MULTIPLE RENAL ANION TRANSPORT SYSTEMS: SEPARATION OF EXCRETORY AND METABOLIC FUNCTIONS

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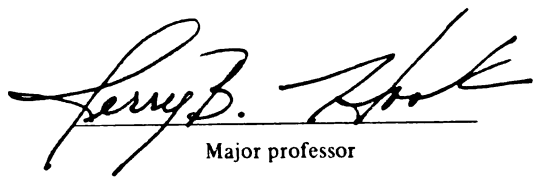

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ABSTRACT

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William Rohl Hewitt

The structural diversity and characteristic tubular handling of actively secreted organic anions suggested that several discrete anion transport systems exist in the kidney. This study was designed to define renal anion secretory systems, identify endogenous substrates and examine modulation of anion transport. The ability of renal proximal tubular tissue to accumulate anions in vitro is low in the newborn and matures in a characteristic pattern. A primary stimulus to development of transport is availability of substrate to be secreted. Transport can be selectively enhanced by challenging immature rabbits with exogenous substrates such as penicillin. Multiple transport systems for ogranic anions could be identified if they mature at different rates or respond differently to substrate challenge during the newborn period. Anions were chosen to represent three proposed transport systems: I) Hippurate-system (p-aminohippurate, PAH); II) urate system (urate, UA); III) L-system (chenodeoxycholate, CDC) or to represent various structural categories of secreted anions. Anions were tentatively placed into three groups based on similar patterns of maturation. PAH, penicillin G (PEN) and phenosulfonphthalein comprised

group I. UA, sulfisoxazole (SULFI) and acetylsalicylate (ASA) were designated group II. CDC was relegated to a third group. In theory, pretreatment with a substrate from one transport system should enhance accumulation of that anion group only. PEN pretreatment markedly enhanced maturation of group I anion accumulation (slice-to-medium (S/M) concentration ratio) without altering maturation of renal cortical slice UA, ASA or CDC accumulation. PEN stimulation of SULFI accumulation was minimal and probably related to accumulation by the overlapping group I system. Pretreatment of neonatal rabbits with most group II or group III anions did not alter group I (PAH) accumulation. ASA stimulation of PAH accumulation was probably secondary to the marked toxicity observed following ASA pretreatment. Kinetic analysis of the rate of PAH accumulation by adult kidney slices suggested that two-energy requiring modes of accumulation existed. These results supported the concept of multiple renal secretory systems for organic anions.

The PAH transport system has been linked to the selective extraction of nonesterified fatty acids (NEFA) from arterial blood. PAH and NEFA may compete for a common intracellular binding site or may be handled by a common enzymatic pathway. Selective maturation of the group I transport system was used to identify interactions between PAH transport and palmitate metabolism. Penicillin treatment increased PAH accumulation by suspensions of proximal tubules and altered distribution of incorporated palmitate-^{14}C within tubule lipid classes. Penicillin increased palmitate-^{14}C esterified to triglycerides (TG) and decreased ^{14}C recovered as NEFA. Age related changes in palmitate esterification to TG correlated with similar changes in tubule PAH

accumulation. Administration of iodipamide (group III) had no effect on PAH accumulation and did not alter palmitate utilization. Penicillin treatment of mature rabbits did not alter either tubule PAH accumulation or palmitate esterification. These results suggested that palmitate and PAH share a common intracellular binding site and that penicillin enhanced PAH accumulation by removing endogenous inhibitors (NEFA).

Acetate increased PAH accumulation by kidney cortex slices from several species. The PAH transport capacity of slices from various species was correlated with the endogenous renal cortical citrate concentration. Preparation and incubation of slices depleted tissue citrate concentration. Acetate increased citrate concentration and PAH accumulation by kidney cortex slices. Physiological concentrations of citrate increased slice PAH accumulation. These data suggested that citrate may act as an intracellular modulator of organic anion transport. Physiological concentrations of α-ketoglutarate or succinate increased slice PAH accumulation. α-Ketoglutarate increased PAH accumulation but had no effect on slice citrate concentration. Slices incubated without substrate were depleted of citrate but not α-ketoglutarate. Acetate significantly increased slice concentration of both α-ketoglutarate and citrate. Thus, the group I anion transport system could be modulated by several metabolic intermediates acting through similar, but separate, mechanisms.

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INTRODUCTION

Introduction of penicillin into clinical medicine stimulated the first major interest in renal organic anion secretion. Two outstanding obstacles, limited supply and rapid renal excretion, restricted the extensive or intensive clinical use of penicillin. Beyer and his colleagues began intensive studies into the mechanisms of renal anion secretion in order to devise stratagems that would modify the rate of penicillin excretion by the kidney and thereby improve the physiological economy of penicillin in man (Beyer et al., 1944; Beyer, 1950). These investigations rapidly led to the clinical use of p-aminohippurate (PAH), and subsequently to the development and use of carinamide and probenecid, as competitive inhibitors of renal penicillin excretion (Beyer et al., 1944; Beyer, 1950). Although the mass production of sufficient quantitites of various penicillins eventually eliminated the need for concurrent administration of competing organic anions, pharmacological interest in the mechanisms of renal anion secretion has been maintained.

