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Physiology of sulfate transport by the crustacean hepatopancreas

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PHYSIOLOGY OF SULFATE TRANSPORT BY THE CRUSTACEAN HEPATOPANCREAS

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ABSTRACT

The hepatopancreas, or digestive gland, of *Homarus americanus* (and other crustaceans), has been shown to play a major role in the digestion and absorption of nutrients. Although the hepatopancreas has been implicated in the manufacture and release of digestive enzymes there have been no reports of its ability to secrete other solutes such as ions. The organ has been suggested as a possible site of excretion due to its cellular morphology and direct access to the lumen of the digestive tract. It has been demonstrated that sulfate is present in lobster hemolymph at a concentration lower than in seawater. The present investigation utilized isolated membrane vesicles to determine the mechanisms and characteristics of sulfate transport across both the apical and basolateral membranes of the hepatopancreas.

The brush border membrane of the hepatopancreatic epithelium appears to contain a transport protein which translocates cytoplasmic sulfate in exchange for luminal chloride. This antiport mechanism operates in an electrogenic fashion by exchanging one sulfate for one chloride, resulting in the movement of a net negative charge out of the epithelial cell. There was no indication of sodium-sulfate cotransport commonly reported for the brush border membrane of vertebrate renal and intestinal epithelia. It was found that the antiporter was stimulated by the presence of a high concentration of luminal protons. This suggested that the sulfate antiporter was regulated by the brush border sodium-proton exchanger which acidifies the hepatopancreas lumen.

Sulfate was found to exchange for the dicarboxylic anion oxalate by an electroneutral antiporter in the hepatopancreatic basolateral membrane. This transporter would allow for movement of sulfate from the hemolymph, which bathes the hepatopancreas, to the cytoplasm of the epithelium. The serosal antiporter did not show a response to manipulation of proton concentrations.

A model of transcellular sulfate secretion by the hepatopancreatic epithelium has been proposed utilizing the two antiporters working in sequence to bring about vectorial sulfate movement. This is the first experimental evidence which implicates the crustacean hepatopancreas as a secretory organ.

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1) Chapter II "Electrogenic, H^+-regulated, sulfate-chloride exchange in lobster hepatopancreatic brush-border membrane vesicles" was submitted to the American Journal of Physiology and accepted on 21 August 1991. It appeared in publication February, 1992.

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3) Chapter IV "Electrogenic coupling of sulfate secretion to chloride transport in lobster hepatopancreas" was submitted to the editors of Advances in Comparative and Environmental Physiology as an invited contribution for a forthcoming volume and currently awaits publication. G. A. Gerencser, Ed. Vol 19, Chapter 6, pp. 109-120, MacMillan India Ltd, Bangacore. (1994).

CHAPTER I

CHARACTERISTICS OF SULFATE TRANSPORT BY THE CRUSTACEAN HEPATOPANCREAS

EPITHELIAL TRANSPORT PROCESSES

The movement of solutes across biological membranes is a ubiquitous process involved in many aspects of cellular physiology. In eukaryotic cells these processes take place in many organelle membranes and across the plasmalemma. Among their many functions are to maintain osmotic, ionic and chemical homeostasis of the cell itself and the organism to which they belong, regulate cell volume, initiate action potentials and facilitate synaptic transmission. The vectorial transport of molecules is accomplished by the concerted action of specific membrane bound proteins which may be very selective, or quite general in their acceptance of a substrate. The epithelial tissue of the digestive and excretory systems provide a boundary between the external environment of organisms and their internal milieu. Portions of this epithelial lining consist of cells which are well-equipped to shuttle numerous solutes in or out of the animal.

There are many important aspects of transport proteins that allow them to carry out their respective roles in membrane transport (Friedman, 1986; Jain, 1988). Transport proteins can act by either passive or active mechanisms according to the nature of the substrate and the importance of the solute to the physiology of the animal. Active

transport mechanisms can utilize energy reserves of the cell (i.e. ATPases) to facilitate movement, or they can harness the electrochemical gradients (especially ions) naturally occurring across cell membranes to provide the driving force to move solutes. These active transport mechanisms have the ability to accumulate substrates to a great extent against a concentration gradient within a cell or the animal's body fluid.

There are different classes of transport proteins which utilize transmembrane chemical gradients to move solutes. Cotransport proteins involve a symport stoichiometry (simultaneous transport of two or more solutes in the same direction), while antiport proteins rely on the exchange of substances in opposite directions across the membrane.

Epithelial cells have a specific morphology which allows them to be very efficient at coming in contact with substrates and moving them across their plasmalemma. The typical epithelial cell consists of apical and serosal surfaces which can have discrete functions in their transport qualities. The apical, or brush border, membrane of the cell usually orients toward the lumen of the organ and is recognized by numerous microvilli. In contrast the serosal, or basolateral, portion of the cell consists of the region of plasmalemma which is found between adjacent epithelial cells and the area at the base, which typically borders the blood of the animal. The microvilli of the apical border and the infolding of the serosal membrane allow for increased surface area to accommodate the multitude of membrane transport proteins.

The accretion of a substance within a cell is usually accomplished by transport proteins located on either the apical or serosal membrane. These solutes taken into the

cell are then utilized by that cell to accomplish its normal physiological activities. On the other hand, the absorption of nutrients and the excretion of wastes which have accumulated in the digestive tract and blood, respectively, are required to pass across both membranes of the cell in series, involving at least two spatially distinct transport proteins.

SULFATE TRANSPORT

There have been quite a number of publications characterizing vertebrate sulfate transport systems, especially mammalian species which need to reabsorb much of the sulfate which passes through their kidneys. These processes have been elucidated from a number of techniques and in a variety of organs such as the intestine, kidneys and respiratory epithelia (Lucke et al., 1979; Schneider et al., 1984; Turner, 1984; Schron et al., 1985; Elgavish et al., 1987; Pritchard, 1987; Renfro et al. 1987). One of the most widely used techniques for studying membrane transport proteins is the isolated membrane vesicle technique (Hopfer et. al., 1973). Based on different surface charges and densities of membrane domains, specific membrane vesicle populations can be fractionated by differential centrifugation and cation mediated precipitation of disrupted epithelial tissues. As the plasmalemma is disrupted via these techniques portions of the lipid bilayer, and included proteins, will spontaneously form into small vesicles due to thermodynamic requirements. After the membrane vesicles have been isolated they can be "loaded" with buffers of known composition and incubated in a separate incubation

buffer. Characteristics of the transport proteins under study can be observed by tracing the movement of radiolabeled substrates which will be transferred into, and retained by, the enclosed vesicular interior. A variety of mechanisms have been clarified which carry out sulfate transport in vertebrate biological membranes. The sulfate anion can be cotransported with cations (sodium or protons), where both substrates move in the same direction. (Lucke et al., 1979; Schneider et al., 1984; Schron et al., 1985). It is also possible to have an antiport mechanism which exchanges other anions for sulfate, in this case the counter ions are usually bicarbonate and chloride (Renfro and Pritchard, 1983; Pritchard, 1987). Sulfate anions carry a negative net charge of 2 so either electroneutral or electrogenic transport is possible depending on the number of co-substrates bound to the carrier protein. To date there has been only one report of a sulfate transport protein which acts in an electrogenic fashion, the sodium-sulfate cotransporter isolated from rabbit ileum epithelium (Ahearn and Murer, 1984).

Sulfate transport involves both reclamation and secretion of sulfate ions depending on the type of animal and the nature of the organ system. Mammalian kidneys are usually involved in sulfate reabsorption where sulfate is removed from the glomerular filtrate and returned to the general circulation (Pritchard, 1987). There is a need for sulfate ions to be incorporated into enzyme cofactors, amino acids and proteins. The movement of sulfate ions can also lead to shifts in acid-base balance when transport involves other ions such as bicarbonate. In contrast, the kidney of the teleost fish has been shown to secrete sulfate as it is transported across renal epithelia and excreted via the urine (Renfro and Pritchard, 1982; 1983). In this case there is a build-up of sulfate

within the blood above the homeostatic level which may result from the animal's metabolism or the relatively high amount of sulfate in seawater. Due to fluctuations in ion concentrations within the body of an animal there can be systems which switch the direction of net transport. This may involve the operation of various transport systems or the reversal of a specific exchange protein.

EXPERIMENTAL ANIMAL

The Atlantic lobster, Homarus americanus, is found along the eastern coast of North America from the Canadian Maritime provinces south to North Carolina (Houdon and Lamarche, 1989). Their typical habitat consists of rocky shelters found from inshore subtidal waters to deep water and over the continental shelf. They are an important predator throughout their range and are extremely significant in the commercial fishing industry. The lobster has the reputation of being a scavenger. While it is true that individuals will consume almost any animal or plant material, at any stage of decomposition, their diet consists mainly of live fish, shellfish and other crustaceans, especially conspecifics (Carter and Steele, 1982; Elner and Campbell, 1987; Houdon and Lamarche, 1989).

Transport proteins allow communication between the external environment and the internal milieu of a specific organism. For an aquatic animal the internal and external environment usually differ markedly in ion and chemical concentration or composition. These animals are faced with the physiological problem of maintaining salt

balance as the excess ions move by diffusion across animal cell membranes to balance their concentrations. Many mechanisms have arisen to deal with this problem and transport proteins are found in a number of sites on the integument and organs which communicate with the surface of the animal. In crustaceans the areas available for the exchange of solutes are limited to gills, digestive tract and excretory structures known as antennal glands (Mantel and Farmer, 1983).

The gills of marine decapods have been shown to be practically impermeable to sulfate which carries a strong negative charge (Lignon, 1987). This leaves two possible sites for sulfate movement in and out of the animal, either the antennal glands, which function as kidneys in crustaceans or via the hepatopancreas, the major site of nutrient absorption. Studies have shown that crustacean urine also varies in urine to plasma sulfate concentration ratio such that some decapods reabsorb sulfate while others secrete the anion (Prosser, 1973). To date there is no conclusive information on the role of the hepatopancreas in the secretion of any solute, however it is a good candidate as a site for elimination as it comes in contact with seawater during the digestive processes. Several physiologists have suggested that the hepatopancreas may indeed be involved with secretion of metabolites and ionic regulation (Gifford, 1962; Dall, 1970; Holliday and Miller, 1984).

In crustaceans the divalent anion, sulfate, is one such solute which is controlled by transport proteins to maintain certain concentrations in the hemolymph. Sulfate concentrations within an organism fluctuate during the synthesis and degradation of amino acids and other proteins; it can also affect the pH of cytoplasm or hemolymph by

its exchange with other ions, such as bicarbonate and protons during transport. Marine decapods vary in their requirement for blood sulfate with some being hyperegulators, hyporegulators, or ion conformers. The lobster Homarus maintains its blood sulfate level below that of the surrounding seawater and therefore must be constantly eliminating some of its sulfate load (Robertson, 1949; Prosser, 1973).

The crustacean hepatopancreas has been extensively studied for more than 100 years; many functions of the organ have been deduced based on histological and morphological techniques (Yonge, 1924; Loizzi, 1971; Dall and Moriarty, 1983). In the lobster the hepatopancreas consists of a diverticulum which connects to the digestive tract between the pyloric region of the stomach and the proximal end of the intestine (Gibson and Barker, 1979). The biiobate organ is situated along the midline of the animal in the dorsal cephalothorax region and is completely surrounded by hemolymph. Structurally the tissue consists of numerous blind ended tubules which have a single layer of epithelial cells surrounding the tubule lumen. Individual tubules have isolated muscle cells wrapped around the exterior surface which aid in movement of solutions into and out of the tubules. Tubules empty into a network of successively larger vessels which drain into the digestive tract. The entire organ is enclosed within a connective tissue sheath which provides a means of maintaining the characteristic shape of the hepatopancreas. The epithelial cells which line the tubules provide the functional boundary between the hemolymph of the animal and the digestive milieu.

There are four major types of epithelial cells which comprise the hepatopancreas; they have been differentiated by structure and position within the length of a tubule and

different developmental strategies have been postulated for the various populations (Loizzi, 1971; Gibson and Barker, 1979). The 'E' cells, which are located at the distal (closed) end of the tubules, are the smallest and are believed to undergo mitosis and give rise to the other cell types within the tubule. The other three cell types are more proximal to the E cells in the tubule and can be considered the active cells of the hepatopancreas. Reabsorptive cells ('R' cells) are thought to actively absorb solutes from the chyme within the lumen and are involved with the storage of materials such as lipids, carbohydrates and minerals within the digestive gland. The 'F' cells, so named for their fibrillar appearance, are thought to be involved in the synthesis of digestive enzymes and may undergo transformation into the 'B' cells. 'B' or blister cells are recognized by their extremely large vacuole, which may encompass most of the entire volume of the cell, and is reputed to contain the digestive enzymes which will be secreted into the lumen to hydrolyze the food particles. The method of secretion may be different in various crustaceans, either holocrine, merocrine or apocrine. There is considerable controversy as to whether these cell types constitute separate populations of cells, or if they represent a developmental sequence through which all hepatopancreas cells pass.

The consensus of many workers is that the primary role of the hepatopancreas is the secretion of digestive enzymes (Dall and Moriarty, 1983). Along with this secretory function it has been suggested that the crustacean hepatopancreas has an absorptive function for nutrients (Yonge, 1924; Loizzi, 1971; Gibson and Barker, 1979). Recently, by the use of membrane vesicles, nutrient transport processes and the mechanisms involved have been well documented for the crustacean hepatopancreas (Ahearn et al.,

1985; Ahearn and Clay, 1987a; 1987b; Ahearn et al., 1992). At this time there has been no direct measurement of nutrient or ion secretion by the crustacean hepatopancreas although this function appears feasible based on other studies.

PROPOSED RESEARCH

The present study involves investigating the physiology of one or more transport proteins found in the hepatopancreas of the Atlantic lobster, Homarus americanus. Preliminary research has determined that there are sulfate transport proteins on both apical and basolateral membranes of the hepatopancreas epithelium (Cattey et al., 1990; Gerencser et al., 1990). These transport proteins appear to utilize anion antiport to move sulfate across the membrane in an electrogenic manner, and the proton concentration, or gradient, may be involved in regulating the magnitude of sulfate translocation (Cattey et al., 1991; 1992).

The first phase of this investigation will involve the characterization of membrane transport mechanisms which are involved in moving sulfate across both basolateral and apical membranes of the hepatopancreas epithelium. Here various factors such as substrate specificity, membrane potential sensitivity, kinetics of transport and the action of transport inhibitors will be determined. This will provide some information on the nature of the mechanism and will allow the development of a model of sulfate transport within the hepatopancreas. Based on this model it should be possible to determine the net flux direction (i.e., absorption or secretion) of the anion. This part of the research

will utilize plasma membrane vesicles and rapid filtration techniques for recovering radioisotopes from vesicles.

Within any cell membrane there are a multitude of transport proteins which simultaneously perform specific operations required to carry out the functions of that cell. Therefore it is feasible that an interaction between the various transport proteins exists such that the substrate of one transport protein can regulate the operation of another transport protein either directly or indirectly. I believe that this may be the case with the sulfate transporter and the sodium/proton exchanger, both localized in the apical membrane of the lobster hepatopancreas (Ahearn et al., 1990). The sodium-proton exchanger has been well-described in many cell types and extensively studied in a variety of physiological roles. Most of these reports involve mammalian cells which utilize an electroneutral system that employs a transport stoichiometry of 1 Na^+ to 1 H^+ (Aronson, 1985). Recently a sodium-proton exchanger has been described in crustacean gill, antennal gland and hepatopancreatic epithelium which operates in an electrogenic fashion exhibiting a transport stoichiometry of 2 Na^+ to 1 H^+ (Towle et al., 1988; Ahearn et al., 1990; Ahearn and Franco, 1990).

SIGNIFICANCE OF PROPOSED RESEARCH

There is a growing body of research on the physiology of the crustacean hepatopancreas which now allows for a comparison to more well known vertebrate

systems. Descriptions of ion transport by the digestive gland of decapods is still in its infancy, with the Na^+/H^+ exchanger as the only example of a transport system which is satisfactorily explained. This is the first time that a transport protein for sulfate has been described in an invertebrate, furthermore it is the first sulfate transporter which acts in an electrogenic manner. This investigation, therefore, may provide some insight into the evolution of transport proteins within the animal kingdom.

In the area of comparative digestive physiology there has been no empirical evidence which supports the role of anion secretion by the digestive gland of a decapod crustacean. This study may shed light on the ability of the digestive gland to carry out transepithelial secretion. If this is the case in Homarus it will be a significant addition to the biology of crustacea.

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CHAPTER II

ELECTROGENIC H^+-REGULATED SULFATE-CHLORIDE EXCHANGE IN LOBSTER HEPATOPANCREATIC BRUSH-BORDER MEMBRANE VESICLES

SUMMARY

Transport of [^{35}S]-sulfate by brush border membrane vesicles (BBMV) of lobster (Homarus americanus) hepatopancreas was stimulated by an outwardly-directed chloride gradient. In contrast, sulfate uptake was not enhanced by inwardly-directed Na^+ or K^+ transmembrane gradients. An inside-positive membrane potential (valinomycin and K^+) stimulated SO_4^{2-}/Cl^- exchange, while an inside-negative membrane potential was inhibitory. Sulfate-sulfate exchange was not affected by alterations of transmembrane potential. An inwardly-directed proton gradient, or the presence of low bilateral pH, enhanced SO_4^{2-}/Cl^- exchange, yet the H^+ gradient alone did not stimulate sulfate uptake in chloride equilibrated BBMV or in vesicles lacking internal Cl^-. The stilbenes SITS and 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS) strongly inhibited SO_4^{2-}/Cl^- exchange. Sulfate influx occurred by a combination of carrier-mediated transfer, exhibiting Michaelis-Menten kinetics, and non-saturable "apparent diffusion". $^{36}Cl^-$ influx into sulfate-loaded BBMV was stimulated by an inside-negative transmembrane potential compared to short-circuited vesicles. These results suggest sulfate-chloride exchange in hepatopancreatic BBMV occurred by an electrogenic carrier mechanism exhibiting a 1:1

flux ratio that was modulated by internal and external H^+-sensitive regulatory sites. The role of this antiport process in anion secretion is discussed.

INTRODUCTION

Gastrointestinal and renal transport of the divalent anion sulfate across epithelial apical membranes has been investigated in various vertebrate groups including mammals (Ahearn and Murer, 1984; Lucke et al., 1979; Pritchard, 1987), teleost fish (Renfro and Pritchard, 1982; Renfro and Pritchard, 1983) and the domestic chicken (Renfro et al., 1987). A number of mechanisms for brush border carrier mediated sulfate transport across epithelial membranes have been proposed and include sodium-sulfate co-transport (Ahearn and Murer, 1984, Lucke et al., 1979, Schneider et al., 1984), anion exchange (Pritchard, 1987; Renfro and Pritchard, 1983; Talor et al., 1987) and pH gradient-dependent transfer (Renfro and Pritchard, 1982; Schron et al., 1985a). These processes contribute to transepithelial regulation of sulfate, which maintains physiological levels of this anion, and may affect acid base balance and cell and plasma osmolarity.

