates the amino acid AIB. This is in agreement with observations on the mammalian lens (Kinoshita, Merola & Hayman, 1965; Kinsey & Reddy, 1965). In the present study it was found that this tissue exhibits all of the classical features of active amino acid transport; uptake against a concentration gradient which is saturable, specificity, as indicated in competition studies, and a dependence on metabolic energy.

AIB uptake by the lens appears to be mediated by both the 'A' and 'L' systems as indicated by the competition experiments. If AIB enters the lens via both systems, then, alanine must also have free access to both systems, as it completely inhibits AIB accumulation. However, it is possible that AIB only enters via an 'A' system for which leucine has an affinity. This could account for the inhibition of AIB accumulation in the presence of high external leucine concentration. It would then appear that AIB is only accumulated via one system, the 'A' system, which is more similar to the classical Na-dependent 'A' system defined by Christensen (1975) as AIB accumulation is markedly inhibited in the absence of Na.

Ouabain inhibits AIB accumulation in the lens, but not immediately. This latency would indicate an indirect effect on uptake and has also been noted in the rabbit lens (Kinsey & Reddy, 1965).

It was found that amino acids are accumulated across the anterior surface of the lens at a greater rate than across the posterior surface. It does, however, appear that the AIB can also be accumulated against

a concentration gradient across the posterior surface. Even though the tissue:medium concentration was not significantly different that 1.0 in these latter experiments, it must be recalled that the nucleus (about one-third of the lens wet weight) does not accumulate a significant amount of AIB so that a tissue: medium concentration of 1.0 for the whole lens represents a much higher concentration in the outer layers where the AIB does accumulate. These observations differ from those of Kinsey and Reddy (1965) in the rabbit lens as they found that amino acids are transported into the lens by the epithelium and without this layer the lens fibers lost their capacity to accumulate amino acids against a concentration gradient. However, in a more recent study by Brassil and Kern (1978) in the rat, it was found that the posterior fiber cells could accumulate amino acids against a gradient when this surface alone was exposed to the amino acids. The experiments in the present study confirm these results. One interesting observation in these experiments was that the T:M ratios found in the free-floating lens after 21 hours incubation was 12:1, whereas in those experiments where the lens was incubated in Ussing-type chambers the T:M was never greater than 3:1. The mounting procedure may cause a decrease in the ability to accumulate AIB.

Sectioning of the lenses preloaded with AIB showed that there was a gradient of concentration of AIB from the outside in and that the anterior section contained the highest concentration of the amino acid. The fiber cells in the periphery, including the posterior region, had a T:M concentration of greater than one. This observation prompted me to measure the ability of the fibers themselves to accumulate AIB.

In the study described previously, Kinsey and Reddy (1965) removed

both the capsule and the epithelium and measured the amino acid transporting properties of the fiber cells of the rabbit lens. They were found to be incapable of active amino acid transport. However, the procedure they used to remove the capsule and epithelium involves an incision with a scalpel after which the loosened capsule is pulled off, the underlying epithelium being removed with it. Since, after this treatment, the authors also showed an abnormal leakiness of both the anterior and posterior surfaces to Na it is likely that the normal functioning of the lens fibers was disrupted. This technique has recently been shown to cause mechanical damage to the underlying fiber cells (Rae & Stacey, 1979). In these histological studies it was demonstrated that mechanical removal of the capsule and epithelium destroys the outer fiber cells since they become leaky to the extracellular space marker, procion yellow. Removal of the capsule using collagenase and subsequently scraping off of the anterior epithelium is apparently a less traumatic way of obtaining viable fiber cells since these cells retain the capacity to accumulate amino acids against a gradient. This activity of the fiber cells would be expected to contribute significantly to the total uptake of AIB by the lens.

Insulin is present in the aqueous humor and it has been demonstrated to have various effects on the lens including actions on gene expression (Milstone & Piatgorsky, 1977) and differentiation (Piatgorsky, Rothschild & Wollberg, 1973). Its effects on amino acid transport have, however, not been studied in the normal lens. Using alloxan diabetic rabbits (Reddy & Kinsey, 1963) it was found that insulin had no effect on amino acid transport in the lens. In the present investigation, insulin was found not to significantly change amino acid accumulation in

the lens. Dexamethasone, a drug that is used topically on the eye, has been associated with the formation of posterior, subcapsular cataract. In this in vitro system, dexamethasone after 16 hour exposure at a high (10^{-6} M) concentration, did not disrupt the amino acid accumulation mechanism in the lens.