Cells of renal proximal tubules in animals and man daily secrete large amounts of anionic compounds produced by normal physiological processes. Included among these compounds are uric acid, glucuronide conjugates of several endogenous steroids and degradation products of cerebral metabolism such as homovanillic acid and 5-hydroxyindolacetic acid. Man is also exposed to a multitude of organic acids in his diet

(e.g., benzoates, quinic acid, butylated hydroxyanisole, saccharin) and his environment (e.g., 2,4,5-trichlorophenoxyacetate; 2,2-bis (chlorophenyl)acetic acid) which are actively secreted by the kidney. In addition, the pharmacologic and/or toxic potential of several anionic drugs used therapeutically (e.g., penicillins, cephalosporins, methotrexate) is determined, in part, by the rate of their renal secretion into the urine. Additionally, diuretic agents such as furosemide, ethacrynic acid and several thiazides require secretion by the anion transport system to attain effective concentrations at their site of action along the luminal aspect of the ascending limb of the loop of Henle. The activity of the renal organic anion transport system in newborn infants and children is of particular relevance. Renal excretion of drugs is less in the newborn than what would be predicted in terms of body weight. This is due, in part, to an immature ability in the kidney of the newborn to transport drugs. Thus, administration of drugs to infants and children based on regimens appropriate for adults, would be expected to produce inappropriate results. The continued impetus provided by these considerations has resulted in a large fund of information pertaining to the mechanisms of renal organic anion secretion.

Characteristics of the Renal Organic Anion Transport System

In general, active transport in biological systems may be regarded as substrate penetration through a membrane against an electrochemical gradient, caused by a process which requires cellular metabolic energy. Interaction of the transported substrate with a cellular

receptor molecule(s) is implicit in this concept of active transport (Despopoulos and Callahan, 1962). The interaction of substrates with finite numbers of receptor molecules confers characteristics upon active transport systems which may be used, in part, to define substrate movement as active. Thus, active transport systems would be expected to exhibit saturation kinetics, substrate specificity and competition of substrates for transport (Despopoulos, 1965; Torretti and Weiner, 1976).

Renal organic anion secretion has been localized anatomically to the proximal tubule by micropuncture (Edwards and Marshall, 1924), stop flow analysis (Malvin et al., 1958) and in vitro perfusion of isolated nephron segments (Tune et al., 1969). Secretion of organic anions by the proximal tubule (as exemplified by PAH transport) fulfills all of the criteria for classification as active transport. Transport of PAH across the peritubular membrane in vitro proceeds uphill, against large concentration gradients (Cross and Taggart, 1950; Foulkes and Miller, 1959; Tune et al., 1969; Carrasquer and Wilczewski, 1971) and follows a kinetic pattern similar to the Michaelis-Menten model of enzyme reactions (Huang and Lin, 1965; Tune et al., 1969). Transport of PAH in vivo exhibits characteristics of saturation, since as plasma PAH concentration increased a maximal PAH transport capacity (T_m) of the renal tubules is reached (Weiner, 1973). Competition between anions for transport and substrate selectivity have also been demonstrated. For example, high plasma concentrations of PAH and probenecid inhibited the secretion of penicillin (Beyer et al., 1944; Beyer, 1950). Similarly, probenecid

penicillin, phenosulfonphthalein (PSP) and numerous other organic acids have been shown to inhibit PAH secretion *in vivo* and PAH accumulation *in vitro* (Weiner, 1973). Kinetic analysis of PAH inhibition by several organic anions has yielded double reciprocal plots consistent with competitive inhibition (Huang and Lin, 1965; Park *et al.*, 1971). Substrate selectivity has been inferred from observations that not all organic anions (e.g., p-aminobenzoic acid, p-nitrobenzylglycine) are transported by the kidney (Despopoulos, 1965; Nielsen and Rassmussen, 1975). In addition, organic cations do not alter secretion of anions, and competitive inhibitors of anion secretion do not interfere with organic cation secretion (Weiner, 1973).

Oxidative metabolism has also been found to be essential for the maintenance of the tubular transport process responsible for organic anion secretion. Incubation of renal cortical slices under anaerobic conditions or at reduced temperature (0°C) markedly depressed PAH accumulation (Cross and Taggart, 1950; Farah and Rennick, 1956). Uncoupling phosphorylation from respiration by addition of dinitrophenol (DNP) or carbonylcyanide-m-chlorophenylhydrazone to incubation medium or by infusion of DNP *in vivo* resulted in a marked depression of PAH transport (Maxild, 1973; Farah and Rennick, 1956; Mudge and Taggart, 1950b). Similarly, organic anion transport is inhibited by a long list of metabolic poisons including cyanide, azide, arsenite, fluoride, dehydroacetate, malonate, monofluoroacetate and oligomycin (Cross and Taggart, 1950; Shideman and Rene, 1951; Shideman *et al.*, 1952; Farah *et al.*, 1953; Farah *et al.*, 1955; Farah and Rennick, 1956; Maxild and Møller, 1969; Maxild, 1973).