The crustacean hepatopancreas has been described as a digestive and absorptive organ based on morphological and histochemical studies of the epithelial cells which comprise most of the tissue. Previous studies also suggest that the organ may in addition play a role in secretion (Gibson and Barker, 1979; Loizzi, 1971; Yonge, 1924). Recently, the use of isolated membrane vesicles provided data to suggest that nutrient absorption across the hepatopancreas of the lobster, Homarus americanus, occurs as a

result of transport activities at the apical border via both Na-dependent and Na-independent mechanisms (Ahearn and Clay, 1987a; 1987b; Ahearn et al., 1985). An electrogenic sodium-proton exchanger, with physiological properties unlike those of the vertebrate antiporter, has also been shown to be present on the brush border of the hepatopancreas epithelium (Ahearn et al., 1990). Other than the occurrence of this Na/H exchanger there is no other direct evidence as to possible functions of the hepatopancreas in inorganic ion transport.

The lobster, being a marine invertebrate is periodically ingesting seawater during both eating and drinking (Mykles, 1980). The sulfate content of seawater is relatively high (25 mM), whereas the blood concentration of this anion in many marine decapods, including the lobster, has been shown to be maintained at lower levels than the surrounding seawater (Prosser, 1973; Robertson, 1949). The present study investigates the role of the brush border membrane of the hepatopancreatic epithelium in the transport of sulfate. Results demonstrate the presence of an anion exchange mechanism in this membrane which is inhibitable by stilbenes, is modified by pH, and operates in an electrogenic fashion.

METHODS

Live atlantic lobsters (Homarus americanus, 0.5 kg each) were purchased from commercial dealers in Hawaii and maintained unfed at 10 °C for up to 1 week in filtered

seawater. All animals were either in intermolt or early premolt as assessed by the molt stage classification scheme introduced by Aiken (1973).

Hepatopancreatic brush border membrane vesicles (BBMV) were prepared from fresh tissue removed from individual lobsters. Each membrane batch was produced from a single organ (15 - 25 g fresh wt) by using a method of combined osmotic disruption, differential centrifugation and magnesium precipitation described previously (Ahearn and Clay, 1987a; Ahearn et al., 1985). Marker enzyme assays confirmed that vesicles prepared by these methods were highly enriched in brush border membranes with minimal contamination from basolateral or organelle membranes (Ahearn et al., 1985).

Transport studies were conducted at 15 °C using the rapid filtration technique developed by Hopfer et al. (1973). For time course experiments, a volume of vesicles (eg. 20 μl) was added to a volume of incubation media (eg. 180 μl) containing 0.1 mM radiolabeled $^{35}SO_4^{2-}$. At various incubation times a known volume (20 μl) of reaction mixture was removed and plunged into 2 ml of ice cold stop solution (stop solution composition varied with experiment and generally consisted of incubation media without any sulfate) to stop the uptake process. The vesicle suspension was then rapidly filtered through 0.65 μm Millipore filters (presoaked in distilled water) and washed with another 5 ml of ice coid stop solution. Filters were transferred to vials containing Beckman Ready Solv HP scintillation cocktail and counted for radioactivity in a Beckman LS-8100 scintillation counter. Transport experiments involving incubations less than 10 seconds were conducted using a rapid-exposure uptake apparatus (Inovativ Labor AG, Adliswil, Switzerland). Uptake was initiated by mixing 5 μl of vesicles with a volume (e.g. 45 μl)

of radiolabeled incubation media, filters were washed and counted for radioactivity as above. For short term incubations a blank was also run for each condition by mixing stop solution, vesicles and radiolabeled incubation media simultaneously, the resulting value was subtracted from corresponding experimental results before determining uptake. For long term time courses an estimation of the nonspecific binding of radioactivity to vesicles and filters was determined. Incubation and intravesicular media varied between experiments and are indicated in the figure legends. Sulfate uptake values were usually expressed as picomoles per milligram protein (Bio-Rad protein assay) per filter using the specific activity of sulfate in the incubation media.

Unless otherwise indicated, valinomycin (50 μM) and bilaterally equal potassium concentrations across the vesicular wall were present to short-circuit the membranes. Each experiment was generally repeated three to five times using membranes prepared from different animals. Within a given experiment each point was determined from three to five replicate samples. Data are presented as means $\pm$ standard errors of a single representative experiment. Standard errors indicate variation due to assay procedure, not individual variation. Similar qualitative experimental findings were obtained in the repetition of an experiment. $^{35}SO_4^{2-}$ as the Na^+ salt was obtained from New England Nuclear. Valinomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 4-acetamido-4'-isothiocyanotostilbene-2,2'-disulfonic acid (SITS), 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS), bumetanide, furosemide, and other reagent grade chemicals were purchased from Sigma Chemical.

RESULTS

Driving force for sulfate uptake

Figure 2.1 indicates that the transport of 0.1 mM $^{35}SO_4^{2-}$ was not affected by an inwardly directed 100 mM Na-gluconate gradient when compared to uptake with bilateral tetramethylammonium (TMA) gluconate. This suggests that sulfate uptake by BBMV was not sodium dependent. In the same experiment a portion of the vesicle preparation was pre-loaded with 25 mM HCO_3^-. As before, there was no increased stimulation of sulfate uptake compared to the TMA-gluconate control. In all conditions there was no accumulation of sulfate above that of the equilibrium value.

Sulfate uptake into BBMV was only slightly stimulated by incubation in media containing 100 mM K-gluconate (no valinomycin or internal K^+), in the presence of 50 μM CCCP (Figure 2.2). However, when vesicles were pre-loaded with 25 mM KCl and incubated in TMA-gluconate media containing equimolar K^+ and valinomycin there was an 'overshoot' accumulation twice that of the equilibrium value. Such results, and those from Figure 2.1, indicate an anion exchange mechanism which can utilize a Cl^- gradient, but not a gradient of HCO_3^-, as a driving force to move sulfate across the apical membrane. The slight K^+-stimulation seen during inwardly directed K^+ gradients may result from electrical coupling between diffusional substrate movements and a small membrane potential not completely eliminated by CCCP short-circuiting.

Effect of membrane potential on sulfate uptake

The possible membrane potential sensitivity of sulfate-chloride exchange was examined by imposing a valinomycin-induced potassium diffusion potential across the vesicular wall and measuring the time course of 0.1 mM sulfate uptake. Transport was determined under both inside-negative and inside-positive conditions and compared to uptake in short-circuited conditions (equal K^+ across membrane). Figure 2.3 shows that when vesicles were incubated in media containing 100 mM K^+ (no internal K^+) sulfate uptake was stimulated above that with bilateral potassium. In contrast, when vesicles were pre-loaded with 100 mM K^+ (no external potassium) sulfate uptake was significantly inhibited. These data suggest that sulfate-chloride exchange is enhanced by an inside-positive membrane potential and inhibited by an inside-negative vesicular interior, supporting the electrogenic nature of the exchange process.

A second series of experiments was designed to investigate the effect of membrane potential on 0.5 mM $^{35}SO_4^{2-}$ transport. In these experiments vesicles were pre-loaded with either 100 mM Cl^- or 10 mM SO_4^{2-} and subjected to valinomycin-induced potassium diffusion potentials as above. Figure 2.4 indicates that sulfate-chloride exchange responds to both inside-negative and inside-positive conditions while sulfate-sulfate exchange appears to be an electroneutral process. At both 15 s and 1 min incubations sulfate-chloride exchange was inhibited by a transmembrane negative potential and enhanced by an imposed positive potential. In contrast, sulfate-sulfate exchange was unaffected by potential manipulations. These data suggest that the

transport protein can accept either 1 Cl^- or 1 SO_4^{2-} molecule during the translocation process.

Effects of pH on sulfate-chloride exchange

Sulfate uptake has been shown to be stimulated by proton (or hydroxyl) gradients in the basolateral membrane of the teleost fish renal epithelium (Renfro and Pritchard, 1982). The possible proton (hydroxyl) gradient stimulation of sulfate-chloride exchange in hepatopancreatic BBMV was investigated in a series of experiments in which the pH of internal and external media was varied and 0.1 mM $^{35}SO_4^{2-}$ uptake was determined.

Figure 2.5 shows that when the pH of the incubation media was maintained at 7.0 and the internal pH was varied from pH 5.0 to 8.0 there was differential transport of sulfate into BBMV pre-loaded with 100 mM Cl^-. The lowest internal pH of 5.0 corresponded to the slowest uptake of sulfate, whereas when the internal pH was greater (pH_{in} = 8.0) than the external pH, sulfate uptake was enhanced over that of all other conditions. Such results suggest that at a constant external pH of 7.0 an increase in internal proton concentration inhibits the transport of sulfate by BBMV. Alternatively, the observed phenomena may have been attributed to competition of internal OH^- with Cl^-.

In a reciprocal experiment where the internal pH was kept constant at pH 7.0 and the pH of the incubation media was raised from 5.4 to 8.0 there was again a gradation of sulfate uptake into vesicles pre-loaded with 100 mM Cl^- (Figure 2.6). With a decrease in external pH there was a corresponding increase in maximal transient sulfate

accumulation. These results indicate that an increase in external proton (or decrease in external hydroxyl) concentration can stimulate sulfate-chloride exchange.

In contrast to the previous two experiments, Figure 2.7 shows the result of sulfate-chloride exchange at various bilateral pH conditions ranging from 5.4 to 8.0. The maximal uptake of sulfate into BBMV occurred at pH 5.4 and decreased as pH was raised to 8.0. Results of this experiment indicate that it was not the proton (or hydroxyl) gradient which stimulated sulfate uptake, but rather the absolute proton (hydroxyl) concentration.

The observation that increased external $[H^+]$, can enhance sulfate uptake suggests a possible regulation of the transport protein by external protons. Information on the regulatory role of protons was investigated by measurement of $^{35}SO_4^{2-}$ transport in vesicles equilibrated with 0.1 mM radiolabelled sulfate, 100 mM TMA-Cl, 50 K-gluconate, 100 mM TMA-gluconate and 50 μM valinomycin at either pH 6.0 or 8.0. Incubation media had the same constituents (same specific activity inside and outside) at pH 6.0. A control condition was included where external TMA-Cl was replaced by TMA-gluconate (Cl gradient) at pH 6.0. In this experiment the only driving force was either the pH gradient or the Cl gradient. Figure 2.8 shows that the Cl- gradient was capable of driving sulfate against a concentration gradient whereas the pH gradient was unable to stimulate a change in sulfate content within the vesicle. These results indicate that neither a pH gradient nor equilibrated chloride were responsible for driving sulfate uptake. The previous experiments addressed the effect of pH on sulfate-chloride exchange, while Figure 2.9 compared the pH effect on vesicles prepared without Cl^- and

vesicles pre-loaded with Cl^-. It was observed that 15 s uptake of 0.1 mM $^{35}SO_4^{2-}$, under the influence of low vs high pH, or opposing pH gradients (in the absence of internal Cl^-) was not stimulated to the level of vesicles pre-loaded with Cl^-. There was also an effect of bilateral pH on Cl^- pre-loaded vesicles where uptake was 4 fold greater at pH 6 vs pH 9. Based on these results the conclusion was made that OH^- ions were unable to substitute for Cl^- as an exchangeable substrate and that a high absolute concentration of protons stimulates SO_4^{2-}/Cl^- exchange.

Kinetic characteristics of sulfate influx

Preliminary investigation determined that $^{35}SO_4^{2-}$ uptake into hepatopancreatic BBMV was linear over the first 12 seconds at the concentrations utilized throughout this study (data not shown). Sulfate influx (7 s uptake) from incubation media to vesicular interior was measured in membranes pre-loaded with 100 mM TMA-Cl, 50 mM K-gluconate and 50 μM valinomycin at pH 7.0 and external media of 100 mM TMA-gluconate, 50 mM K-gluconate and variable sulfate (0.1 - 20 mM) at pH 7.0. Figure 2.10 shows that sulfate influx was a curvilinear function of external sulfate concentration. An influx relationship such as this can be described as the sum of at least two independent processes acting simultaneously: 1) a Michaelis-Menten carrier mechanism illustrating saturation kinetics, and 2) a linear entry system with a rate proportional to the external sulfate concentration. These two processes operating together can be described by the equation

$$J = \frac{J_{max}\,[S]}{K_t + [S]} + P[S] \qquad (1)$$

where J is total $^{35}SO_4^{2-}$ influx in nanomoles per mg protein per 7 s, J_{max} is apparent maximal carrier mediated influx, K_t is the apparent sulfate concentration resulting in half-maximal uptake, [S] is the external sulfate concentration and P is the rate constant of the linear entry component, which can be defined as apparent diffusional permeability.

A nonlinear iterative best fit computer program was utilized to analyze the data in Fig. 2.10 by equation (1). Apparent transport parameters calculated in this manner are as follows: apparent K_t = 0.27 mM, apparent J_{max} = 1.28 nmol . mg protein^{-1} . 7 s^{-1} and P = 0.36 nmol . mg protein^{-1} . 7 s^{-1} .mM^{-1}.

In an attempt to determine the internal binding coefficient of sulfate to hepatopancreatic BBMV during sulfate-sulfate exchange vesicles were pre-loaded with various concentrations of SO_4^{2-} (0.05 - 10 mM) and incubated in media which contained 0.5 mM radiolabeled sulfate. Figure 2.11 shows that under these conditions influx versus internal sulfate concentration exhibited a hyperbolic relationship which can be described by the Michaelis-Menten equation,

$$J = \frac{J^S_{max}\,[S_i]}{K^S_i + [S_i]} \qquad (2)$$

where K^S_i is the binding coefficient of sulfate to vesicular interior, $[S_i]$ corresponds to the internal sulfate concentration and other symbols are the same as equation (1). Transport

parameters were calculated as above and were: apparent K^S_i = 3.68 $\pm$ 1.8 mM and apparent J^S_{max} = 125.9 $\pm$ 25.7 pmol.mg protein^{-1} . 7 s^{-1}.

Figure 2.12 indicates the influx of 0.1 mM $^{35}SO_4^{2-}$ into BBMV which were pre-loaded with various concentrations of Cl^- (10 - 400 mM). These data approximated a hyperbolic relationship which was fit to the Michaelis-Menten equation,

$$J = \frac{J^{Cl}_i [Cl_i]}{J^C_{max} + K^C_i} \qquad (3)$$

where K^C_i is the apparent binding coefficient of Cl^- to the vesicle interior, $[Cl_i]$ is the internal Cl^- concentration and other symbols are as in equation (1). Analysis indicates that the apparent K^C_i = 54.5 $\pm$ 14 mM and apparent J^C_{max} = 173 $\pm$ 13 pmol . mg protein^{-1} . 6 s^{-1} during sulfate-chloride exchange.

This series of experiments indicates that there was a higher apparent affinity of the carrier for sulfate on the external membrane surface with significant apparent diffusion of $^{35}SO_4^{2-}$ into the vesicles. It was observed that the influx of 0.1 mM sulfate reaches half-maximal rate at approximately 55 mM Cl^- (intravesicular) during sulfate-chloride exchange.

<u>^{36}Cl-sulfate exchange in BBMV</u>

An experiment was designed to determine if $^{36}Cl^-$ would exchange for internal sulfate and respond to a membrane potential in hepatopancreatic BBMV. A portion of the vesicle preparation was pre-loaded with 50 mM K_2SO_4 (50 μM valinomycin) and incubated in media containing 10 mM $^{36}Cl^-$, with or without 100 mM K-gluconate.

Another portion was pre-loaded with 100 mM KCl (valinomycin) and incubated in media containing $^{35}SO_4^{2-}$, with or without 100 K-gluconate. These conditions provided vesicles which were either short-circuited or were inside-negative. Figure 2.13 indicates that $^{35}SO_4^{2-}$ influx (Fig 13a) was inhibited by an inside-negative diffusional potential as compared to short-circuited vesicles (as in Fig 2.4), while $^{36}Cl^-$ influx (Fig 2.13b) was enhanced by an inside-negative K^+ diffusion potential over the short circuited condition. These data suggest that the chloride-sulfate exchange occurred in a 1:1 ratio and that the excess negative charge, of the internal SO_4^{2-}, was repelled by a negative intravesicular space.

Effect of inhibitors and competitors on sulfate influx

Potential anion exchange transport inhibitors and competitive ions were tested in BBMV pre-loaded with 100 mM TMA-gluconate, 100 mM KCl and 50 μM valinomycin (Figure 2.14). Incubation media consisted of 100 TMA-gluconate, 100 K-gluconate and either 10 mM of Cl^-, HCO_3^- or $S_2O_3^-$, or 1 mM DIDS, SITS, bumetanide or furosemide. The 15 second uptake of $^{35}SO_4^{2-}$ was strongly inhibited by DIDS, SITS and $S_2O_3^-$, while Cl^-, HCO_3^-, bumetanide and furosemide slightly, but significantly, reduced uptake.

DISCUSSION

In the current investigation we presented evidence for the existence of a carrier mediated sulfate-chloride exchange on brush border membrane vesicles isolated from

lobster hepatopancreatic epithelium. Sulfate carriers have been described for the brush border of several vertebrate tissues. Both sodium-sulfate co-transport and sulfate-hydroxyl exchange mechanisms, in the same BBMV, have been demonstrated in rabbit ileal brush border (Schneider et al.,1984; Schron et al., 1985). In avian renal BBMV multiple pathways were shown to transport sulfate; sodium-sulfate co-transport, sulfate-bicarbonate exchange and proton-dependent sulfate transport (Renfro et al., 1987). Marine teleost renal tubule BBMV have been shown to contain a sulfate-anion exchange mechanism which is most effective with bicarbonate (Renfro and Pritchard, 1983), whereas no sodium or proton stimulation of sulfate transport was observed in this brush border (Renfro and Pritchard, 1982).