The effect of DIDS in decreasing AIB accumulation in the lens and its parallel to the effects of this compound on corneal amino acid transport was, indeed, very interesting. It is possible that it has a direct action on the carrier here. However, I cannot rule out indirect such as changes in metabolism of the lens.

4. Lens: AIB efflux

The inability of compounds that markedly stimulate amino acid efflux in the cornea to have such effects on the lens was initially puzzling. Treatment of the cornea with ouabain, amphotericin B and the addition of exchanger amino acids, such as alanine and AIB, caused the release of almost all of the preloaded amino acid within 3 hours. Such treatment of lenses preloaded with AIB only caused a small increase in the amount of AIB leaving the tissue. The magnitude of this response was also disconcerting because the mechanism for AIB accumulation in the lens was responsive to the presence of ouabain just as it was in the cornea. These results prompted a reconsideration of the original efflux data to determine in what ways the lens may differ from other tissues, including the cornea, in its normal physiological amino acid transport properties. The first consideration was the specialized morphology of the lens. Where do amino acids enter and accumulate in this organ? The results

showed that AIB enters the lens across all surfaces and that the fiber cells are capable of accumulating amino acids, even in the absence of the capsule and epithelium. It seems likely that AIB can also leave the lens across all surfaces. The capsule did not impede the movement of amino acids out of the lens as observed in the calf lens by Kern (1970). It is important to consider the anatomy of the lens in relation to amino acid transport. As the lenticular epithelial cells divide, they push equatorially to a site of differentiation. The cells then transform into fiber cells which are laid down on top of already existing fibers that are pushed towards the center of the lens; no cells are lost. Thus the lens has an 'onion-like' appearance, one layer on top of another. There can be several hundred layers of fibers extending from the outer surface to the central, nuclear, region of the lens (Rae & Stacey, 1979). The fibers become more and more packed until there is virtually no extracellular space in the central core of the lens.

Amino acids not only enter the surface fiber cells but also penetrate into deeper layers. However, since the extracellular space is so small the AIB may be expected to move from cell to cell on its journey towards the inner parts of the lens. As a T:M of 1.0 was never reached in the inner one-third of the lens a concentration gradient exists from the outside to the inside of the lens, with the highest concentration in the outer layers. It is possible, in such a system, to envisage the process the efflux of AIB after preloading with the amino acid. When the lenses are placed in amino acid-free media the AIB present in the surface layers leaves first; hence the rapid initial rate of efflux. As the first layer empties, the concentration gradient shifts for the

next layer of cells (Fig. 33) and they, in turn, unload into the surface

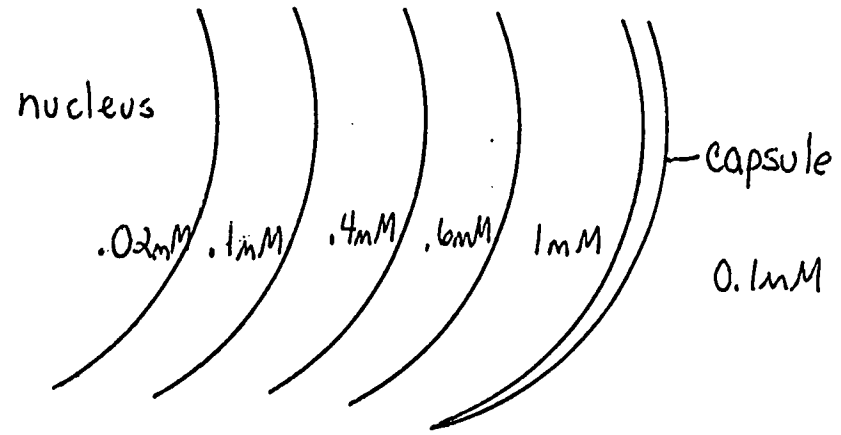


Fig. 33

layer. However, the more centrally located cells are protected against loss of amino acid until the layer next to them is depleted to the extent that the concentration gradient is favorable. If much of the amino acid, in order to leave the lens, must pass out of one cell into the next, in a series arrangement, and each cell has a resistance to the efflux of the amino acid (as we observed in the cornea where very few cell layers are involved and the rate of exodus is very slow compared to the rate of uptake) the total resistance when many cell layers must be traversed would be expected to be great. Hence the rate of exodus will decrease with time and also with the number of layers of cells the amino acid must traverse. This is indeed what was found. Although the rate of exodus increases with increased loading time, if efflux is calculated over the course of an experiment, the rate decreases sharply, even though the concentration of AIB in the lens changes little (Fig. 27).