Although the organic anion transport system as a whole exhibits characteristics of active transport, one or more of its components may operate passively. Sperber (1959) suggested that the movement of an anionic substrate through a secreting cell may be logically divided into three major components: 1) uptake (passage from extracellular to intracellular fluid across the peritubular membrane), 2) accumulation and/or transcellular movement, and 3) passage from the cell to the tubular lumen. In accordance with this suggestion, Foulkes and Miller (1959) demonstrated the existence of two intracellular compartments for PAH; one formed by facilitated diffusion across the peritubular membrane, the second formed by an energy-dependent intracellular concentrating mechanism, possibly involving binding to cellular components. The two compartments were considered to be linked in series, that is, facilitated diffusion of PAH provided an intracellular substrate pool for the concentrating mechanism (Foulkes and Miller, 1959). These conclusions were the result of analyses of PAH flux into and efflux from rabbit renal cortical slices. The first compartment rapidly equilibrated with extracellular PAH but contained PAH in excess of that found in the interstitial space. The amount of PAH accumulated into this compartment approximated that expected, assuming distribution of PAH throughout the intracellular water at a concentration equal to that of the incubation medium. The second compartment equilibrated at a slower rate and was responsible for the high slice-to-medium PAH ratios (Foulkes and Miller, 1959). Subsequent investigations (Foulkes, 1963; Farah et al., 1963; Anderson et al., 1969; Welch and Bush, 1970) confirmed the existence of multiple compartments for intracellular PAH (and other organic anions) and extended this

observation to isolated proximal tubule suspensions as well (Sheikh and Møller, 1970). However, some controversy as to the energy requirements of each compartment and the link (if any) between compartments has arisen. Sheikh and Møller (1970) concluded that PAH was actively transported across the peritubular membrane (in contrast to facilitated diffusion) since the rapid PAH influx in their experiments reached concentrations 3-4 fold greater than the medium PAH concentration. The slow phase of PAH influx (compartment 2) was thought to represent passive diffusion (binding) to subcellular components (Sheikh and Møller, 1970). Nitrofurantoin accumulation by rabbit kidney slices also proceeds by formation of two intracellular compartments, one by passive diffusion and one by an energy-requiring mechanism (Anderson et al., 1969). However, the compartments were apparently arranged in parallel (i.e., both had access to extracellular nitrofurantoin) rather than existing in series as had been postulated by Foulkes and Miller (1959) and Sheikh and Møller (1970).

Recently, transport of PAH into basal-lateral plasma membrane vesicles derived from rat proximal tubule cells has been demonstrated (Berner and Kinne, 1976). Vesicle PAH uptake exhibited saturation kinetics and was competitively inhibited by probenecid. PAH uptake varied inversely with medium osmolality and destruction of vesicles (heat inactivation, osmotic shock) markedly reduced PAH uptake thus demonstrating that PAH uptake primarily represented transport into an osmotically reactive intravesicular space rather than simple adsorption by vesicle membranes (Berner and Kinne, 1976). Ross and his colleagues (Holohan et al., 1975; 1976) have reconstitited a solubilized, PAH specific binding protein(s) derived from dog kidney plasma

membrane proteins in phospholipid vesicles and obtained results similar to those of Berner and Kinne (1976). Transport of PAH was saturable and was inhibited by known competitors of PAH secretion (Holohan et al., 1976). Since both vesicle preparations are free of metabolism and devoid of intracellular components, PAH transport across vesicular membranes likely represents facilitated diffusion across the peritubular membrane of proximal tubules (Berner and Kinne, 1976; Holohan et al., 1975; 1976). Although the possibility of active transport of PAH across the peritubular membrane cannot be eliminated on the basis of these experiments, the model of PAH transport proposed by Foulkes and Miller (1959) is strengthened.

Binding of organic anions to intracellular organelles and/or cytoplasmic proteins has often been considered an integral component of the organic anion transport system. Foulkes and Miller (1959) and Farah et al. (1963) initially postulated that the slow component of PAH diffusion observed during efflux experiments represented binding to intracellular components which constituted the PAH concentrating mechanism. Competitive inhibitors of transport were believed to act, in part, by prohibiting access of PAH to the trapping mechanism thereby increasing efflux by increasing PAH content in the freely diffusible pool (compartment 1) (Foulkes and Miller, 1959; Farah et al., 1963). Acceptance of this hypothesis has been hindered by the failure to demonstrate evidence of quantitatively important tissue binding of PAH (Berndt, 1976). One group of investigators have attributed this failure to use of methods inadequate for demonstration of the relatively weak tissue binding of PAH (Farah et al., 1963). However, a second group including Foulkes (1963) and Tune et al.

(1969) have accepted the alternate hypothesis. That is, these investigators believe that intracellular binding is not a requisite step in the accumulation and transcellular movement of PAH. Foulkes (1963) determined that secretion of PAH into the urine occurred from an intracellular pool coextensive with total cellular content. When a known inhibitor of PAH secretion (Diodrast) was infused, no effect on the size of the PAH secretory pool was observed. If Diodrast competed for the intracellular binding sites responsible for PAH accumulation as suggested earlier (Foulkes and Miller, 1959), then Diodrast infusion should have decreased the PAH secretory pool. The absence of such competition was believed to contradict the concept of intracellualr binding of these anions (Foulkes, 1963). Tune et al. (1969) studied PAH transport using single segments of proximal tubule dissected from a rabbit kidney. Each tubule segment was immersed in a bath which represented the peritubular blood and perfused via micropipets with an artifical "urine" of any desired composition. Tubules actively accumulated PAH when it was added to the surrounding medium. The intracellular PAH concentration was found to be greater than that of either the surrounding medium or the luminal perfusate. The movement of PAH from the cell into the luminal fluid was therefore consistent with a process of simple diffusion (Tune et al., 1969). In addition, Tune et al. (1969) estimated the relative permeability of the luminal and peritubular membranes to PAH. The luminal membrane was estimated to be at least 10-fold more permeable to PAH than the peritubular membrane (Tune et al., 1969).