In the lobster hepatopancreas in vivo there is a pH gradient maintained across the epithelium with a lower pH in the lumen than in the blood (Gifford, 1962). We therefore tested the effect of pH gradients on the uptake of sulfate into BBMV. Sulfate-chloride exchange was diminished when the internal pH was less than that of the external pH and sulfate uptake was enhanced as the external pH was decreased (constant internal pH). This suggested that an extravesicular pH lower than internal pH could stimulate sulfate-chloride (or sulfate/hydroxyl) exchange. When pH was held constant on both sides of BBMV (Fig 2.7) there was increased uptake at lower pH, an effect similar to that obtained in Figure 2.6. This result suggested that it was not the pH gradient, but the lower pH (increased external protons), which stimulated sulfate-chloride exchange. An equilibrium shift experiment was designed to determine if a pH gradient (pH_{in} = 8.0, pH_{out} = 6.0) could provide the driving force to accumulate $^{35}SO_4^{2-}$ into BBMV under

equilibrated $^{35}SO_4^{2-}$ and chloride conditions (Fig 2.8). A pH gradient or varying absolute pH did not stimulate SO_4^{2-} uptake in the absence of internal Cl^- (Figure 2.9).

This series of experiments suggests that a proton (or hydroxyl) gradient does not act as a driving force during sulfate-chloride exchange, nor could it stimulate uptake alone, yet there were significant effects of varying pH on the magnitude of sulfate-chloride exchange. All of the experiments were short-circuited by the presence of valinomycin and equimolar potassium across the BBMV so that H^+-generated transmembrane diffusion would be unlikely to cause the results observed in Figures 2.5 and 2.6. Protons have been shown to act as allosteric activators of the Na/H exchanger in rabbit renal BBMV (Aronson et al., 1982) and Na-SO_4 co-transport in rabbit ileum BBMV (Ahearn and Murer, 1984). The results observed in Figs 2.7 and 2.9 would support the idea of external protons having a modifier role of stimulating the sulfate-chloride exchanger. There may also be a pH-sensitive regulatory site on the internal surface of the transport protein which can inhibit sulfate-chloride exchange. One must be careful in comparing results from different vesicle preparations, but the magnitude of the external pH effect (Fig 2.6 and 2.7) is twice that of the internal effect (Fig 2.5) which suggests that the external modifier site is more important in transport regulation.

The existence of internal pH-sensitive regulatory sites for the Cl-HCO_3 exchanger has been demonstrated on the rabbit ileal brush border membrane using membrane vesicles (Mugharbil et al., 1990), and in isolated cell preparations of lymphocytes (Mason et al., 1989) and Vero cells (Olsnes, 1986). An external pH-sensitive regulatory site on the sulfate-chloride exchanger in the hepatopancreatic brush border membrane

would be physiologically important due to the 2 Na/H exchanger also present in the membrane (Ahearn et al., 1990). The 2 Na/H exchanger operates during luminal acidification following ingestion of a meal, and would provide a stimulus for enhanced sulfate-chloride exchange. When the sodium-proton exchanger is operating there would be an increase in the $[H^+]$ in the hepatopancreatic lumen, and a corresponding decrease of protons in the cytoplasm. This would tend to enhance the sulfate-chloride exchanger due to modification at an external site as in Figure 2.6, and regulation at an internal site as in Figure 2.5 (via a decrease in internal $[H^+]$).

The effect of valinomycin-induced K^+ diffusion potentials on sulfate-chloride exchange was investigated to determine the effect of membrane potential on transport in BBMV. Uptake was measured under both inside-negative and inside-positive membrane potentials (100 mM K^+ on respective side of vesicle) and compared to short-circuited conditions with bilaterally equimolar potassium. Figure 2.3 indicates that sulfate-chloride exchange is stimulated by a positive vesicular interior while inhibited by a negative vesicular interior. These data suggest that there is an excess of negative charge transferred into the vesicle during the exchange process. The effect of membrane potential on sulfate-sulfate exchange is compared to sulfate-chloride exchange in Fig 2.4. Vesicles were pre-loaded with either 100 mM Cl^- or 10 mM SO_4^{2-} and the uptake of $^{35}SO_4^{2-}$ was measured under the above membrane potentials. It was observed that alteration of transmembrane potential had no effect on sulfate-sulfate exchange while sulfate-chloride exchange was affected as above (Fig 2.3). These data support the idea that the carrier can accommodate either one SO_4^{2-} ion or one Cl^- ion which would result

in electroneutral sulfate-sulfate exchange or electrogenic sulfate-chloride exchange. These findings are in contrast with the reports of other investigations on sulfate carriers and electrical coupling in BBMV. Sulfate-bicarbonate exchange was unaffected by valinomycin-induced potassium diffusion potentials in both flounder renal brush border (Renfro and Pritchard, 1983) and rat renal cortex BBMV (Pritchard, 1987). Electrical coupling was not supported in pH-gradient stimulated SO_4^{2-} uptake in rabbit ileal BBMV (Schron et al., 1985a), while Na^+-SO_4^{2-} co-transport, in the same organ, was variably affected depending on pH and Na^+ concentration (Ahearn and Murer, 1984). In rat and rabbit renal cortical BBMV Na-dependent sulfate transport was shown to be an electroneutral process (Lucke et al., 1979; Schneider et al., 1984).

The influx of $^{35}SO_4^{2-}$ in hepatopancreatic BBMV occurred by at least one carrier mediated mechanism exhibiting Michaelis-Menten kinetics and a second process which may be simple diffusion (Fig 2.10). The apparent binding constant for sulfate association with the vesicular interior is an order of magnitude higher than that observed for the vesicular exterior. The estimated J_{max} of both sulfate and chloride loaded vesicles was similar, while the estimated binding of sulfate to vesicle interior was considerably less than that for chloride. The cellular concentrations of these ions is unknown, hence it is not possible to determine which of these binding constants is physiologically relevant. It is possible that the high internal Cl^- would have caused a cis-inhibition of sulfate/sulfate exchange due to the experimental conditions which resulted in a spill-over of unlabeled chloride. The magnitude of cis-inhibition by Cl^- has not yet been determined.

The sulfate-chloride exchanger was significantly inhibited by thiosulfate and the disulfonic stilbenes DIDS and SITS (Fig 2.14) as reported by various investigators (Pritchard, 1987; Renfro et al., 1987; Renfro and Pritchard, 1983). Furosemide and bumetanide were not as effective as with rabbit ileal BBMV (Schron et al., 1985b) or bovine kidney tubule BBMV (Talor et al., 1987). The strong inhibition of DIDS and SITS provides further evidence for the presence of an anion exchanger in hepatopancreatic BBMV.

Under the assumption that exchange mechanisms such as this can operate in both directions it should be possible to measure the uptake of $^{36}Cl^-$ into vesicles in exchange for internal sulfate. If the exchange operated at a 1:1 ratio it would be expected to also react to a membrane potential. It was observed that $^{36}Cl^-$ influx did respond to an inside-negative potential (Fig 2.13b) by enhancing the uptake at 1 min incubation. With this arrangement there would be a secretion of sulfate from the vesicular interior in exchange for luminal chloride which is further driven by the inside negative potential that is characteristic of epithelial cells (e.g. Ahearn, 1982; Gerencser, 1985). Given the above binding constants there is a higher apparent affinity of the vesicular interior for sulfate which supports the idea of sulfate being transferred from the cell interior to the lumen. This phenomenon would support the work of some investigators who have postulated that the crustacean gut can provide an excretory function in the elimination of certain solutes (Dall, 1970; Gifford, 1962).

From this study it can be tentatively suggested that the hepatopancreas of the intermolt lobster may play a role in the secretion of the divalent anion sulfate. The

mechanism of sulfate transport across the basolateral membrane needs to be characterized in order to more clearly ascertain the net flux direction of this ion across the epithelium. It is very likely that the hepatopancreas can transport sulfate in both directions depending on physiological, environmental and even hormonal conditions.

FIGURE 2.1

Time course of 0.1 mM $^{35}SO_4^{2-}$ uptake by hepatopancreatic BBMV. Vesicles contained (in mM) 100 TMA-gluconate and 50 K- gluconate (■,□) or 100 TMA-gluconate, 25 K-gluconate and 25 $KHCO_3$ (●). Incubation media contained 100 TMA-gluconate and 50 K- gluconate (□,●) or 100 Na-gluconate and 50 K-gluconate (■). All media contained 40 HEPES-Tris and 50 μM valinomycin at pH 7.0.

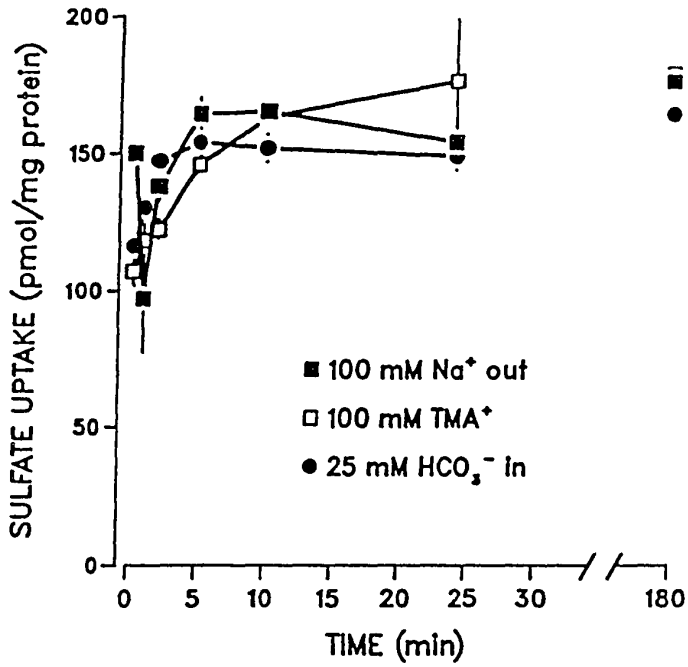

SULFATE UPTAKE (pmol/mg protein)
200
150
100
50
0
0
5
10
15
20
25
30
180
TIME (min)
100 mM Na+ out
100 mM TMA+
25 mM HCO3- in

FIGURE 2.2

Time course of 0.1 mM $^{35}SO_4^{2-}$ uptake by hepatopancreatic BBMV. Vesicles were pre-loaded with (in mM) 100 TMA-gluconate, 25 K-gluconate, 25 KCl and 50 μM valinomycin (■), 150 TMA-gluconate and 50 μM CCCP (□) or 100 TMA-gluconate, 50 K-gluconate and 50 μM valinomycin (●). Incubation media contained 100 TMA-gluconate and 50 K-gluconate (■, ●) or 100 K-gluconate and 50 TMA-gluconate (□). All media contained 40 HEPES-Tris at pH 7.0.

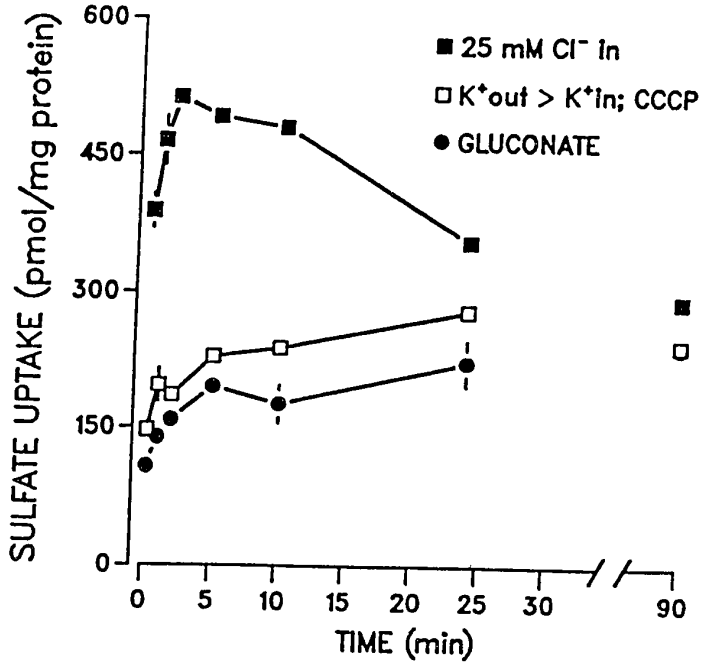

600
450
300
150
0
SULFATE UPTAKE (pmol/mg protein)
25 mM Cl⁻ in
K⁺out > K⁺in; CCCP
GLUCONATE
0
5
10
15
20
25
30
90
TIME (min)

FIGURE 2.3

Effect of membrane potental on exchange driven uptake of 0.1 mM $^{35}SO_4^{2-}$ uptake into hepatopancreatic BBMV. Vesicles were pre-loaded with (in mM) 100 TMA-gluconate and 100 TMA-Cl (■) or 100 K- gluconate and 100 TMA-Cl (□, ●). Incubation media contained 100 K- gluconate and 100 TMA-gluconate (■, □) or 200 TMA-gluconate (●). All media contained 40 HEPES-Tris and 50 μM valinomycin at pH 7.0.

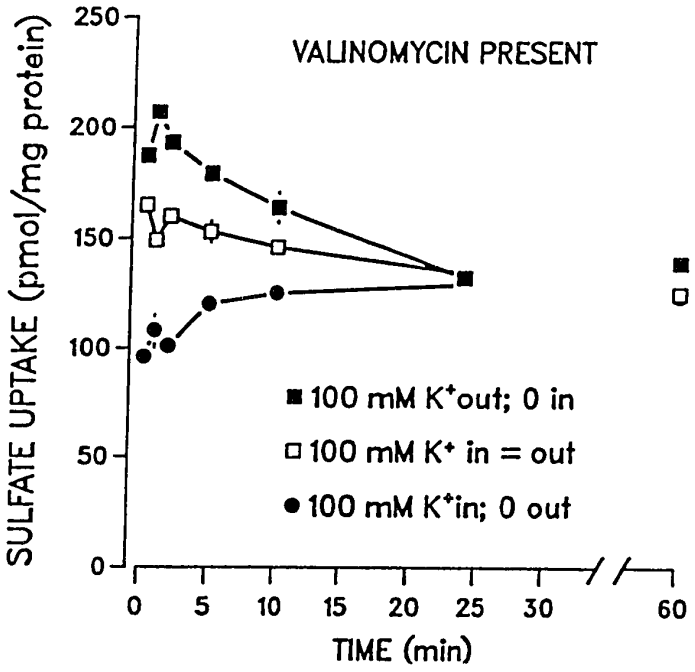

VALINOMYCIN PRESENT
SULFATE UPTAKE (pmol/mg protein)
250
200
150
100
50
0
0
5
10
15
20
25
30
60
TIME (min)
■ 100 mM K⁺out; 0 in
□ 100 mM K⁺ in = out
● 100 mM K⁺in; 0 out

FIGURE 2.4

Effect of membrane potential on sulfate-chloride and sulfate-sulfate exchange in hepatopancreatic BBMV. Media as follows (in mM): (Cl/NEG) inside, 100 TMA-Cl and 100 K-gluconate; outside, 200 TMA-gluconate. (Cl/SC) in, 100 TMA-Cl and 100 K-gluconate; out, 100 TMA-gluconate and 100 K-gluconate. (Cl/POS) in, 100 TMA-Cl and 100 TMA-gluconate; out, 100 K-gluconate and 100 TMA-gluconate. (SO_4/NEG) in, 10 Na_2SO_4, 100 K-gluconate and 85 TMA-gluconate; out, 200 TMA-gluconate. (SO_4/SC) in, 10 Na_2SO_4, 100 K-gluconate and 85 TMA-gluconate; out, 100 K-gluconate and 100 TMA-gluconate. (SO_4/POS) in, 10 Na_2SO_4, 185 TMA-gluconate; out, 100 K-gluconate and 100 TMA-gluconate. All media contained 40 HEPES-Tris and 50 μM valinomycin at pH 7.0. All incubation media contained 0.5 mM $^{35}SO_4^{2-}$.

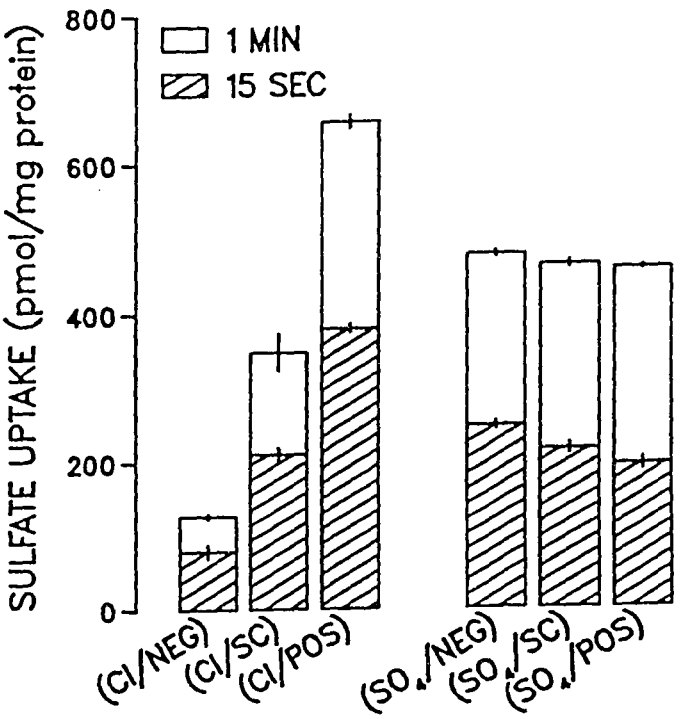

800
600
400
200
0
SULFATE UPTAKE (pmol/mg protein)
1 MIN
15 SEC
(Cl/NEG)
(Cl/SC)
(Cl/POS)
(SO4/NEG)
(SO4/SC)
(SO4/POS)

FIGURE 2.5

Influence of internal pH on the uptake of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BBMV. Vesicles contained (in mM) 100 TMA-Cl, 50 K-gluconate, 50 μM valinomycin and pH adjusted to 7.0 (□) or 8.0 (■) with 40 HEPES-Tris, or pH 5.0 (○) or 6.0 (●) with 40 MES-Tris. Incubation media contained 100 TMA-gluconate, 50 K-gluconate and 40 HEPES-Tris at pH 7.0.