A model was proposed in an attempt to describe the movements of amino acids within the lens (Fig. 34). In this model there are two cellular compartments, an outer layer which completely envelops the sphere (B)

and an inner core (A). When the lens is removed from the preloading media and placed in an amino acid free environment (C) the accumulated amino acids can move either out of B into C or move into A, the gradient is probably favorable for movements in either of these directions.

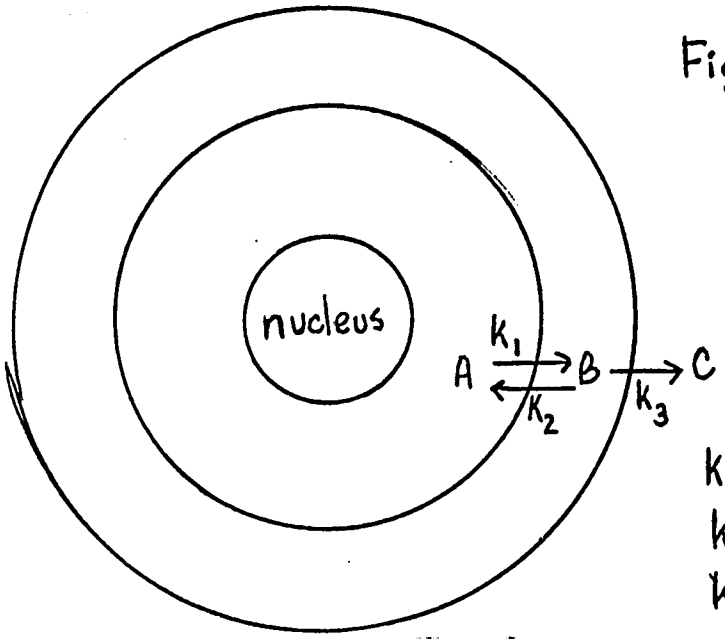


Fig. 34

$k_1 = .001\ min^{-1}$
$k_2 = .100\ min^{-1}$
$k_3 = .200\ min^{-1}$

Also, amino acids that have been accumulated in A may also move into B, although transfer in this direction may not be favored by the concentration gradient. The following equations describe the interactions of the three compartments and can be usd to define the rates at which transfer between the compartments occur:

$$\frac{dY_A}{dt} = -k_1 Y_A + k_2 Y_B$$

$$\frac{dY_B}{dt} = k_1 Y_A - (k_2 + k_3) Y_B$$

$$\frac{dY_C}{dt} = -k_3 Y_B$$

When these equations are fitted to the measured efflux results it is found that k_2 is 100-times k_1 and is 0.5 k_3. Movement of amino acids out of the surface region of the lens is therefore favored, however movement deeper into the lens, as described by this model, is much greater than movement from the deeper layers to the surface.

That the data obtained in these experiments fits such a theoretical model indicates that amino acid efflux from the lens is a very complex process, as would be thought, when one considers the anatomical arrangement of this tissue. It is highly likely that many more than two compartments exist in the lens, especially if each cell layer represents a compartment. Schultz and Curran (1970) pointed out the difficulty of determining the the kinetics of amino acid transport in the lens both because unidirectional fluxes and intracellular concentrations are so difficult to determine. When Kern (1970) studied the efflux of neutral amino acids from the calf lens, he also found that the rate of exodus was much less than that of uptake. He postulated that efflux took place via a non-saturable carrier mediated system which was equivalent to the non-saturable portion of the uptake system. The inability to saturate efflux carriers in the calf lens is not surprising in light of the above discussion.

The addition of substances that increase amino acid efflux, such as ouabain and amino acid exchangers will probably only act at the surface layer. If they enter and act in the deeper parts of the lens they could even decrease the efflux by driving the amino acid down the concentration gradient in the opposite direction, or further into the lens. If they acted primarily at the surface it could explain the initial small increase

in efflux which is observed in their presence. This anatomical arrangement of the lens cells may function as a conservation and protective mechanism to maintain the intralenticular amino acid levels, such as if the lens is transiently exposed to toxic compounds or reduced levels of nutrients.

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