Tune and his colleagues also conducted experiments in which PAH was present only in the luminal perfusate. When PAH was added only to

the perfusate, it moved across the luminal membrane into the cell and eventually appeared in the surrounding bath. However, the intracellular PAH concentration determined in these tubules was not different than the PAH concentration of the luminal perfusate. If intracellular binding of PAH occurred, the tissue concentration should have been greater than that in the perfusing fluid. Thus, Tune et al. (1969) concluded that PAH was not bound significantly within the tubule cell, and that the measured tissue PAH concentrations were representative of a free transport pool. In spite of the persuasive negative argument provided by these data, a causative relationship between intracellular binding and kidney anion accumulation has continued to be stressed.

The continued emphasis on intracellular binding is due, in part, to recent examinations of the binding of phenolsulfonphthalein (phenol red, PSP) to homogenates of renal cortical tissue (Sheikh, 1972; Eveloff et al., 1976). Extensive binding of PSP to microsomal and mitochondrial fractions of rabbit kidney homogenates has been observed (Sheikh, 1972). However, only that fraction bound to microsomes was depressed by competitive inhibitors (e.g., probenecid) and thus could be considered as representing binding to a specific protein(s). Phenol red found in the cytosolic fraction was completely ultrafilterable. Thus, cytoplasmic proteins were apparently incapable of binding phenol red (Sheikh, 1972). More detailed analyses of PSP binding to the microsomal fraction of rabbit kidney cortex demonstrated the existence of two independent populations of binding sites (Eveloff et al., 1976). Probenecid inhibited binding to only one population (the high affinity population) of binding sites whereas a Scatchard analysis suggested that dinitrophenol competitively inhibited PSP

binding to both high and low affinity sites. Dual populations of binding sites are of particular relevance to the model of PAH transport proposed by Foulkes and Miller (1959). Thus, it is attractive to speculate that the high affinity population of binding sites represents a carrier system for the initial facilitated diffusion step across the peritubular membrane (Eveloff et al., 1976). The near identity of association constants calculated for the high affinity PSP binding sites and for PAH uptake into basal-lateral membrane vesicles (Berner and Kinne, 1976) adds further credence to this hypothesis (Eveloff et al., 1976). The low affinity population of PSP binding sites might then participate in the intracellular concentrating mechanism proposed by Foulkes and Miller (Eveloff et al., 1976). However, this last speculation must be tempered with the observation that cytoplasmic proteins bind significant amounts of organic anions. Using different techniques to study anion binding, Arias and his colleagues have postulated that the cytoplasmic protein, ligandin (GSH-S-transferase B, Y-protein), serves as the major organic anion acceptor in renal proximal tubule cells (Kirsch et al., 1975; Arias et al., 1976). Several lines of evidence support the hypothesis that ligandin (and related proteins) functions as a transport carrier within the cell. The GSH-S-transferase fraction isolated from homogenates of renal cortex binds several transported organic anions including PAH and penicillin (Kirsch et al., 1975). Probenecid administration can inhibit the binding of penicillin to renal ligandin following in vivo injection (Kirsch et al., 1976). Administration of inducers of renal drug metabolizing enzymes increased the concentration of renal GSH-S-transferases and concomitantly increased renal binding, plasma disappearance and urinary excretion of organic anions (Kirsch et al.,

1975; Arias et al., 1976; Kaplowitz and Clifton, 1976). These observations appear to support the hypothesis that ligandin is a component of the renal organic anion transport system. However, Pegg and Hook (1977) were unable to demonstrate that binding of PAH to ligandin was the rate-limiting step in PAH transport. In general, the contradictory nature of the observations and conclusions presented by various investigators do not permit a definitive conclusion regarding the contribution of intracellular binding to the overall transport process.

Evidence Supporting the Concept of Multiple Transport Systems for Organic Anion Secretion in the Kidney