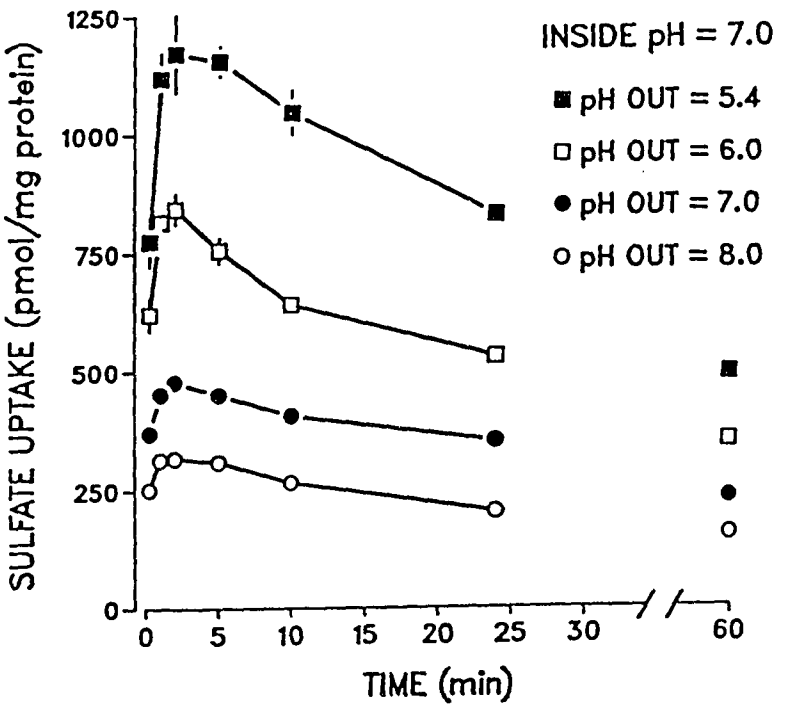

INSIDE pH = 7.0
pH OUT = 5.4
pH OUT = 6.0
pH OUT = 7.0
pH OUT = 8.0
SULFATE UPTAKE (pmol/mg protein)
1250
1000
750
500
250
0
0
5
10
15
20
25
30
60
TIME (min)

FIGURE 2.6

Influence of external pH on the uptake of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BBMV. Vesicles contained (in mM) 100 TMA-Cl, 50 K-gluconate, 50 μM valinomycin and 40 HEPES-Tris at pH 7.0. Incubation media 100 TMA-gluconate, 50 K-gluconate and pH was adjusted to 7.0 (●) or 8.0 (○) with 40 HEPES-Tris, or pH 5.4 (■) or 6.0 (□) with MES-Tris.

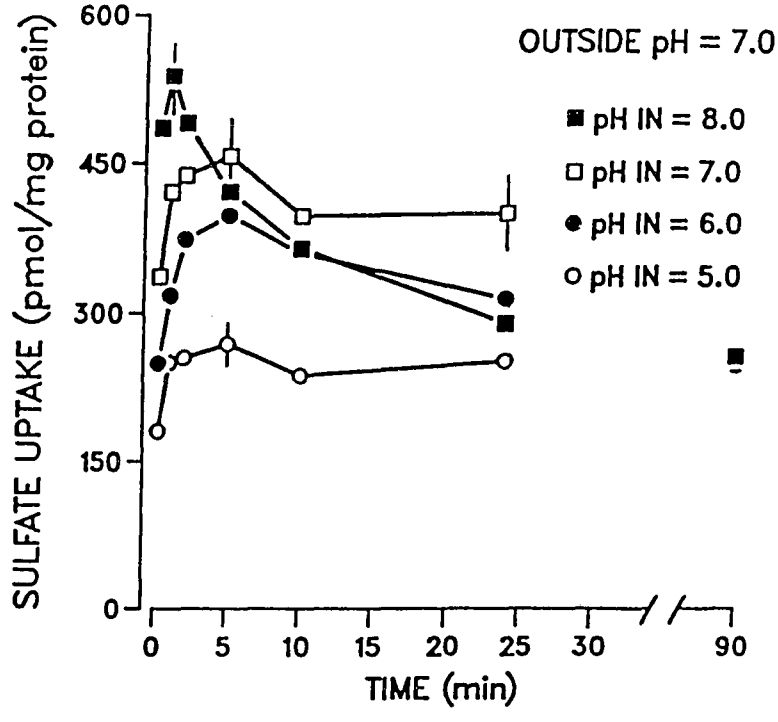

SULFATE UPTAKE (pmol/mg protein)
600
450
300
150
0
0
5
10
15
20
25
30
90
TIME (min)
OUTSIDE pH = 7.0
pH IN = 8.0
pH IN = 7.0
pH IN = 6.0
pH IN = 5.0

FIGURE 2.7

Effect of various bilateral pH conditions on the uptake of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BBMV. Vesicles contained (in mM) 100 TMA-Cl, 50 K-gluconate, 50 μM valinomycin and adjusted to pH 7.0 (●) or 8.0 (○) with 40 HEPES-Tris, or to pH 5.4 (■) or 6.0 (□) with 40 MES-Tris. Incubation media contained 100 TMA-gluconate and 50 K-gluconate with pH adjusted as above.

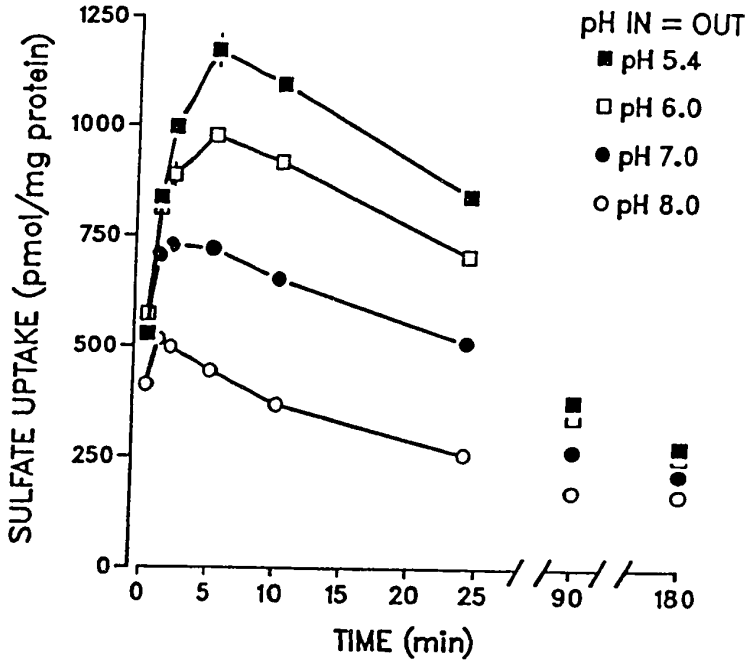

SULFATE UPTAKE (pmol/mg protein)
1250
1000
750
500
250
0
0
5
10
15
20
25
90
180
TIME (min)
pH IN = OUT
pH 5.4
pH 6.0
pH 7.0
pH 8.0

FIGURE 2.8

Effect of a pH gradient combined with equilibrated Cl and a Cl gradient alone on the time course of 0.1 mM $^{35}SO_4^{2-}$ uptake into hepatopancreatic BBMV. Vesicles were pre-equilibrated for 2 hours with (in mM) 0.1 $K_2[^{35}S]O_4$, 100 TMA-Cl, 100 TMA-gluconate, 50 K- gluconate, 50 μM valinomycin and 40 HEPES-Tris at either pH 6.0 (□) or 8.0 (■). Incubation media contained the same salts (□,■) or the replacement of TMA-Cl by TMA-gluconate (●) to establish a Cl gradient. All incubation media maintained at pH 6.0.

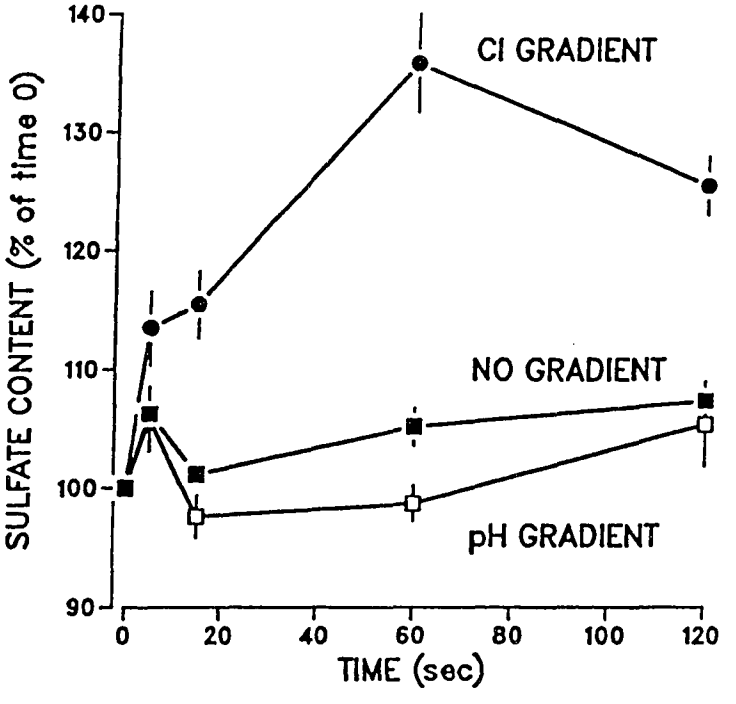

SULFATE CONTENT (% of time 0)
140
130
120
110
100
90
0
20
40
60
80
100
120
TIME (sec)
Cl GRADIENT
NO GRADIENT
pH GRADIENT

FIGURE 2.9

Effect of pH and internal Cl^- on 0.1 mM $^{35}SO_4^{2-}$ uptake into hepatopancreatic BBMV. Vesicles were pre-loaded with (in mM) 100 TMA-gluconate, 100 K-gluconate, 50 μM valinomycin and adjusted to pH 9 with 40 HEPES-TRIS, or pH 6 with 40 MES-TRIS. Chloride pre-loaded vesicles (hatched bars) contained 100 KCl replacing K-gluconate. Incubation media contained 0.10 $^{35}SO_4^{2-}$, 100 TMA-gluconate, 100 K-gluconate and adjusted to pH 9 with 40 HEPES-TRIS, or pH 6 with 40 MES-TRIS.

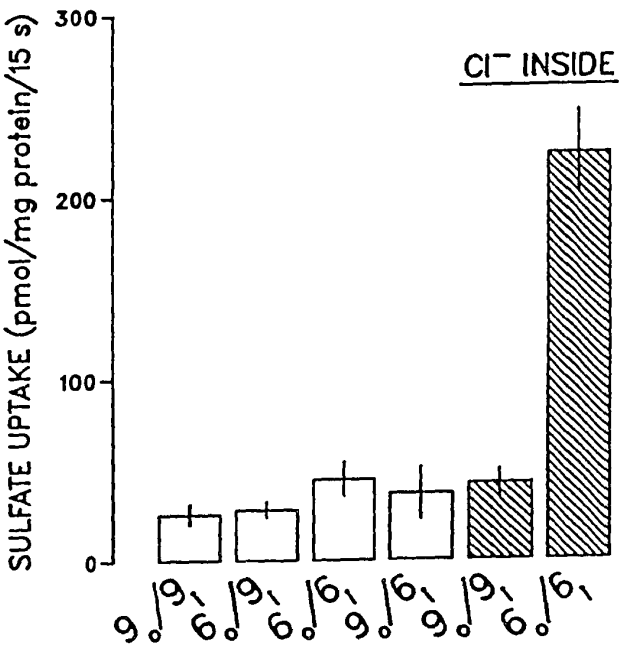

SULFATE UPTAKE (pmol/mg protein/15 s)
300
200
100
0
Cl⁻ INSIDE
9
9
6
9
6
6
9
6
9
9
6
6

FIGURE 2.10

Effect of external sulfate concentration on the uptake of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BBMV. Vesicles were pre-loaded with 100 mM TMA-Cl, 50 K-gluconate, 50 μM valinomycin. Incubation media contained 100 mM TMA-gluconate, 50 mM K-gluconate and indicated sulfate concentrations. All media contained 40 mM HEPES-Tris at pH 7.0.

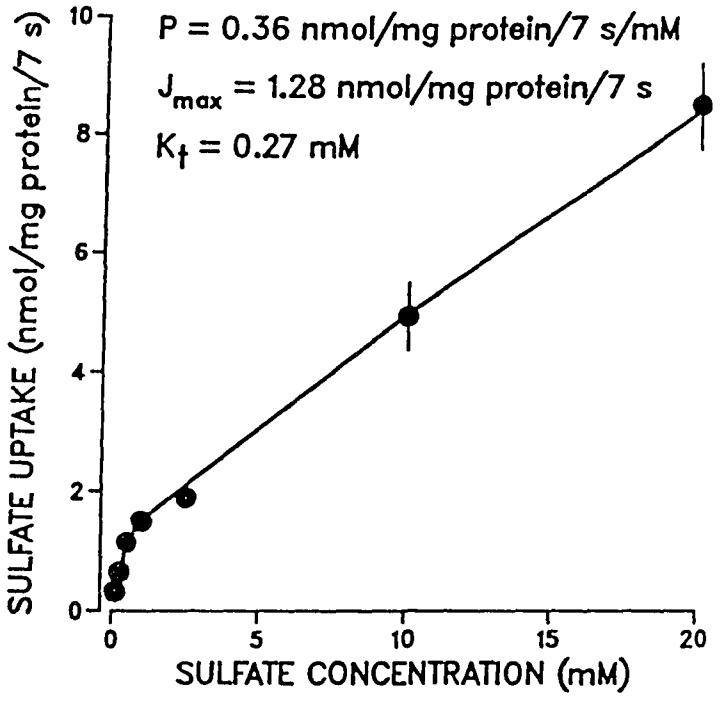

P = 0.36 nmol/mg protein/7 s/mM
J_{max} = 1.28 nmol/mg protein/7 s
K_t = 0.27 mM
SULFATE UPTAKE (nmol/mg protein/7 s)
SULFATE CONCENTRATION (mM)
0
2
4
6
8
10
0
5
10
15
20

FIGURE 2.11

Effect of internal sulfate concentration on the uptake of 0.5 mM $^{35}SO_4^{2-}$ into hepatopancreatic BBMV. Vesicles were pre-loaded with (in mM) 125 TMA-gluconate, 50 K-gluconate and indicated K_2SO_4 concentrations (K-gluconate was altered to maintain 50 mM K+ and osmolality). Incubation media contained 125 TMA-gluconate, 50 K-gluconate and 0.5 mM ^{35}S-sulfate. All media contained 40 HEPES-Tris at pH 7.0.

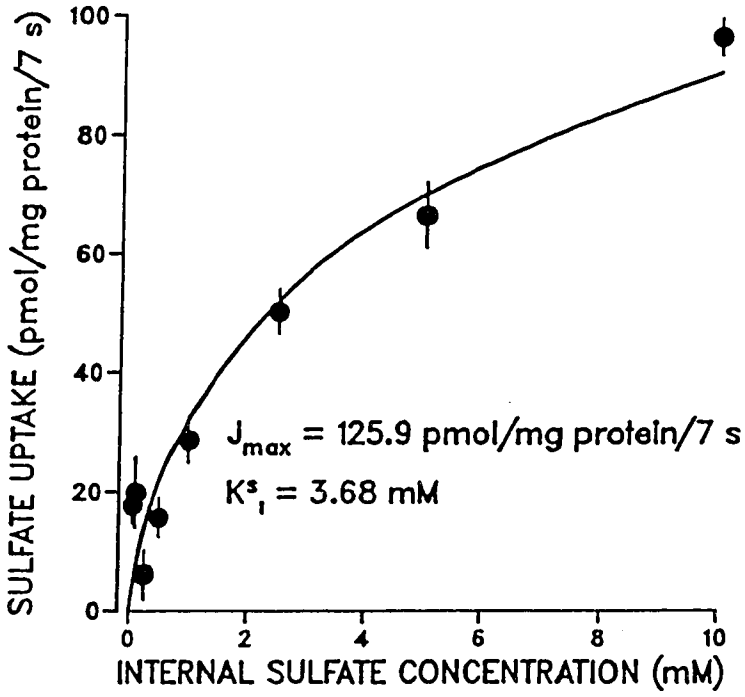

SULFATE UPTAKE (pmol/mg protein/7 s)
100
80
60
40
20
0
0
2
4
6
8
10
INTERNAL SULFATE CONCENTRATION (mM)
J_{max} = 125.9 pmol/mg protein/7 s
K^s_i = 3.68 mM

FIGURE 2.12

Effect of internal chloride concentration on the influx of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BBMV. Vesicles were pre-loaded with 100 K-gluconate, 10 - 250 mM TMA-Cl, 50 - 290 mM TMA-gluconate and 50 μM valinomycin. Incubation media contained 300 mM TMA-gluconate, 100 mM K-gluconate and 0.1 mM $^{35}SO_4^{2-}$. All media contained 40 mM HEPES-Tris at pH 7.0.

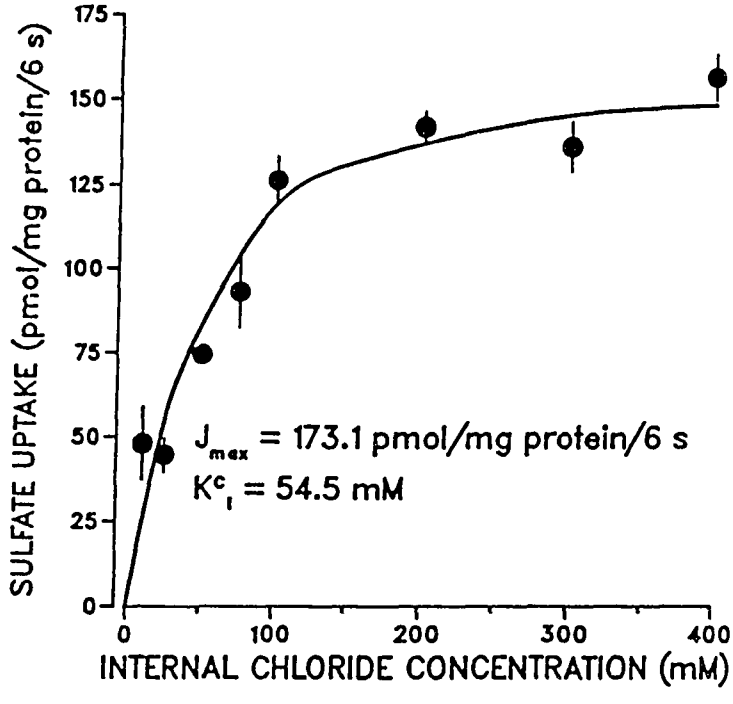

SULFATE UPTAKE (pmol/mg protein/6 s)
175
150
125
100
75
50
25
0
0
100
200
300
400
INTERNAL CHLORIDE CONCENTRATION (mM)
J_{max} = 173.1 pmol/mg protein/6 s
K^c_i = 54.5 mM

FIGURE 2.13

Effect of inside-negative vesicular interior on (A) $^{35}SO_4^{2-}$-chloride exchange and (B) $^{36}Cl^-$-sulfate exchange in hepatopancreatic BBMV. A: vesicles were pre-loaded with (in mM) 100 KCl and 100 TMA-gluconate and incubated in media containing 0.1 mM $^{35}SO_4^{2-}$ and either 100 K-gluconate and 100 TMA-gluconate (open bar) or 200 TMA- gluconate (hatched bar). B: vesicles were pre-loaded with 50 K_2SO_4 and 75 TMA-gluconate and incubated in media containing 10 mM $^{36}Cl^-$ and either 100 K-gluconate and 100 TMA-gluconate or 200 TMA-gluconate. All media contained 40 HEPES-TRIS at pH 7.5.