The structural requirements for transport by the renal organic anion secretory system have not as yet been elucidated (Rennick, 1972; Weiner, 1973). Several classes of endogenous and exogenous organic acids, including mono- and dicarboxylic acids, sulfonic acids, substituted benzoic acids, ethereal sulfates and sulfonamides are actively secreted by the cells of the renal proximal tubule (Despopoulos, 1965; Weiner, 1973; Nielsen and Rasmussen, 1976). Two proposals regarding the structural requirements necessary for transport have been based on the structure of PAH, a model substrate of the anion transport system. Taggart (1958), concluded that the carboxyl moiety alone was responsible for substrate specificity since occlusion of the carboxyl by formation of a hydroxamate resulted in a non-transported compound. However, the carboxyl group above cannot account for substrate specificity since several carboxylic anions (e.g., paminobenzoic acid) are not actively transported by the system (Despopoulos, 1965). Subsequently, Despopoulos (1965) suggested that transport specificity

relied on a 3 point drug-receptor attachment involving a reinforced ionic bond between the two carboxyl oxygens and a supporting hydrogen bond formed with the carbonyl oxygen. Sulfonic acid and sulfonamide groups were considered to be equally reactive in these transport reactions (Despopoulos, 1965). This theory explained the transport of a large number of organic anions, but could not satisfactorily account for the active transport of several anions such as diodrast and phenoxyacetate (Weiner, 1973). Although additional proposals have been advanced, the structural heterogeneity of these transported anions has made it impossible to delineate the structural requirements for transport (assuming substrate interaction with a single receptor molecule). This has led to the suggestion that several different anionic transport systems with differing specificities exist within the cells of the proximal tubule (Weiner, 1973).

The characteristics of tubular secretion of two well studied organic anions, uric acid (UA) and PAH are consistent with the hypothesis of multiple anion transport systems (Weiner and Fanelli, 1974). Uric acid is the major end product in the degradative metabolism of purines in man and higher apes (Rastegern and Their, 1972). In most other species, uric acid is of quantitatively less importance due to formation of allantoin by the uricolytic enzyme, uricase (Mudge et al., 1973). Renal handling of uric acid includes glomerular filtration, active tubular reabsorption and secretion, the relative magnitude of each varying from one species to another (Mudge et al., 1973). Both reabsorption and secretion of urate occur along the proximal tubule, with transport in either direction being carrier mediated (Mudge et al., 1973). Proximal tubule secretion of uric acid has

several characteristics in common with secretion of PAH (Weiner and Fanelli, 1975). Secretion of both anions is remarkably potassium dependent and several substances that inhibit PAH transport also inhibit urate secretion (Weiner and Fanelli, 1975). However, several investigations with various species suggested that uric acid and PAH are secreted by separate, overlapping transport mechanisms.

May and Weiner (1971) demonstrated that the hyperuricemic agent, m-hydroxybenzoic acid (m-HBA), inhibited excretion of uric acid by Cebus kidney. Since m-HBA was actively secreted, competitive inhibition of urate secretion was suggested as the mechanism of action (May and Weiner, 1971). These investigators also demonstrated that m-HBA was a more powerful inhibitor of uric acid secretion than PAH. These observations could have resulted from differing affinity of m-HBA and PAH for a single transport system (May and Weiner, 1971). However, m-HBA completely suppressed urate excretion at concentrations that caused only slight inhibition of PAH transport, results which were not consistent with a single secretory mechanism for PAH and uric acid. Therefore, May and Weiner (1971) postulated that two overlapping transport systems were involved, one system with high affinity for m-HBA was also the anion secretory system utilized by uric acid, whereas the second system was the major secretory system for PAH and had little affinity for m-HBA. Weiner and Tinker (1972) and Fanelli and Weiner (1973) subsequently demonstrated that pyrazinoic acid, the active metabolite of pyrazinamide, effectively blocked urate secretion by monkey (Cebus albifrons) and chimpanzee (Pan troglodytes verus) kidney at doses which did not influence the clearance or the T_m of PAH. The authors concluded that these findings supported the previous

suggestion that two secretory systems with different affinity for PAH and urate exist in the mammalian renal tubule.

Tubular secretion of PAH and uric acid also appear to be separate in man. Boner and Steele (1973) demonstrated that infusion of sufficient PAH to produce plasma concentrations of 10 mg/100 ml did not significantly decrease urate secretion. Suppression of urate secretion with pyrazinamide did not inhibit tubular secretion of PAH nor did PAH inhibit the antiuricosuric effect of pyrazinamide (Boner and Steele, 1973). Meisel and Diamond (1977) demonstrated that both PAH and pyrazinamide inhibited the uricosuric response to probenecid administration in man by separate mechanisms. Induction of uricosuria by probenecid is dependent upon secretion of probenecid into the renal tubule in concentrations sufficient to competitively inhibit the urate reabsorptive mechanism (Kippen et al., 1974). PAH infusion significantly decreased probenecid secretion without altering urate secretion. The decreased uricosuric response to probenecid was proportionate to the reduction in probenecid excretion. In contrast, pyrazinamide had no effect on probenecid excretion but did significantly inhibit urate secretion, thus reducing the concentration of urate available for inhibition of reabsorption by probenecid (Meisel and Diamond, 1977).

Evidence of separate secretory mechanisms for PAH and urate exist in lower species as well. Dantzler (1970) observed no effect of PAH infusion on urate clearance in water snakes. Subsequently, Dantzler (1974) demonstrated that high bath urate concentrations ([urate] = 20[PAH]) had no effect on PAH secretion by isolated perfused garter snake tubules. Although both the PAH and urate transport systems were saturable, PAH secretion reached saturation at a bath concentration

approximately 85% lower than the concentration needed to saturate the urate secretory system (Dantzler, 1974). Zmuda and Quebbeman (1975) demonstrated that tubular secretion of urate in chickens genetically selected for a high incidence of articular gout and hyperuricemia was approximately 40% that of nongouty controls. In contrast, secretion of PAH in gouty chickens was not different from control. Foulkes (1975) and Kramp and Lenoir (1975) demonstrated little interaction between urate and PAH transport at the peritubular membrane of rat kidney.

Bárány (1972; 1973a,b; 1974a,b; 1975) postulated the existence of a group of separate, but overlapping, transport systems to explain the effects of various anions on the slice accumulation of iodipamide. One system (H-system), sensitive to iodohippurate inhibition, was assumed to be the classical hippurate transport system (Bárány, 1972). The second, hippurate insensitive system (L-system), was inhibited by anions normally excreted by the liver (Bárány, 1973a,b). Thus, bile acids (e.g., chenodeoxycholate), cholographic agents and flavaspidic acid produced marked inhibition of iodipamide accumulation by the L-system (Bárány, 1973b; 1974b; 1975). Benzylpenicillin and PAH had little effect on iodipamide accumulation by the L-system. In addition, pyrazinoic acid, m-hydroxybenzoic acid and salicylic acid also had little effect on iodipamide accumulation via the L-system, suggesting that the L-system was not involved in uric acid accumulation in rabbit kidney cortex (Bárány, 1973b). Several straight chain, dicarboxylic TCA cycle intermediates (e.g., α-ketoglutarate, succinate, malate) had little or no inhibitory effect on iodipamide

accumulation indicating that transport of these metabolic intermediates was not mediated by the L-system (Bárány, 1973b).

Comparative studies of iodipamide secretion in rabbit and dog also support the concept of multiple organic anion transport systems. Berndt et al. (1968) used both in vivo (iodipamide/inulin clearance ratio = 3.7) and in vitro (iodipamide S/M ratio = 32.3±2.06) techniques to demonstrate the existence of an active secretory system for iodipamide in rabbit kidney cortex. Iodipamide accumulation was oxygen and pH dependent, saturable and was depressed by both metabolic poisons and competitive inhibitors. In contrast, iodipamide handling by dog kidneys was not consistent with the concept of an active secretory system. Renal clearance ratios were not different from unity nor did stop-flow techniques demonstrate a secretory peak for iodipamide. In vitro accumulation of iodipamide by dog renal cortex slices was negligible (S/M = 1.21±0.06). Dinitrophenol, cyanide and iodoacetamide had no effect on iodipamide accumulation by dog kidney slices. In contrast, kidney slices from the same dogs accumulated PAH against a concentration gradient (PAH S/M = 3.5 without acetate; 9.8 with acetate). Thus, the lack of iodipamide accumulation by dog slices could not be ascribed to nonviability (Berndt et al., 1968). Bárány (1972, 1973a) subsequently confirmed these results and concluded that dog kidney completely lacked the L-transport system for iodipamide while retaining the classical hippurate (H-system) transport system.

These observations support the hypothesis that there exists within the renal proximal tubule at least 3 separate, overlapping transport systems for organic anions: 1) a system with high relative

affinity for hippurates; 2) a system with high relative affinity for anions like iodipamide; and, 3) a third system with high relative affinity for uric acid.

The ability of rabbit renal cortical tissue to accumulate PAH in vitro is low in the newborn and matures in a characteristic pattern. PAH transport can be selectively stimulated by challenging newborn rabbits with large doses of substrates of the transport system (Hirsch and Hook, 1969, 1970). If multiple transport systems for organic anions exist in the kidney they might mature at different rates or respond differently to substrate challenge during the newborn period. Therefore, an analysis of the factors modulating the in vitro handling of several organic anions by neonatal and adult rabbit renal cortical slices was undertaken to provide evidence which would support or refute this hypothesis. Three criteria were utilized to identify transport systems and classify member substrates: 1) The pattern of maturation of intrinsic transport capacity measured as accumulation of organic anions into renal cortical slices; 2) changes in anion accumulation by renal cortical slices after pretreatment of neonatal rabbits with large doses of penicillin; and, 3) changes in PAH accumulation by renal cortical slices following pretreatment of neonatal rabbits with various anions. Accumulation of PAH by renal cortical slices was used as the standard of reference during each phase of the investigation.

Evidence Supporting Metabolic Modulation of PAH Secretion

The net PAH secretory capacity of the organic anion transport system may be influenced by alterations in several physiological variables. An analysis of the effects produced by altering these

variables may serve to illuminate the mechanism(s) controlling renal PAH transport. For example, the profound enhancement of renal PAH secretion produced by acetate, and its precursors, lactate and pyruvate has suggested that the PAH transport system normally operates at less than maximal capacity (Cross and Taggart, 1950; Mudge and Taggart, 1950; Schachter et al., 1955; Cohen and Randall, 1964). These metabolic intermediates (and, in particular, acetate) have been shown to increase the intrinsic slice capacity for PAH accumulation in vitro and to increase the maximum tubular transport capacity for PAH (T_m PAH) when infused in vivo. Similarly, renal cortical slices from alkalotic rats accumulate more hippurate under normal incubation conditions (i.e., pH 7.40) than slices from nontreated controls (Weiss and Preuss, 1970). Production of acute metabolic alkalosis in vivo has also been found to increase T_m PAH in dogs (Mudge and Taggart, 1950).