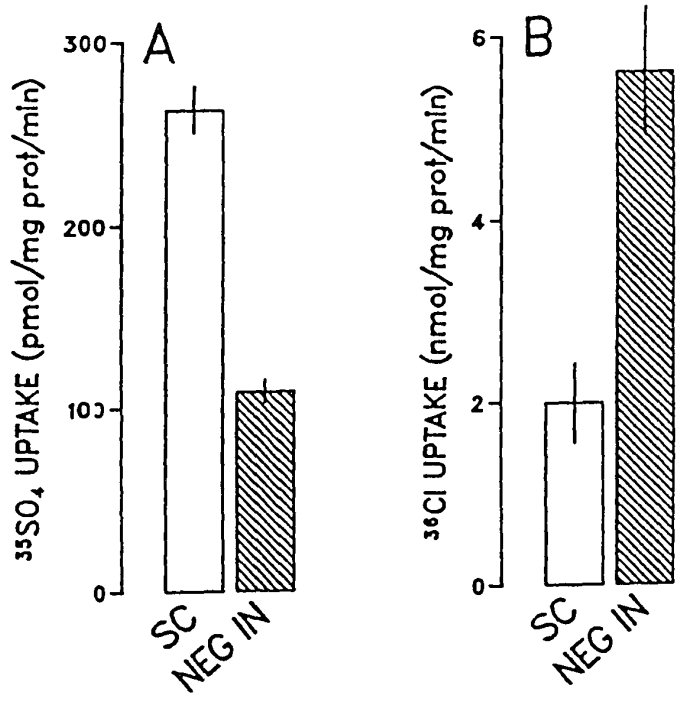
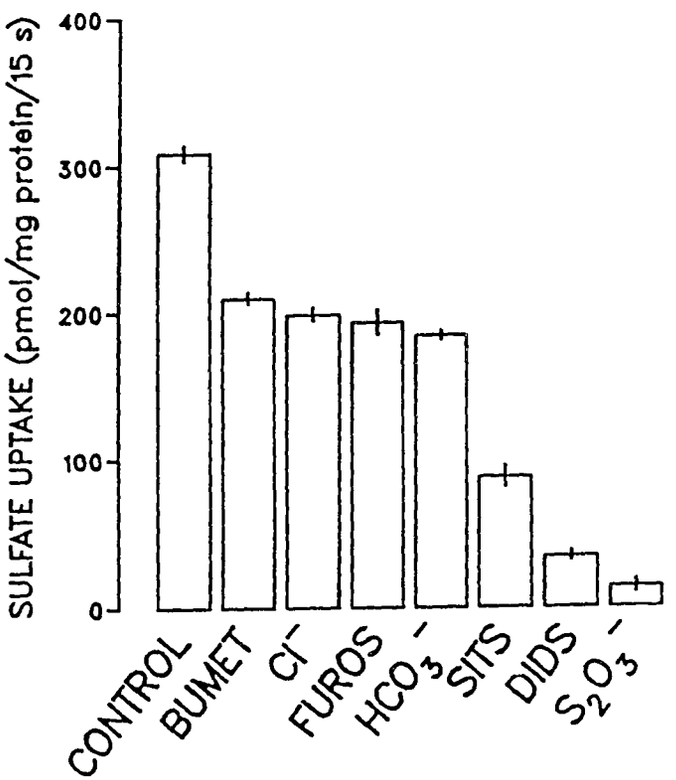

A
300
200
100
0
$^{35}SO_4$ UPTAKE (pmol/mg prot/min)
SC
NEG IN
B
6
4
2
0
^{36}Cl UPTAKE (nmol/mg prot/min)
SC
NEG IN

FIGURE 2.14

Effect of potential inhibitors on $^{35}SO_4^{2-}$-Cl^- exchange in hepatopancreatic BBMV. Vesicles were pre-loaded with (in mM) 200 TMA-gluconate, 100 KCl, 50 μM valinomycin. Incubation media contained either 0.1 $K_2[^{35}S]O_4$, 200 mM TMA-gluconate, 100 K-gluconate and either 1 SITS, DIDS, bumetanide or furosemide, or 190 mM TMA-gluconate, 100 K-gluconate and either 10 NaCl, $NaHCO_3$, or NaS_2O_3. All media contained 40 HEPES-TRIS at pH 7.0.


SULFATE UPTAKE (pmol/mg protein/15 s)
400
300
200
100
0
CONTROL
BUMET
Cl⁻
FUROS
HCO3⁻
SITS
DIDS
S2O3⁻

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CHAPTER III

SULFATE-OXALATE EXCHANGE BY LOBSTER HEPATOPANCREATIC BASOLATERAL MEMBRANE VESICLES

SUMMARY

Purified basolateral membrane vesicles (BLMV) were prepared from lobster hepatopancreas by osmotic disruption and discontinuous sucrose gradient centrifugation. ^{35}[S]ulfate uptake was stimulated by 10 mM intravesicular oxalate when compared to gluconate loaded vesicles. Sulfate-oxalate exchange was not affected by transmembrane valinomycin-induced potassium diffusion potentials (inside-negative or inside-positive) suggesting electroneutral anion transport. Sulfate uptake was not stimulated by the similar carboxylic anions formate, succinate, oxaloacetate or ketoglutarate. Sulfate (0.1 mM) uptake by BLMV was a hyperbolic function of internal oxalate concentration (0.10 - 10 mM) with apparent kinetic constants of $K_m = 0.18 \pm 0.06$ mM and $J_{max} = 268 \pm 18$ pmol/mg protein/7 s. Sulfate influx occurred by at least one saturable Michaelis-Menten carrier system, apparent $K_m = 6 \pm 1.7$ mM and $J_{max} = 382.3 \pm 17$ pmol/mg protein/7 s. Sulfate-oxalate exchange was significantly reduced by the anion antiport inhibitors DIDS and SITS, but not affected by bumetanide or furosemide. The possible role of this exchange mechanism in transepithelial sulfate transport across the crustacean hepatopancreas is discussed.

INTRODUCTION

Sulfate transport across serosal plasma membranes has been described in a wide variety of organisms and cell types including intestinal (Hagenbuch et al., 1985; Knickelbein et al., 1986; 1990; Langridge-Smith and Field, 1981; Schron et al., 1987), renal (Pritchard and Renfro, 1983; Renfro et al., 1987; Renfro and Pritchard, 1982; Shiu-Ming and Aronson, 1988) and liver (Hugentobler et al., 1987; Hugentobler and Meier, 1986; Meier et al., 1987) epithelia. The mechanisms involved in sulfate uptake differ considerably among the many systems which have been investigated. Transport can be coupled to proton (Hugentobler and Meier, 1986; Renfro and Pritchard, 1982) and sodium (Langridge-Smith and Field, 1981) cotransport, or anion exchange, i.e., bicarbonate or chloride, (Knickelbein and Dobbins, 1990; Pritchard and Renfro, 1983; Renfro et al., 1987; Schron et al., 1987). In many of these reports it has been reported that sulfate transport can be stimulated by exchange with a number of organic anions, especially oxalate, (Hugentobler et al., 1987; Hugentobler and Meier, 1986; Knickelbein et al., 1986; Knickelbein and Dobbins, 1990; Meier et al., 1987; Shiu-Ming and Aronson, 1988; Ullrich et al., 1987). Although carrier-mediated sulfate transport is observed in the basolateral membrane of numerous vertebrate systems, the net handling of this anion can be either absorptive or secretory in nature, depending on the specific animal and organ. The physiological properties associated with sulfate regulation in these diverse organisms include cell and plasma osmoregulation, maintenance of ion composition and acid-base balance.

In crustaceans, the hepatopancreas is involved in both digestion and absorption of nutrients (Gibson and Barker, 1979; Loizzi, 1971; Yonge, 1924), in addition some investigators have implicated this organ as a site of excretion as well (Dall, 1970; Gifford, 1962; Holliday and Miller, 1984). Recently the use of isolated membrane vesicles has led to the definition of absorptive and exchange mechanisms for a number of solutes in the hepatopancreas brush-border (Ahearn and Clay, 1987a, 1987b; Ahearn et al., 1985, 1990; Cattey et al., 1992) and a chloride-bicarbonate exchange process (Ahearn et al., 1987) in the basolateral membrane. To date there have been no empirical studies which suggest the transepithelial secretion of a solute by the lobster hepatopancreas.

The present study utilizes hepatopancreatic basolateral membrane vesicles (BLMV) to characterize a sulfate-oxalate exchange mechanism which is specific with respect to organic anions, is inhibited by stilbenes and is unaffected by pH. This investigation in combination with our recent report of sulfate-chloride exchange in the brush border of lobster hepatopancreas (Cattey et al., 1992) can provide a mechanism for the net epithelial secretion of this divalent anion.

METHODS

Live Atlantic lobsters (*Homarus americanus*, 0.5 kg each) were purchased from commercial dealers in Hawaii and maintained unfed at 10°C for up to 1 wk in filtered

seawater. All animals in this study were either in intermolt or early premolt as assessed by the molt stage classification scheme introduced by Aiken (1973).

Hepatopancreatic basolateral membrane vesicles were prepared from fresh tissue removed from individual lobsters. Each membrane batch was produced from 10 g fresh wt from one organ using a modification of the method described by Ahearn et al. (1987). In the present study hepatopancreatic tissue was homogenized for 30 s with a Polytron (Brinkmann Instruments) in 300 ml of bomb buffer [25 mM NaCl, 10 mM Tris(hydroxymethyl)aminomethane-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonicacid (Tris-HEPES), pH 8.0] containing 0.2 mM phenylmethylsulfonyl fluoride (PSMF). The homogenate was centrifuged for 15 min at 500 g with a Sorvall RC-5C high-speed centrifuge with an SS-34 fixed angle rotor. After suction removal of a surface lipid layer, the supernatant was centrifuged for 33 min at 100,000 g with a Beckman L8-55 ultracentrifuge with an SW-28 swinging bucket rotor. The resulting pellet consisted of a dark, highly condensed central region and a fluffy, light external area. The light portion was separated from the remainder of the pellet and collected by pipet, brought up in 10 ml of 50% sucrose in gradient buffer (12.5 mM NaCl, 10 mM HEPES-Tris and 0.5 mM ethylenediaminotetraacetic acid, pH 7.4) and resuspended by passage 10 times through a small-bore Pasteur pipette. The solution was brought to a final volume of 36 ml and placed in the bottom of 4 centrifuge tubes (9 ml each) and overlayed with successive 9-ml volumes of 40, 30 and 20% sucrose in gradient buffer. The discontinuous sucrose gradient was centrifuged at 100,000 g for 93 min in a Beckman ultracentrifuge. Vesicles were collected from the 30-40% sucrose interface and brought

up in a small volume of inside buffer (composition indicated in figure legends). Vesicles were allowed to equilibrate in internal buffer at 4°C for 2 h and the solution was centrifuged at 100,000 g for 33 min. Final vesicle pellets were brought up in a small volume of inside buffer and homogenized with 15 passes through a 22-gauge needle.

Spectrophotometric assays of marker enzymes were used to establish the relative purity of the membrane preparation. Alkaline phosphatase activity was determined using Sigma kit no. 104, leucine aminopeptidase was measured by the technique of Hasse et al. (1978), and cytochrome-c oxidase activity was assessed by the method of Cooperstein and Lazarow (1951). Na-K-stimulated ATPase was measured using the phosphate liberation technique of Ames (1966) with the modifications described by Murphy and Riley (1962).

Transport studies were conducted at 15°C using the rapid filtration technique developed by Hopfer et al. (1973). For time course experiments 20 ul of vesicles was added to 180 ul of incubation media containing 0.1 mM radiolabeled $^{35}SO_4^{2-}$. At various incubation times a known volume (20 ul) of reaction mixture was removed and plunged into 2 ml of ice cold stop solution (composition varied with experiment and generally consisted of incubation media without sulfate) to stop the uptake process. The vesicle suspension was then rapidly filtered through 0.65 um Millipore filter and washed with another 7 ml of ice-cold stop solution. Filters were transferred to vials containing Beckman Ecolume scintillation cocktail and counted for radioactivity in a Beckman LS-8100 scintillation counter. Transport experiments involving incubations of < 10 s were conducted using a rapid-exposure uptake apparatus (Inovativ Labor, Adliswil,

Switzerland). Uptake was initiated by mixing 5 ul of vesicles with 95 ul of radiolabeled incubation media, and filters were washed and counted for radioactivity as above. Blank values were run for each condition by mixing stop solution, vesicles, and radiolabeled incubation media simultaneously; the resulting value was subtracted from corresponding experimental results before uptake was determined. Incubation and intravesicular media varied between experiments and are indicated in the figure legends. Sulfate uptake values were expressed as picomoles per milligram protein (Bio-Rad protein assay) per filter using the specific activity of sulfate in the incubation media.

Unless otherwise indicated, valinomycin (50 *u*M) and bilaterally equal potassium concentration across the vesicular wall were present to short-circuit the membranes. Each experiment was repeated at least three times using membranes prepared from different animals. Within a given experiment each point was determined from three to five replicate samples. Data are presented as means $\pm$ SE of a single representative experiment. Similar quantitative experimental findings were obtained in the repition of an experiment.

$^{35}SO_4^{2-}$ as the Na^+ salt was obtained from New England Nuclear. Valinomycin, 4-acetamido-4'-isothocyanostilbene-2,2'-disulfonic acid (SITS), 4,4'-diisothiocyanostilbene-2,2'- disulfonic acid (DIDS), bumetanide, furosemide, and other reagent grade chemicals were purchased from Sigma Chemical.

RESULTS

Physical characteristics of hepatopancreatic BLMV

Purity of BLMV, prepared by the centrifugation methods used in this study, was assessed by comparing the activities of membrane-bound marker enzymes assayed in the initial hepatopancreas homogenate with activities of the same enzymes in the final vesicle suspension (Table 3.1). Putative brush-border marker enzymes alkaline phosphatase and leucine amino peptidase did not exhibit increased activity in the BLMV suspension. Likewise, cytochrome-*c* oxidase, a mitochondrial marker enzyme, was not enriched in the vesicle fraction. In contrast, the activity of the basolateral membrane marker enzyme, Na-K-ATPase, was found to be increased in the vesicle suspension, with respect to the initial homogenate. These observations suggest that vesicles, prepared by the above technique, contained predominately basolateral membranes with minimal contamination from brush border or organelle membrane sources.

To confirm the closure of hepatopancreas BLMV, formed by the above preparation, and to determine the movement of sulfate into an osmotically reactive space, equilibrium (120 min) uptake of 0.1 mM ^{35}Sulfate was determined at a variety of transmembrane osmotic gradients. Vesicles were loaded with 90 mM tetramethylammonium gluconate (TMA-gluconate), 100 mM K-gluconate, 10 mM Na-oxalate, pH 7, and incubated in media containing 100 mM TMA-gluconate, 100 mM K-gluconate, 0.1 mM radiolabeled sulfate and variable quantities of sucrose at the same pH. Figure 3.1 indicates a linear association between sulfate uptake and the reciprocal of

external osmolarity, suggesting the uptake into a defined vesicular space. Extrapolation of the curve to the vertical intercept shows negligible nonspecific isotope binding at infinite osmolarity.

Transport Properties of hepatopancreatic BLMV

Figure 3.2 shows the stimulation of an outwardly directed oxalate gradient on the uptake of 0.1 mM $^{35}SO_4^{2-}$ by BLMV, when compared with vesicles lacking an exchangeable anion (gluconate only). Additionally, the possible effect due to alteration of membrane potential by valinomycin-induced potassium diffusion potentials across the vesicular wall was observed. An inside-negative potential was established using 100 mM intravesicular potassium and incubation media without K^+; and an inside-positive potential using 100 mM external potassium and no internal K^+. The short-circuited condition contained bilaterally equal potassium. Fig. 2 indicates that these manipulations of membrane potential had no effect on sulfate-oxalate exchange. This would suggest that the antiport process is electroneutral in nature.

To further ascertain the electrogenic nature of sulfate-oxalate exchange another experimental protocol was employed. Vesicle batches were loaded with 10 mM oxalate, sulfate, carbonate, or chloride respectively. Both internal and external media contained 100 mM potassium, a portion of each membrane suspension was short-circuited by exposure to valinomycin. The remaining vesicle suspension was not incubated in the ionophore. Fig 3.3 indicates that only vesicles which had been loaded with chloride exhibited differential sulfate uptake. These results suggest that, in the absence of a freely

moveable charge, there is a build up of negative potential that inhibited the movement of sulfate which exchanged for the monovalent anion. When vesicles were loaded with divalent anions there was no significant difference between the experimental conditions. These results suggest that sulfate will exchange at a 1 : 1 ratio with the above anions and that sulfate-oxalate exchange was electroneutral. It is uncertain as to whether the reduced uptake with carbonate was due to the divalent species or the monovalent bicarbonate.

Whether sulfate would exchange with a wide range of organic anions as transferable substrates was investigated by trans-stimulation with 5 mM oxalate, oxaloacetate, succinate, formate, ketoglutarate and citrate (Fig. 3.4). Only oxalate was able to stimulate 0.1 mM $^{35}SO_4^{2-}$ uptake greater than the response obtained in the presence of the non-exchangeable anion gluconate. This experiment indicates that in BLMV the antiporter is relatively specific and will not accept similar dicarboxylic acids, or the tricarboxylic acid citrate, as a substrate.

Information about the effect of various bilateral pH conditions and proton (hydroxyl) gradients was obtained for sulfate-oxalate exchange. Vesicles were loaded with 10 mM oxalate, equilibrated to pH 6, 7 or 8 and incubated in media of the same respective pH. All conditions were significantly stimulated when compared to gluconate loaded vesicles (pH 7) but the absolute proton (hydroxyl) concentration did not differentially effect sulfate uptake (Fig 3.5). Likewise, a transmembrane proton gradient established across the vesicular wall, pH 8 out and pH 6 in, or pH 6 out and pH 8 in, had no effect on sulfate-oxalate exchange. These results suggest that the simultaneous

imposition of transmembrane proton (hydroxyl) gradients, in addition to an outwardly directed oxalate gradient do not enhance sulfate uptake into lobster BLMV.

Potential anion exchange inhibitors of sulfate-oxalate antiport were tested in BLMV loaded with 10 mM oxalate (Fig 3.6). Inhibitors were added to the external media at 1 mM concentration, while the control condition was sulfate-oxalate exchange without any inhibitor. The potent stilbene compounds DIDS and SITS significantly reduced sulfate uptake while both furosemide and bumetanide had no inhibitory effect. This is further evidence that sulfate uptake into BLMV was an antiport mechanism.