The ability of the kidney to secrete PAH can also be reduced below normal. Weiss and Preuss (1970) observed that chronic metabolic acidosis depressed the ability of rat cortical slices to accumulate hippurate. Marked depression of in vitro PAH accumulation and/or T_m PAH can also be produced by various dicarboxylic TCA-cycle intermediates (e.g., α-ketoglutarate, succinate, fumarate), fatty acids of intermediate carbon chain length (C_5-C_{10}) and by certain amino acids (e.g., alanine, glutamate). In addition, Hirsch (1974) found that renal organic anion transport was markedly depressed during the chronic potassium depletion produced by feeding rats a low potassium diet. Renal cortical slices from potassium repleted rats exhibited a normal ability to accumulate PAH.

The relationship between these random observations and a mechanism(s) for control of renal PAH secretion is not well understood. Disturbances in normal acid-base balance or in K^+ homeostasis are known to alter several metabolic parameters, including plasma and renal cortical concentrations of several of the aforementioned metabolic intermediates (Relman, 1972; Adler et al., 1974). Thus, an examination of the effect(s) of one or more of these metabolites might aid in the characterization of a common mechanism(s) by which any of the variables mentioned alter PAH secretion. The two carbon fragment, acetate has been the substrate most extensively studied in relationship to PAH transport.

Several mechanisms have been proposed for the acetate-induced stimulation of PAH accumulation. Cross and Taggart (1950) suggested that acetate might play a unique role in PAH transport since only acetate and certain of its precursors to a lesser degree, uniformly stimulated PAH accumulation by rabbit kidney cortex slices. Since PAH accumulation was not correlated with the respiratory stimulation obtained with several substrates, Cross and Taggart (1950) further postulated that acetate acted as one of the rate-limiting cellular components of the transport system, rather than by producing general acceleration of oxidative metabolism.

An alternate mechanism arose from investigations regarding the conjugation of benzoic acid derivatives with glycine to form hippurates and related N-acylglycines. After demonstrating the presence of enzymes capable of synthesizing and degrading aromatic and aliphatic acylglycines in rabbit kidney cortex, Schachter et al. (1955) evaluated the ability of several aliphatic acylglycines to inhibit slice

accumulation of PAH, an aromatic acylglycine. Acylglycines formed from fatty acids of intermediate carbon-chain length (C_5-C_{10}) depressed PAH accumulation to variable degrees. The inhibition was a function of the affinity of the acylglycine for the PAH transport system and the rate of hydrolysis of the compound to a non-competitive metabolite. Although the appropriate acylglycines were formed from acetate, propionate and butyrate, the rapid hydrolysis and/or low affinity of the transport system for these compounds prevented inhibition of PAH accumulation.

The lack of a competitive interaction between acetylglycine and PAH is the basis for the mechanism of acetate-induced stimulation of PAH secretion proposed by Schachter et al. (1955). Schachter et al. (1955) concluded that addition of exogenous acetate would yield acetyl-CoA as the most abundant acyl-CoA available to glycine acyltransferase, thereby reducing the amount of inhibitory acylglycines (e.g., octanoylglycine) by competitive formation of acetylglycine. This hypothesis was strengthened when a comparative study of acetate stimulation demonstrated enhancement of PAH accumulation only in those species containing a glycine acyltransferase enzyme system. Interestingly, the converse relationship was not valid since PAH accumulation by rat renal cortical slice was not responsive to exogenous acetate although an acylglycine synthetic mechanism was present (Schachter et al., 1955). Based on the proposed mechanism Schachter et al. (1955) interpreted the acetate stimulation of PAH transport observed in in vitro and in vivo preparations devoid of exogenous fatty acid as indicating that PAH transport was partially inhibited at all times by acylglycines formed from endogenous fatty acids.

Although the mechanism proposed by Schachter et al. (1955) was attractive, sufficient contradictory evidence exists to make this hypothesis unacceptable. For example, renal acylglycine synthesis was thought to be limited by cortical glycine concentration. Acetate stimulation would therefore be related to reduction of the rate-limiting component. However, Murdaugh and Elliot (1969) demonstrated that acetate stimulation of slice PAH accumulation persists in the presence of excess glycine. These results could be interpreted as an indication that the competitive site occurred earlier in the reaction sequence for acylglycine formation (i.e., competition for coenzyme A or the acylating enzyme) (Weiner, 1973). However, addition of excess acetate to rat renal cortex homogenates did not influence acylglycine synthesis (Kim and Hook, 1972). Acetate also had no effect on acylglycine synthesis in experiments where glycine concentration had been made rate-limiting (Kim and Hook, 1972). During the course of this study, Kim and Hook (1972) also observed acetate-induced stimulation of PAH accumulation by rat and chicken slices although only rat tissue was capable of acylglycine synthesis. Kim and Hook (1972) concluded that there was no relationship between the effect of acetate on p-aminohippurate transport and the acetylglycine synthesis system.