The kinetic characteristics of sulfate-oxalate exchange was examined by two experimental protocols. Preliminary investigation determined that sulfate uptake into BLMV was linear for the first 15 s at the concentration employed in this study (data not shown). First, 0.1 mM sulfate influx (7 s uptake) was measured in BLMV loaded with 100 TMA gluconate, 100 mM K gluconate and various oxalate concentrations (0.1 - 10 mM) at pH 7. Incubation media contained 0.1 mM $^{35}SO_4^{2-}$, 100 TMA gluconate, 100 mM K gluconate, pH 7. Figure 3.7 shows that sulfate influx was a hyperbolic function of internal oxalate concentration. Such a relationship can be described by a Michaelis-Menten carrier mechanism illustrating saturation kinetics. This process can be described by the equation

$$J = \frac{J_{max}\,[Ox]}{K_m + [Ox]} \qquad (1)$$

where J is total sulfate influx in pmol per mg protein per 7 s, J_{max} is apparent maximal carrier mediated influx, K_m is the apparent binding coefficient of oxalate to vesicular interior, [Ox] is the internal oxalate concentration.

A non-linear iterative best-fit computer program was utilized to analyze the data in Fig. 3.7 by Eq. 1. Apparent transport parameters derived in this manner were as follows: apparent K_m = 0.18 $\pm$ 0.06 mM and apparent J_{max} = 267.9 $\pm$ 18 pmol·mg protein^{-1}·7 s^{-1}.

The second experiment assessed the role of external sulfate concentration on sulfate-oxalate exchange in BLMV. Vesicles were loaded with 10 mM K oxalate, and incubated in media containing various concentrations of sulfate (0.25 - 30 mM). Initial 7 s uptake was measured and found to be a hyperbolic function of external sulfate concentration. Figure 3.8 shows this relationship which again can be described by the Michaelis-Menten equation

$$J = \frac{J_{max}\,[S]}{K_t + [S]} \qquad (2)$$

where K_t = the apparent sulfate concentration resulting in half maximal uptake, [S] is the external sulfate concentration, and the other symbols are the same as in Eq. 1. Transport parameters were calculated as above and were apparent K_t = 6.0 $\pm$ 1.7 mM, and apparent J_{max} = 382.3 $\pm$ 37 pmol·mg protein^{-1}·7 s^{-1}.

DISCUSSION

The present study provides evidence for the occurrence of a carrier-mediated sulfate-oxalate exchanger found in BLMV isolated from lobster hepatopancreas. A number of marine invertebrates, including the lobster, have been shown to maintain plasma levels of sulfate below that which is found in seawater (Prosser, 1973; Robertson, 1949). This suggests that the organism is faced with a constant need to eliminate sulfate against a concentration gradient. The mechanism elucidated here could be utilized as one means of removing sulfate from the blood to assist in maintaining anion homeostasis.

Sulfate-dicarboxylic acid exchange has been observed in the basolateral membrane of a variety of tissues such as the rabbit ileum (Knickelbein and Dobbins, 1990), rabbit renal cortex (Shiu-Ming and Aronson, 1988) and elasmobranch liver (Hugentobler and Meier, 1986). As shown in Fig. 3.2, when BLMV are loaded with 10 mM oxalate there is stimulation of sulfate uptake over gluconate loaded vesicles. Other sulfate-oxalate exchangers will accept similar dicarboxylic acids as trans-stimulators. Oxaloacetate (Knickelbein and Dobbins, 1990) and succinate (Hugentobler and Meier, 1986) were able to exchange with sulfate in the rabbit ileum and the skate liver, respectively. Lobster BLMV were found to be conservative in the acceptance of a suitable substrate and did not utilize oxaloacetate, succinate, formate, α-ketoglutarate or citrate. It appears that in hepatopancreas BLMV the sulfate exchanger does not extend its substrate specificity to other important physiological anions.

Recently, we have described an electrogenic sulfate-chloride exchanger in brush border membranes of hepatopancreas (Cattey et al., 1992). To determine if the BLMV antiporter was similar in nature we employed experiments which altered membrane potential. First, sulfate-oxalate exchange was measured in the presence of both inside-negative and inside-positive conditions and compared to short-circuited vesicles. Figure 3.2 indicates that sulfate uptake was unaffected by these manipulations of transmembrane potential. In the second experiment we compared sulfate uptake in the presence of internal monovalent versus divalent anions under short-circuited conditions (Fig. 3.3). Sulfate was found to exchange for oxalate, sulfate, carbonate and chloride. Uptake was similar when each of the divalent anions were utilized, with or without the ionophore valinomycin present. Sulfate-chloride exchange was inhibited in vesicles which were not short-circuited suggesting a build-up of intravesicular negative charge. From this set of experiments we conclude that sulfate-oxalate exchange in BLMV is electroneutral and consists of 1 sulfate : 1 oxalate antiport.

To determine if pH had an effect on sulfate-oxalate exchange we measured uptake in the presence of various bilateral pH conditions and under both inward and outward proton gradients (Fig. 3.5). No effects of these manipulations were observed, this is in contrast to the proton stimulation of sulfate-chloride exchange described for the hepatopancreas brush border (Cattey et al., 1992).

Sulfate-oxalate exchange was strongly inhibited by the stilbene derivatives DIDS and SITS which have been shown to inhibit sulfate anion exchange in other systems (Hugentobler et al., 1987; Knickelbein and Dobbins, 1990; Meier et al., 1987; Pritchard

and Renfro, 1983). Furosemide and bumetanide will inhibit sulfate antiport in rat liver (Meier et al., 1987) and rabbit ileum (Knickelbein and Dobbins, 1990) BLMV but were not effective in our lobster preparation.

Sulfate influx into BLMV occurred by at least one carrier-mediated mechanism which exhibited Michaelis-Menten kinetics. When uptake was measured as a function of internal oxalate concentration an apparent K_m of 0.18 mM was observed suggesting a relatively high affinity of the vesicular interior to the organic anion (Fig. 3.7). Sulfate association with the vesicle exterior was shown to have an apparent K_m = 6 mM, this is in agreement with the level of sulfate (18 mM) reported in lobster plasma (Prosser, 1973; Robertson, 1949). These observations suggest that the high sulfate concentration in lobster blood could act as the driving force for sulfate-oxalate exchange across the BLMV.

Epithelial sulfate transport in mammals has been shown to be primarily absorptive in nature, yet a brush border sulfate-anion exchange pathway has been described for both rabbit proximal tubule (Brazy and Dennis, 1981) and rat renal cortex (Pritchard, 1987). This brush border antiport mechanism is similar to sulfate-anion exchangers observed in nonmammalian vertebrates (Renfro et al., 1987; Renfro and Pritchard, 1983) and may allow for bidirectional sulfate transport in these cells.

In contrast to mammals, the marine teleost kidney has been shown to be predominately involved in sulfate secretion. It has been proposed that sulfate crosses the basolateral membrane of the renal tubule epithelium via a pH-dependent, electroneutral

process (Renfro and Pritchard, 1982) and exits by way of a sodium-independent sulfate-anion exchanger located at the brush border membrane (Renfro and Pritchard, 1983).

Multiple transport pathways have been described for sulfate in the avian kidney (Renfro et al., 1987) which are suggested to play a part in different functional roles. On the brush border membrane there is a strong sodium-sulfate cotransport system similar to that found in mammals which may be involved in reabsorption, and a bicarbonate-sulfate exchanger which could mediate sulfate secretion. The basolateral membrane contains a bicarbonate-sulfate exchanger which could function in either direction depending on the respective ion concentrations. Thus, the chick renal tubule maintains transport processes which allow for both sulfate absorption and secretion.

The basolateral sulfate-oxalate exchanger described in the present investigation, in concert with the sulfate-chloride exchanger on the brush border of lobster hepatopancreas (Cattey et al., 1992) can provide a mechanism for transepithelial transport of this divalent anion. This system of transporters, arranged in series, would allow for the elimination of sulfate from the lobster hemolymph as a means of anion regulation. This proposed secretory model is the first description of a possible excretory process found within the hepatopancreas which has previously been known largely for its digestive and absorptive importance.

Table 3.1. Enzyme characterization of Atlantic lobster (*Homarus americanus*) hepatopancreatic basolateral membrane vesicles.

Enzyme	Homogenate Activity, ($umol \cdot mg^{-1} \cdot h^{-1}$)	BLMV Activity, ($umol \cdot mg^{-1} \cdot h^{-1}$)	Purification Factor	n
Alkaline phosphatase	1.21 $\pm$ 0.37	1.29 $\pm$ 0.38	1.07 $\pm$ 0.16	8
Leucine anminopeptidase	488 $\pm$ 35.1	804 $\pm$ 113	0.66 $\pm$ 0.12	3
Cytochrome-*c* oxidase	65.6 $\pm$ 2.6	15.5 $\pm$ 1.9	0.24 $\pm$ 0.03	3
Na^+-K^+-ATPase	3.81 $\pm$ 0.71	47.3 $\pm$ 5.7	13.9 $\pm$ 2.2	5

Values are means $\pm$ SE. Enzyme activities are in umol product released per mg protein per h. Purification factors are means of individual basolateral membrane vesicle (BLMV) activities / individual homogenate activities. n, number of preparations.

FIGURE 3.1

Effect of transmembrane osmotic gradients on 120 min equilibrium uptake of 0.1 mM $^{35}SO_4^{2-}$ by hepatopancreatic BLMV. Vesicles were loaded with 100 mM TMA-gluconate, 80 mM K-gluconate and 10 mM K_2-oxalate and incubated in external media containing 100 mM TMA-gluconate and 100 mM K-gluconate and variable quantities of sucrose. All media contained 40 HEPES-Tris and 50 μM valinomycin at pH 7.0.

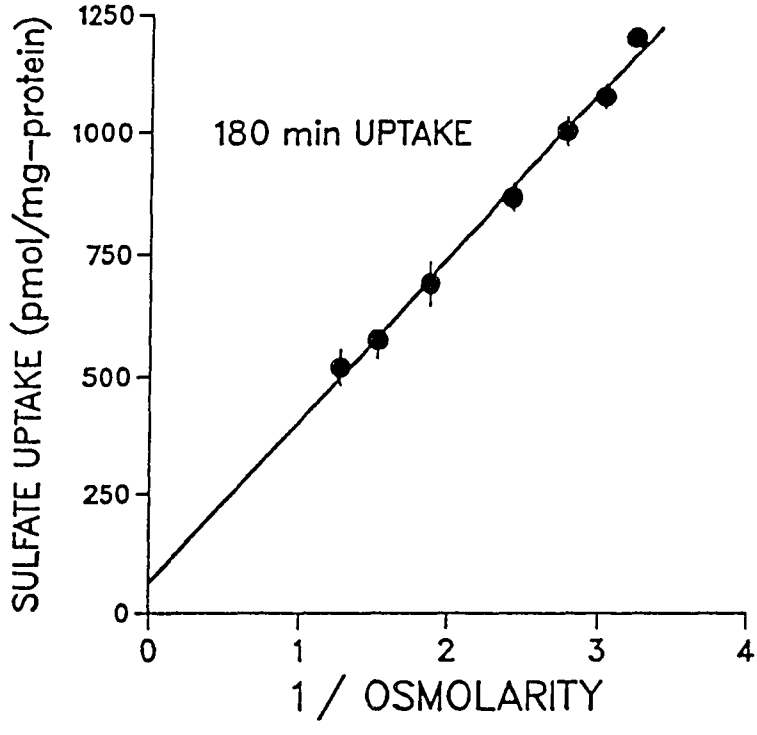

SULFATE UPTAKE (pmol/mg-protein)
1250
1000
750
500
250
0
180 min UPTAKE
0
1
2
3
4
1 / OSMOLARITY

FIGURE 3.2

Time course of 0.1 mM $^{35}SO_4^{2-}$ uptake by hepatopancreatic BLMV. Vesicles contained (OX/POS) 180 mM TMA-gluconate 10 mM K_2-oxalate; (OX/NEG, OX/SCC) 100 mM TMA-gluconate, 80 mM K-gluconate and 10 mM K_2-oxalate; (CONTROL) 100 mM TMA-gluconate, 100 mM K-gluconate. Incubation media contained (OX/POS, OX/SCC, CONTROL) 100 mM TMA-gluconate and 100 mM K-gluconate, or (OX/NEG) 200 TMA-gluconate. All media contained 40 HEPES-Tris and 50 μM valinomycin at pH 7.0.

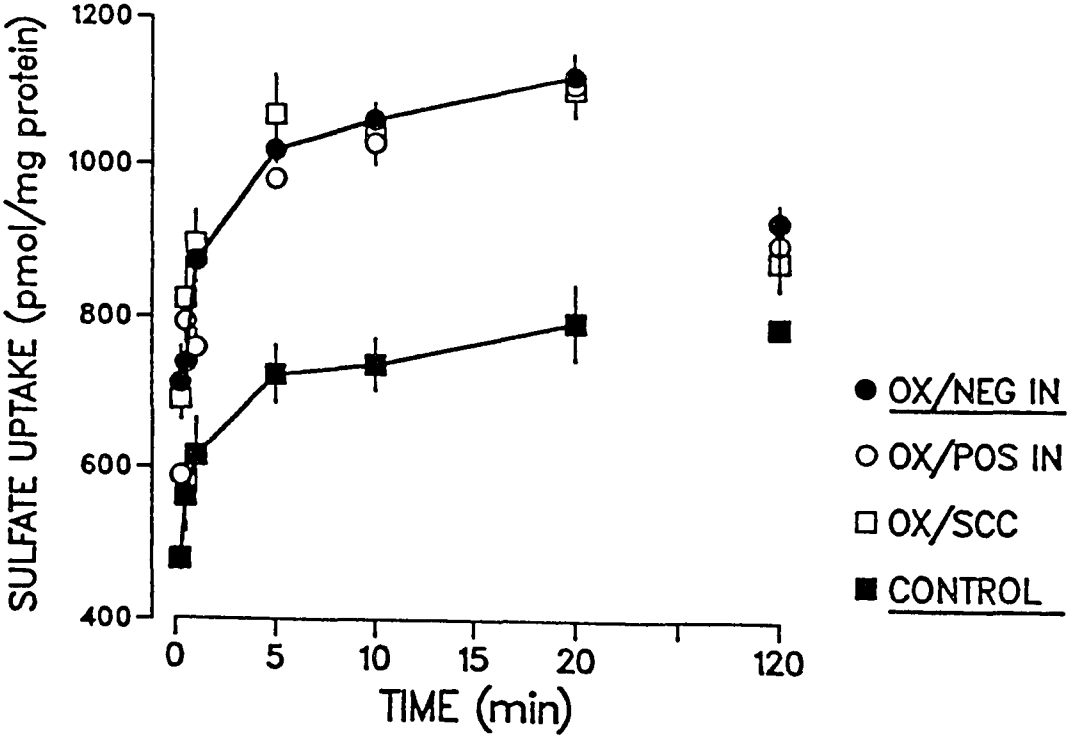

SULFATE UPTAKE (pmol/mg protein)
1200
1000
800
600
400
0
5
10
15
20
120
TIME (min)
OX/NEG IN
OX/POS IN
OX/SCC
CONTROL

FIGURE 3.3

Effect of membrane potential on exchange driven uptake of 0.1 mM $^{35}SO_4^{2-}$ uptake into hepatopancreatic BLMV. Vesicles were loaded with 100 mM TMA-gluconate and 100 mM K-gluconate and 10 mM of indicated anion (Substituted isomotically for K-gluconate). Incubation media contained 100 TMA-gluconate and 100 K- gluconate. SCC conditions also contained 50 μM valinomycin. All media contained 40 HEPES-Tris at pH 7.0.

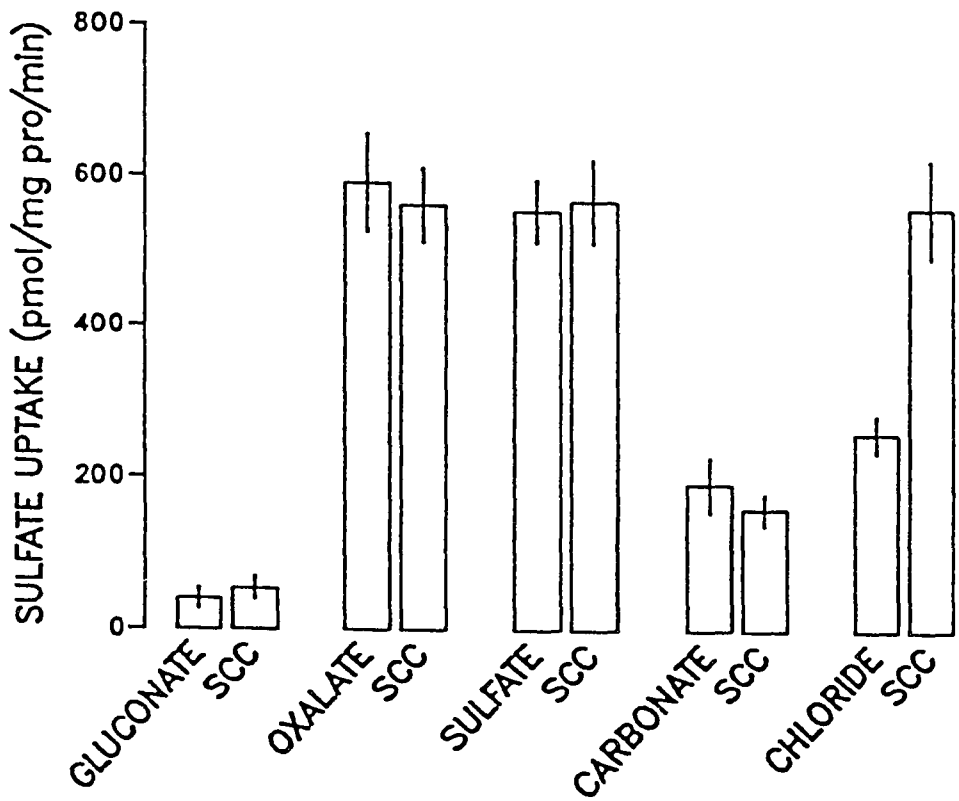

SULFATE UPTAKE (pmol/mg pro/min)
800
600
400
200
0
GLUCONATE
SCC
OXALATE
SCC
SULFATE
SCC
CARBONATE
SCC
CHLORIDE
SCC

FIGURE 3.4

Effect of internal organic anion on 0.1 mM $^{35}SO_4^{2-}$ uptake into hepatopancreatic BLMV. Vesicles were loaded with 100 mM TMA-gluconate, 100 K-gluconate and 5 mM of indicated anion (Substituted isosmotically for TMA-gluconate). External media contained 100 mM TMA-gluconate and 100 K-gluconate. All media contained 40 HEPES-Tris and 50 μM valinomycin at pH 7.0.

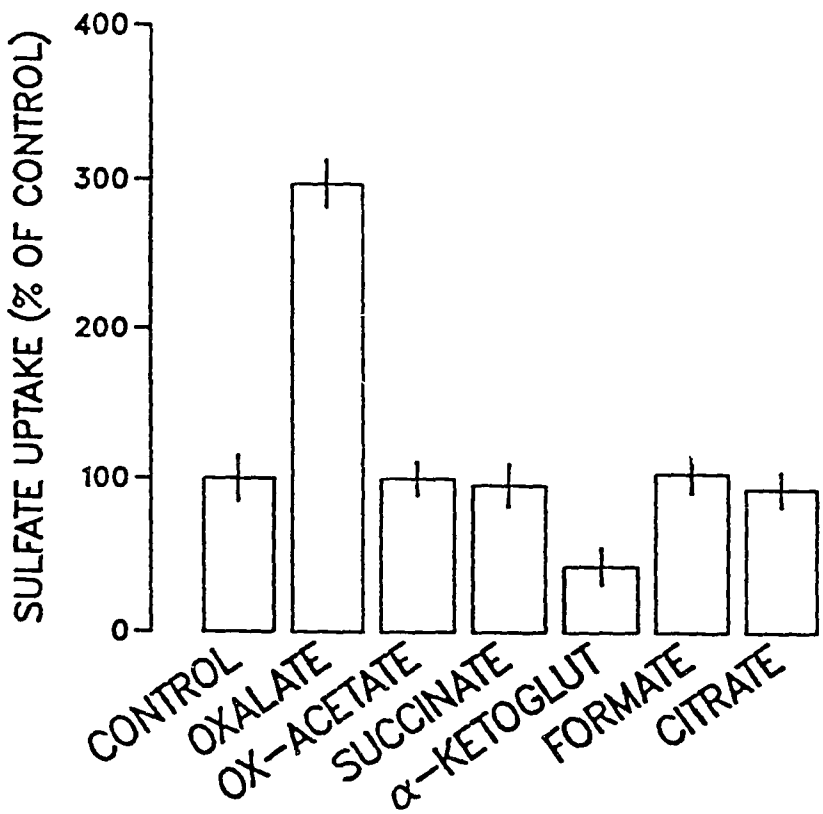

SULFATE UPTAKE (% OF CONTROL)
400
300
200
100
0
CONTROL
OXALATE
OX-ACETATE
SUCCINATE
α-KETOGLUT
FORMATE
CITRATE

FIGURE 3.5

Effect of various bilateral pH conditions and pH gradients on the uptake of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BLMV. Vesicles contained 100 mM TMA-gluconate, 80 mM K-gluconate, 10 mM K_2-oxalate adjusted to pH 7.0 or 8.0 with 40 mM HEPES-Tris, or to pH 6.0 with 40 mM MES-Tris. Incubation media contained 100 TMA-gluconate and 100 K-gluconate with pH adjusted as above. All media contained 50 uM valinomycin.

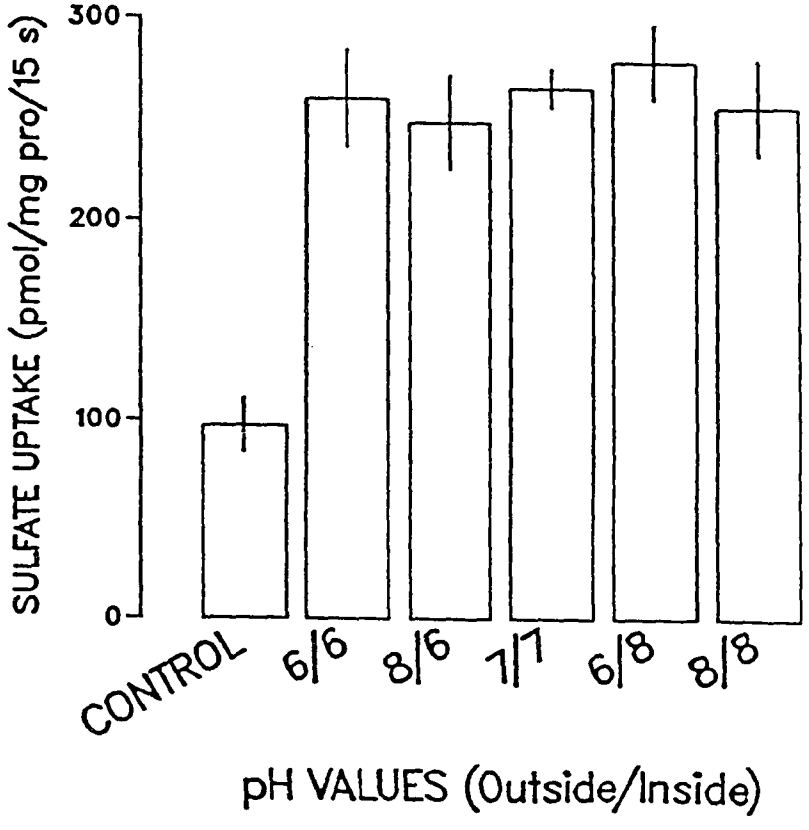

SULFATE UPTAKE (pmol/mg pro/15 s)
300
200
100
0
CONTROL
6|6
8|6
7|7
6|8
8|8
pH VALUES (Outside/Inside)

FIGURE 3.6

Effect of potential inhibitors on $^{35}SO_4^{2-}$-oxalate exchange in hepatopancreatic BLMV. Vesicles were loaded with 100 TMA-gluconate, 80 K-gluconate and 10 mM K_2-oxalate. Incubation media contained 0.1 $K_2[^{35}S]O_4$, 100 mM TMA-gluconate, 100 mM K-gluconate and either 1 mM SITS, DIDS, bumetanide or furosemide. All media contained 50 μM valinomycin and 40 HEPES-TRIS at pH 7.0.

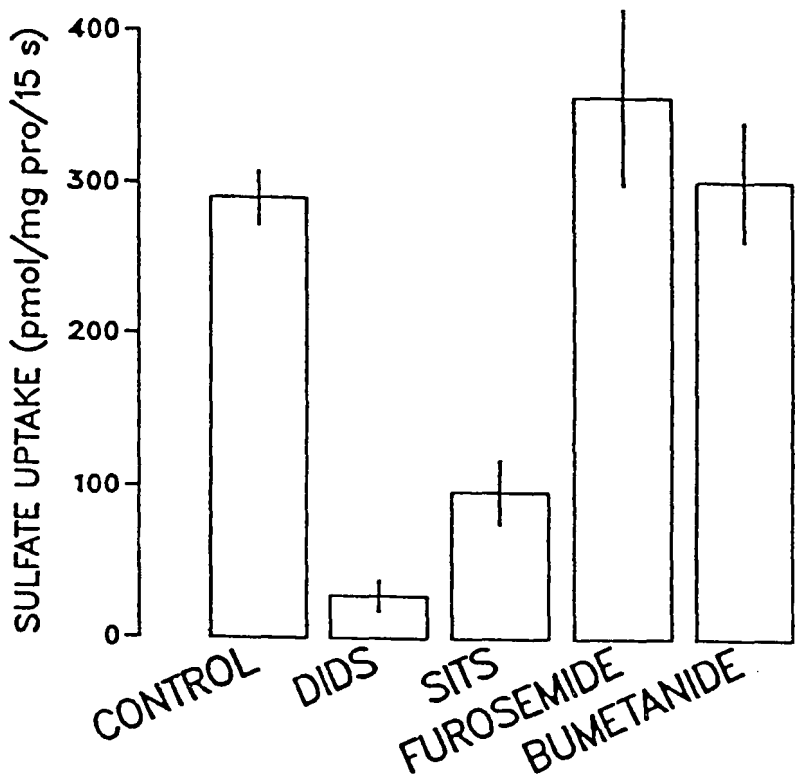

SULFATE UPTAKE (pmol/mg pro/15 s)
400
300
200
100
0
CONTROL
DIDS
SITS
FUROSEMIDE
BUMETANIDE

FIGURE 3.7

Effect of internal oxalate concentration on the influx of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BLMV. Vesicles were loaded with 100 K-gluconate, 0.10 - 10 mM Na_2-oxalate, 90 - 100 mM TMA-gluconate. Incubation media contained 100 mM TMA-gluconate, 100 mM K-gluconate and 0.1 mM $^{35}SO_4^{2-}$. All media contained 50 μM valinomycin and 40 mM HEPES-Tris at pH 7.0.

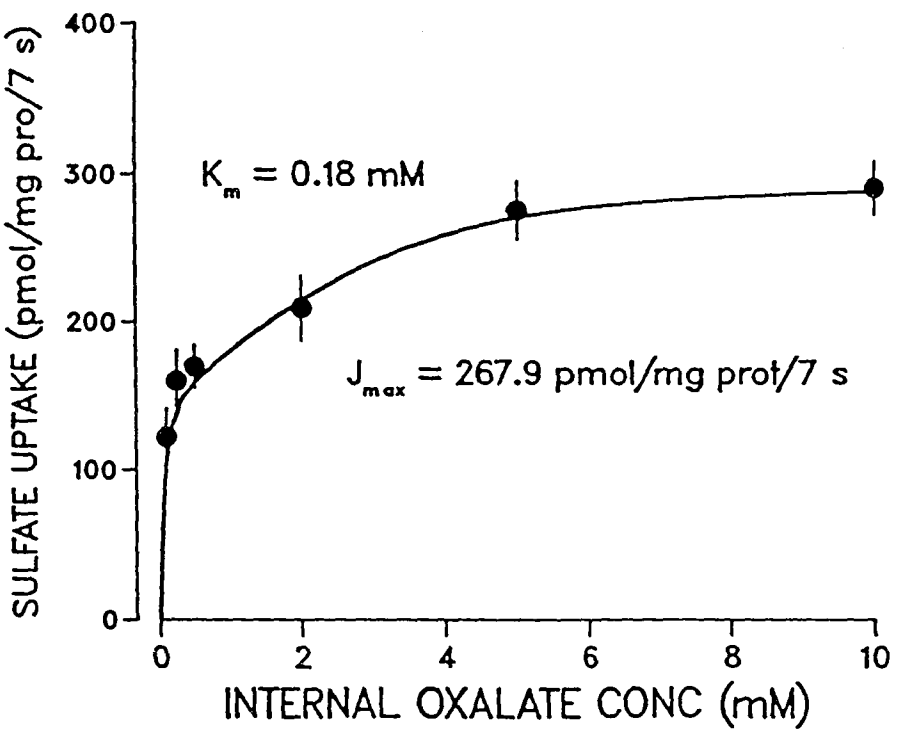
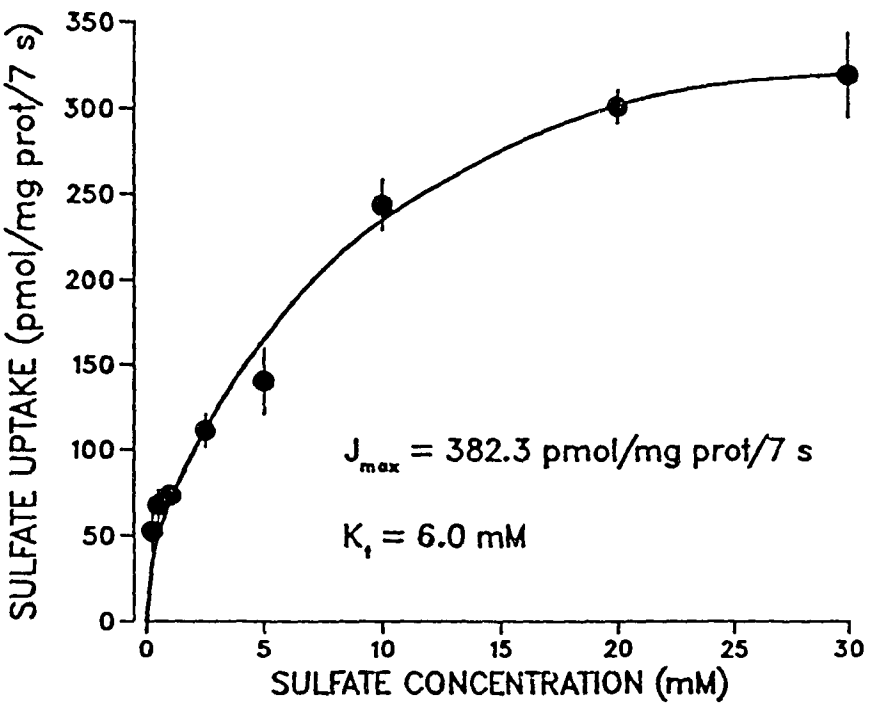

400
300
200
100
0
SULFATE UPTAKE (pmol/mg pro/7 s)
K_m = 0.18 mM
J_{max} = 267.9 pmol/mg prot/7 s
0
2
4
6
8
10
INTERNAL OXALATE CONC (mM)

FIGURE 3.8

Effect of external sulfate concentration on the uptake of 0.1 mM $^{35}SO_4^{2-}$ into hepatopancreatic BLMV. Vesicles were loaded with 100 mM TMA-gluconate, 80 mM K-gluconate, 10 mM K_2-oxalate. Incubation media contained 100 mM TMA-gluconate, 100 mM K-gluconate and indicated sulfate concentrations. All media contained 50 uM valinomycin and 40 mM HEPES-Tris at pH 7.0.


SULFATE UPTAKE (pmol/mg prot/7 s)
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
SULFATE CONCENTRATION (mM)
J_{max} = 382.3 pmol/mg prot/7 s
K_t = 6.0 mM

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CHAPTER IV

ELECTROGENIC COUPLING OF SULFATE SECRETION TO CHLORIDE TRANSPORT IN LOBSTER HEPATOPANCREAS

INTRODUCTION

The present report describes a proposed transcellular secretory mechanism for the divalent anion sulfate which has been characterized in the hepatopancreas of a decapod crustacean, Homarus americanus. The driving force for the overall transcellular movement of sulfate is a sulfate-chloride antiporter present in the brush border membrane which operates in an electrogenic fashion, exhibiting a 1 : 1 coupling ratio. The brush border antiporter is regulated by external protons, a physiologically relevent characteristic due to the generally low luminal pH of the crustacean hepatopancreas during digestion and absorption functions. There is a separate anion exchanger on the hepatopancreatic basolateral membrane which provides for sulfate entry into the epithelial cell from the hemolymph. The processes involved with sulfate transport will be discussed with reference to the current models of sulfate transport in vertebrate tissues.

HEPATOPANCREATIC EPITHELIUM

The decapod hepatopancreas, also known as the digestive or midgut gland, is a multilobed structure located in the cephalothorax comprised of many epithelial-lined blind-ended tubules. The gland exists as an outpocketing of the digestive tract in the pyloric region of the stomach and the anterior intestine (Loizzi, 1971; Van Weel, 1974; Gibson and Barker, 1979; Dall and Moriarty, 1983). Much research has been done which implicates the organ as a site for secretion of digestive enzymes, the site of final digestion of ingested materials and the major site of nutrient absorption. Although the hepatopancreas has been largely known for its digestive and absorptive properties, it has been speculated that it may also play a role in excretion of ions or metabolic wastes (Gifford, 1962; Dall, 1970; Gibson and Barker, 1979; Holliday and Miller, 1984).

VERTEBRATE SULFATE TRANSPORT MECHANISM

Most of our current knowledge of sulfate transport mechanisms has been accumulated from investigations on many vertebrate organisms, with insects being the only invertebrate group where sulfate regulation has been studied (Maddrell and Phillips, 1975 and 1978). Mosquito larvae, existing in sulfate-rich hyperosmotic lakes, appear to absorb sulfate through the gut wall and secrete the anion, via a saturable system against a concentration gradient, by the malpighian tubules (Maddrell and Phillips, 1975).

Intestinal and hepatic transport

Sulfate is actively reabsorbed across the apical border of the small intestine of the rabbit (Langridge-Smith et al., 1983; Ahearn and Murer, 1984), the rat (Cardin and Mason, 1975), and the pig (Wolffram et al., 1988) by a Na-cotransport system. Intestinal brush border membrane vesicles have also been shown to possess pH dependent sulfate uptake (Schron et al., 1985) and an anion exchange mechanism (Knickelbein et al. 1985). The only mechanism reported for sulfate transport across the intestinal basolateral membrane is anion exchange (Langridge-Smith and Field, 1981; Hagenbuch et al., 1985; Schron et al., 1987; Knickelbein and Dobbins, 1990).

Anion exchange of inorganic and organic anions with sulfate has also been described in liver sinusoidal and canalicular plasma membrane vesicles from the rat (Hugentobler and Meier, 1986; Meier et al., 1987) and sinusoidal (basolateral) membrane vesicles of the elasmobranch (Hugentobler et al., 1987).

Renal sulfate handling

Sulfate reabsorption in the mammalian kidney has been observed by numerous investigations. Luminal uptake is predominately via the sodium dependent pathway (Lucke et al., 1979; Schneider et al., 1984; Turner, 1984), with anion exchange exhibited in both perfused tubules (Brazy and Dennis, 1981) and membrane vesicles (Pritchard, 1987; Talor et al., 1987). The transport systems described for sulfate exit from renal epithelia involve a basolateral exchanger which can accept both inorganic and

organic anions (Pritchard and Renfro, 1983; Low et al., 1884; Hagenbuch et al., 1985; Kuo and Aronson, 1988).

Other vertebrate renal systems have been investigated with respect to sulfate regulation. In contrast to the mammalian reabsorption paradigm, teleost fish exhibit a net secretory sulfate mechanism. The driving force for transepithelial sulfate movement appears to be proton-dependent sulfate cotransport at the basolateral membrane (Renfro and Pritchard, 1982). No effect of sodium on sulfate uptake was observed in basolateral or brush border membrane vesicles. The efflux of sulfate from flounder renal tubule occurred by the anion exchanger demonstrated on the brush border (Renfro and Pritchard, 1983).

Sulfate transport in the chick renal tubule demonstrated characteristics of both the mammalian and teleost systems described above (Renfro et al., 1987). Brush border membrane vesicles were shown to maintain multiple pathways for sulfate transport. Sulfate uptake was stimulated by both sodium and proton cotransport and anion exchange with bicarbonate. Basolateral membrane vesicles did not respond to sodium or proton cotransport, but sulfate-bicarbonate exchange produced concentrative uptake. It appears that the avian renal tubule maintains the capacity for potential bidirectional sulfate transport.

Isolated cells and cell cultures

Isolated red blood cells and a variety of cultured epithelial cells also exhibited sulfate transport systems. Red blood cells showed sulfate-chloride exchange and proton-

sulfate cotransport (Milanick and Gunn, 1984). Sulfate anion exchange systems have been demonstrated in confluent cultures of human lung fibroblasts (Elgavish and Meezan, 1989) and human ureteral epithelia (Elgavish et al., 1991), brush border membrane vesicles from bovine trachea (Elgavish et al., 1987), and isolated Ehrlich ascites tumor cells (Levinson, 1978).

TRANSMEMBRANE SO_4/Cl EXCHANGE IN VERTEBRATE EPITHELIA

Among the many sulfate anion exchange mechanisms described for epithelial cells there exists a wide variety of specificities for acceptable substrates which can drive the antiporter. Sulfate exchangers are saturable with respect to external sulfate and internal anions. Most of the antiporters are suppressed by the classic disulfonic stilbene exchange inhibitors SITS and DIDS and different tissues are sensitive to various other transport inhibitors such as furosemide, bumetanide and probenecid. There are few studies which directly address the coupling ratio of sulfate anion exchange and experiments involving the manipulation of membrane potential which indicate electroneutral exchange. Table 4.1 provides a comparison of apparent kinetic constants for sulfate antiport in brush border and basolateral membranes of several vertebrate species and the lobster.

Luminal sulfate transport

Talor and coworkers (1987) demonstrated the presence of an anion antiporter at the luminal membrane of the beef kidney epithelium by combined methods. First they showed [^{3}H]DIDS binding to the brush border membrane exhibiting kinetics which

suggested a single class of binding sites. Secondly they observed stimulated sulfate uptake in the presence of outwardly-directed chloride, bicarbonate and hydroxyl gradients which were inhibited by DIDS and furosemide. Based on potassium/valinomycin-induced diffusion potentials they concluded that the exchange process was electroneutral. It was suggested that the brush border antiporter may be involved in the secretory flux of sulfate (Talor et al., 1987). At the brush border membrane of the rat kidney epithelium a sulfate-bicarbonate antiporter has been shown which accepted chloride, nitrate and hydroxyl ions (Pritchard, 1987) which was inhibitable by both DIDS and SITS. Flounder renal brush border membrane vesicles demonstrated a concentrative sulfate-anion antiporter which was stimulated strongly by bicarbonate and to a lesser extent by chloride, thiosulfate and thiocyanate (Renfro and Pritchard, 1983). A significant inhibition of sulfate-bicarbonate exchange occurred when challenged by DIDS and probenecid. Due to the fact that this antiporter did not respond to the manipulation of membrane potential the authors proposed an exchange of 1 sulfate to 2 monovalent anions.

Basolateral sulfate transport

The rat renal sulfate-anion exchanger in the basolateral membrane showed a very broad range of substrate specificity with respect to inorganic and organic anions (Low et al., 1984). Stimulated uptake occurred in the presence of chloride, bicarbonate, phosphate, thiosulfate, formate, acetate, lactate, p-aminohippuric acid and oxalate. Probenecid was able to block the effects of the above anions. Pritchard and Renfro

(1983) working with the same tissue also observed sulfate anion exchange; though the carrier did not utilize chloride it was found to be electroneutral and probenecid-sensitive. The chick renal tubule basolateral membrane also exhibited SITS-sensitive sulfate-bicarbonate exchange which is not cis-inhibited by chloride (Renfro et al., 1987).

The rabbit ileum exhibited a carrier mediated sulfate-chloride exchange in the basolateral domain (Schron et al., 1987). Among the anions tested oxalate was the most effective in cis-inhibition of the antiporter. The same membrane was shown to contain a sulfate-bicarbonate antiporter which also transported oxalate (Kuo and Aronson, 1988). This appeared to be a separate exchanger than the sulfate-chloride antiporter because chloride had no effect on sulfate-bicarbonate exchange (Knickelbein and Dobbins, 1990).

Sulfate-oxalate exchange appeared to be a common mechanism in both sinusoidal and canalicular membrane vesicles of liver epithelia (Hugentobler and Meier, 1986; Hugentobler et al., 1987; Meier et al., 1987). In rat sinusoidal membrane vesicles sulfate uptake was stimulated by oxalate and succinate but not by chloride or bicarbonate (Hugentobler and Meier, 1986). In skate sinusoidal membrane vesicles oxalate and sulfate cis-inhibited pH gradient stimulated sulfate uptake but chloride was ineffective (Hugentobler et al., 1987). In rat liver canalicular membrane vesicles there was a sulfate-bicarbonate antiporter which was trans-stimulated by sulfate, oxalate and thiosulfate but not by chloride or other dicarboxylic acids (Meier et al., 1987). All of the sulfate antiporters which accepted oxalate in liver membrane vesicles were suppressed by the anion exchange inhibitor DIDS.

TRANSMEMBRANE SULFATE ANTIPORT IN HEPATOPANCREATIC EPITHELIA

Electrogenic sulfate/chloride exchange at the luminal membrane

Unlike sulfate transport mechanisms in vertebrate renal and intestinal brush border membranes the lobster hepatopancreas was not shown to demonstrate a sodium-sulfate cotransport system (Cattey et al., 1992). Sulfate exchange in the luminal membrane was stimulated by chloride and sulfate but not by bicarbonate gradients. Due to the orientation of brush border membrane vesicles the translocation of sulfate into the vesicular interior represented net sulfate movement into the hepatopancreatic epithelium. There are several lines of evidence which suggest that the physiological role of this antiporter is the secretion of cytoplasmic sulfate into the lumen of hepatopancreatic tubules. First, the antiporter was found to respond strongly to the imposition of a potassium-valinomycin induced membrane potential. When the vesicular interior was positive there was a stimulation of sulfate influx and an inhibition in the presence of a negative interior. Based upon the negative transmembrane potential typical of intact epithelial cells (see Gerencser, 1985) the movement of a net negative charge into an electro-negative cytoplasm seems unlikely. A second line of evidence for the electrogenicity of this antiporter was provided when comparing chloride-sulfate exchange to sulfate-sulfate self exchange under opposing membrane potentials. There was no effect of membrane potential on sulfate-sulfate exchange, while sulfate exchange with the monovalent anion reacted as described above. This suggested that the carrier protein operated in a 1 to 1 ratio regardless of substrate charge. Finally, radiolabeled chloride uptake into sulfate-loaded vesicles was stimulated in the presence of a negative interior

when compared to short-circuited vesicles which would imply the net movement of a negative charge out of the vesicle.

Electroneutral sulfate-oxalate exchange at the basolateral membrane

In lobster hepatopancreatic epithelium basolateral membrane we observed another antiport mechanism which accepted the divalent carboxylic anion oxalate (Cattey et al., 1993). This exchange mechanism was electroneutral and transported one sulfate for one oxalate ion. There appears to be a quite specific acceptance of substrates with respect to other carboxylic acids. Formate, succinate, oxaloacetate, α-ketoglutarate and citrate did not stimulate sulfate uptake as they do in other sulfate exchange systems. Sulfate-oxalate antiport did not respond to the imposition of transmembrane pH gradients as observed in the brush border exchanger. The sulfate-oxalate exchanger was extremely sensitive to the anion antiport inhibitors SITS and DIDS (Cattey et al., 1993).

Proton regulation of hepatopancratic chloride-sulfate exchange

As mentioned above the lumenal contents of the crustacean hepatopancreas have been recorded at a low pH value (ie. pH 4.0; Gibson and Barker, 1979) due to the digestive processes of the organ. In light of the reports of pH dependent sulfate transport in different epithelial membranes (eg; Renfro and Pritchard, 1982; Schron et al., 1985), and this natural proton gradient maintained across the hepatopancreas brush border, it seemed likely that there might be a similar system in the lobster. Although no pH dependent sulfate transport was shown in hepatopancreatic vesicles, there was a marked

effect of low pH on chloride-sulfate exchange. In the presence of low extravesicular pH (high proton concentration), chloride-sulfate exchange was significantly enhanced. This effect was observed in the presence of both a transmembrane proton gradient or bilaterally equal pH conditions.

Regulation of membrane bound transport proteins by protons at either internal or external modifier sites has been described for several different cell types. Chloride-bicarbonate exchange in rabbit ileal brush border membranes was stimulated by a cytoplasmic pH modifier site which was presumed to be the effect of internal hydroxyl ions (Mugharbil et al., 1990). In lobster basolateral membrane vesicles chloride-bicarbonate exchange was stimulated by protons (Ahearn et al., 1987), but due to the response of this antiporter to transmembrane chloride and pH gradients, it was suggested that the process involved simultaneous movements of a proton with chloride. Lymphocytes also demonstrated an internal pH regulatory site which adjusted chloride-bicarbonate exchange in response to sodium-proton exchange (Mason et al., 1989). A kidney cell line, Vero cells, showed a pH regulation of chloride antiport (either chloride-bicarbonate or chloride-chloride). This investigation performed on intact cells suggested that in response to internal hydroxyl ions chloride uptake was significantly enhanced.

TRANSCELLULAR SULFATE TRANSPORT IN LOBSTER HEPATOPANCREAS

The epithelial cells of the hepatopancreas operate in the secretion of digestive enzymes and the absorption of nutrients from the luminal contents (Gibson and Barker, 1979). There have been a variety of nutrient transporters described for the brush border

of the lobster hepatopancreas which confirm the absorptive nature of the organ (Ahearn et al., 1992). The lobster controls the concentration of sulfate in the hemolymph (18 mM) at a value lower than found in seawater (25 mM) (Prosser, 1973). The lobster, as with other crustaceans, ingests seawater during both eating and drinking which provides for a high sulfate concentration in the hepatopancreas lumen. Based on our studies with sulfate transport in isolated membrane vesicles we propose a secretory pathway for this anion by epithelial cells of the hepatopancreas (Fig 4.1).

The initial step for sulfate movement from hemolymph to cytoplasm of the hepatopancreas cells occurs by an anion exchange system located in the basolateral membrane. The exchange of cytoplasmic oxalate for sulfate occurs by a saturable carrier mediated mechanism which does not accept similar dicarboxylic acids. The cytoplasmic concentration of sulfate is unknown and assumed to be relatively low so that the driving force for this antiporter would be the transmembrane sulfate gradient. The kinetic characteristics of the exchanger support this observation with the apparent binding constant for sulfate on the extracellular surface of the carrier being physiologically relevant (K_m = 6.0 mM) to the blood concentration. The apparent binding coefficient for cytoplasmic oxalate is relatively low (K_m = 0.18 mM) suggesting a high apparent affinity of the antiporter for oxalate. The physiological role of oxalate in the lobster is uncertain, it is possible that the anion, in the form of calcium oxalate, is involved with the sequestrating of, or in the deposition of, calcium that occurs prior to molting and the formation of the new exoskeleton.

The sulfate-oxalate antiporter is an electroneutral carrier which exchanges one sulfate for one oxalate ion. The basolateral carrier also exhibits self exchange and appears to accept chloride as a substrate. There is no stimulation of the carrier due to manipulations of transmembrane pH gradients. The carrier is very sensitive to the anion exchange inhibitors DIDS and SITS which provides support for an antiport system.

The movement of sulfate from the hepatopancreas cytoplasm into the tubular lumen occurs by a separate antiporter present in the brush border membrane of the epithelial cells. This electrogenic chloride antiporter appears to be the overall driving force for the transcellular movement of sulfate by this epithelium. The combined transmembrane chloride gradient and the extrusion of a net negative charge from the cytoplasm of the epithelial cell would drive the carrier. When a potassium/valinomycin induced membrane potential was established across the vesicle wall, sulfate uptake was stimulated by a positive, and inhibited by a negative, interior. In contrast, when measuring chloride uptake into sulfate-loaded vesicles there was enhanced chloride uptake due to a inside-negative membrane potential. Based on this observation and the normal gradients established in the lumen and the cytoplasm of hepatopancreas cells, the evidence suggests that the predominant movement of sulfate is out of the cell. The luminal membrane of hepatopancreas lacks the sodium-sulfate cotransport system typical of vertebrate renal (Lucke et al., 1979; Schneider et al., 1984) and intestinal (Lucke et al., 1981) epithelia. Along with the absence of a sodium cotransport system, the brush border does not exhibit proton (hydroxyl)-stimulated transport such as that of the rabbit ileal brush border (Schron et al., 1985) or flounder basolateral renal tubule (Renfro and

Pritchard, 1982). This was observed in the presence of either inward or outward directed pH gradients and under equilibrated sulfate conditions with a simultaneous inwardly-directed proton gradient (Cattey et al., 1992).

It was interesting to find that chloride-sulfate exchange was enhanced by elevated external protons, the lower the extravesicular pH, the greater the sulfate uptake. This was observed under pH gradient or equilibrated pH conditions. This is important when considering other physiological properties of the crustacean hepatopancreas. The gastrointestinal contents are acidic and are continuous with the hepatopancreas lumen thereby providing an environment relatively high in protons during digestion and absorption. Until recently it was unclear as to the mechanism of tubular acidification. Recently, a sodium-proton exchanger was described in both the lobster and freshwater prawn hepatopancreatic brush border which could adequately account for observed luminal proton concentrations (Ahearn et al., 1990). This acid environment would allow for increased activity of the electrogenic chloride-sulfate antiporter and play an important role in the regulation of sulfate (Fig 4.1).

The net secretion of sulfate by the lobster hepatopancreas represents an example of sulfate handling very different from that of vertebrate sulfate handling. Although there are sulfate anion exchange mechanism present on the brush border of renal tubules (Pritchard, 1987; Talor et al., 1987), the potent sodium cotransport system prevails driving tubular reabsorption. The antiporters in the lobster hepatopancreas share many properties with those found in mammalian epithelia yet the overall function appears to differ markedly. Sulfate secretion, also driven by anion exchange at the brush border,

has been observed in the flounder renal tubules which also shares attributes of the lobster antiporter. It appears that the lobster hepatopancreas acts in a manner similar to the teleost kidney with respect to the regulation of sulfate homeostasis.

There are two other sites among crustaceans which are involved in ion transport, the gills and the antennal or green glands which are analogous to the kidneys (Mantel and Farmer, 1983). The gills demonstrate sodium-proton antiport (Towle, 1990), sodium-ammonium exchange (Pressley et al., 1981), and chloride-bicarbonate exchange (Lee and Pritchard, 1985). Antennal gland epithelium functions as the excretory organ of crustaceans and is involved in nutrient reabsorption (Behnke et al., 1990) and solute elimination. The antennal gland has been shown to secrete magnesium (Holliday, 1980), the organic anion p-aminohippuric acid (Holliday and Miller, 1984), and an organic cation, tetraethylammonium (Miller and Holliday, 1987). Both of these tissues are potential sites of sulfate transport and regulation, but to date there have been no studies characterizing the transport mechanisms for this anion by these epithelia.

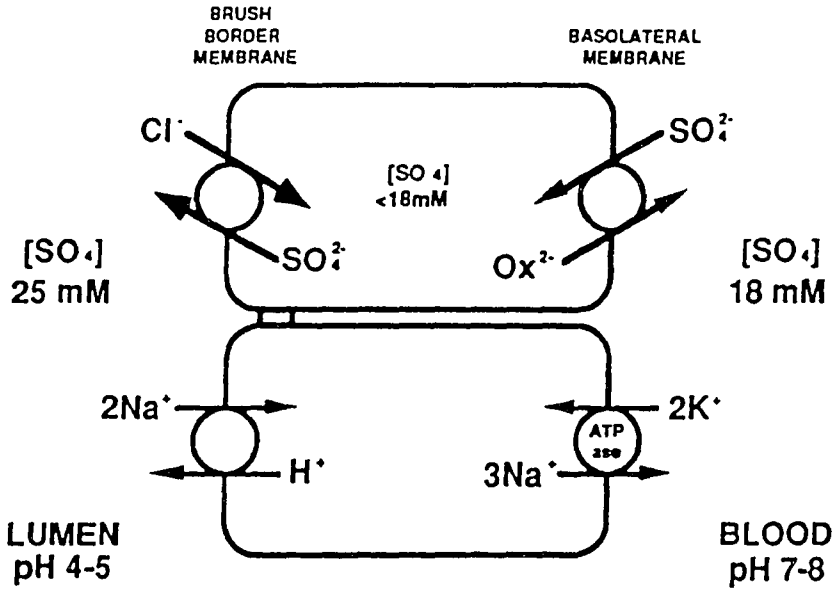
Table 4.1. Comparison of apparent kinetic characteristics of sulfate-anion antiport.

Antiporter	Epithelia	Km	Jmax	Reference
SO_4/HCO_3	Rat Kidney BBM	0.4	1.1	Pritchard, 1987
SO_4/OH	Rabbit Ileal BBM	0.48	4.1	Schron et al., 1985
SO_4/Cl	Lobster Hepatopancreas BBM	0.27	11.0	Cattey et al., 1992
SO_4/OH	Rat Liver BLM	16	12.2	Hugentobler and Meier, 1986
SO_4/HCO_3	Rabbit Ileal BLM	0.12	539	Langridge-Smith and Field, 1981
SO_4/Cl	Rabbit Ileal BLM	0.30	1.6	Schron et al., 1987
SO_4/Oxalate	Lobster Hepatopancreas BLM	6.0	3.3	Cattey et al., 1993

Km expressed in mM, Jmax expressed as nmol $SO_4 \cdot$ mg protein$^{-1} \cdot$ min^{-1}.
BBM, brush border membrane; BLM, basolateral membrane.

FIGURE 4.1

Schematic diagram of hepatopancreatic epithelium illustrating the proposed model of sulfate secretion. The approximate concentrations of sulfate in the blood and tubular lumen are indicated and the intracellular sulfate concentration is assumed to be less than that of the blood and lumen. In the upper cell sulfate initially enters the cytoplasm by moving down a concentration gradient in electroneutral exchange for oxalate. Sulfate then exits the cell via electrogenic exchange with chloride. The secretion of sulfate against its concentration gradient is driven by chloride moving down its concentration gradient. The net movement of a negative charge out of the epithelial cell facilitates the action of the transporter. The lower cell indicates the brush border sodium-proton exchanger which is driven by the transmembrane sodium gradient and membrane potential. During digestion and absorption the lumen is acidified by the protons which in turn stimulate the chloride-sulfate antiporter by an external regulatory site.


BRUSH
BORDER
MEMBRANE
BASOLATERAL
MEMBRANE
Cl⁻
SO₄²⁻
[SO₄]
<18mM
[SO₄]
25 mM
SO₄²⁻
Ox²⁻
[SO₄]
18 mM
2Na⁺
2K⁺
ATP
H⁺
3Na⁺
LUMEN
pH 4-5
BLOOD
pH 7-8

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