What, then, is the mechanism of the stimulatory effect of acetate on PAH transport? Although PAH transport is dependent on oxidative energy metabolism, neither acetate, lactate or pyruvate alter slice ATP concentration despite a pronounced stimulation of PAH accumulation (Maxild, 1973). Indeed, recent studies by Gerencser et al. (1977) demonstrated a significant reduction of ATP concentration in renal cortical slices incubated with acetate. These and other (Weiner,

1973), observations indicate a lack of correlation between the magnitude of renal PAH accumulation and the absolute amount of ATP available in renal cortical tissue (Maxild, 1973; Weiner, 1973).

Cohen and Randall (1964) have postulated that alterations in renal metabolism produced by disturbances in acid-base balance might affect PAH transport if specific substrates (either stimulatory or inhibitory) were to accumulate or disappear from within the kidney. Similarly, Weiner (1973) suggested that metabolic inhibitors cause a shift in patterns of metabolic products resulting in accumulation of specific inhibitors of PAH transport. In this respect, organic anion transport appears to be remarkably sensitive to inhibition of TCA cycle activity. Depression of citrate (monofluoroacetate) and succinate (dehydroacetate, malonate, chlorguanide) oxidation markedly decreased PAH transport *in vitro* and *in vivo* (Shideman and Rene, 1951; Shideman *et al.*, 1952; Farah *et al.*, 1953; Farah *et al.*, 1955; Farah and Rennick, 1956). The effect of these metabolic poisons was specific since organic cation accumulation was not altered at inhibitor concentrations that essentially eliminate PAH accumulation. In addition, dehydroacetate has also been shown to selectively inhibit PAH secretion without altering the energy dependent reabsorption of glucose or phosphate (Seevers *et al.*, 1950). As might be expected, acetate protects against inhibition of PAH transport produced by these inhibitors (Shideman *et al.*, 1952; Farah *et al.*, 1953).

The specific inhibition of PAH transport resulting from decreased TCA cycle activity and acetate stimulation of PAH secretion can be interpreted in several ways, each consistent with the hypotheses of

Cohen and Randall (1964) and Weiner (1973). However, one hypothesis, capable of accounting for both stimulation and inhibition of PAH transport, deserves particular attention. If a modulator (with concentration dependent stimulatory and inhibitory properties) was maintained at a low, substimulatory concentration by normal TCA cycle activity, acetate could stimulate PAH transport by increasing modulator concentration. Alternatively, TCA cycle inhibition at a point subsequent to modulator synthesis could elevate the concentration into the inhibitory range and thus specifically inhibit PAH transport. Inhibition might also occur if sufficient acetate was metabolized to elevate modulator concentration into the inhibitory range. This model for metabolic regulation of PAH transport was chosen for further study for several reasons. Cross and Taggart (1950) demonstrated that several TCA cycle intermediates markedly inhibited PAH accumulation when present at high concentration in the incubation medium. In contrast, low concentrations of these same intermediates significantly stimulated PAH accumulation (Cross and Taggart, 1950; Farah et al., 1955). Finally, incubation of rat renal cortical slices with acetate (10 mM) at 37°C, or incubation of dog kidney slices with acetate (100 mM) at 25°C resulted in a small but significant depression of PAH accumulation (Koishi, 1959; Despopoulos, 1956).

Teleologically, it seems unlikely that the primary function of an elaborate metabolic regulatory mechanism such as that proposed above would exist merely to control the excretion rates of exogenous organic acids (Forster, 1967). Systems of this complexity, if they do indeed exist, seem better adapted to serve as a mechanism that may control

delivery rates or maintain intracellular levels of oxidative intermediates or metabolites (Forster, 1967).

Relationship Between Transport of PAH and Transport and Utilization of Endogenous Anionic Metabolic Substrates

The kidney is capable of considerable substrate specificity in that it selectively extracts specific metabolic substrates from arterial blood (Cohen and Barac-Nieto, 1973). The substrates present in normal arterial blood which the kidney extracts and utilizes at significant rates *in vivo* are: free fatty acids (in particular, palmitate), lactate, glutamine, glucose and citrate (Cohen and Kamm, 1976). In addition, the kidney extracts several substrates, including inositol, pyruvate, α-ketoglutarate, fructose glycerol, acetoacetate and β-OH butyrate, which are present at such low concentration in arterial blood that they could not make a substantial contribution to the energy requirements of the kidney even if they were completely extracted and oxidized (Cohen and Kamm, 1976).

That several of these metabolic substrates are organic anions suggested the possibility that the organic anion transport system serves as a mechanism to extract anionic metabolites from arterial blood and deliver them to sites of intracellular dissimilation (Barac-Nieto and Cohen, 1973). Indeed, the literature contains experimental evidence suggesting that lactate, citrate, α-ketoglutarate and palmitate enter the proximal tubule cell from the peritubular blood by a mechanism(s) other than simple passive diffusion.

Dies *et al.* (1969) demonstrated that lactate was filtered at the glomerulus and subsequently was completely reabsorbed (<1% of the

filtered load was excreted) along the proximal tubule. The lactate reabsorptive process operated against an electrochemical gradient and exhibited a maximal rate, characteristics consistent with transport by a saturable, energy-requiring mechanism (Dies et al., 1969). Barac-Nieto and Cohen (1973) recalculated data from experiments performed by Dies et al. (1970) and concluded that renal uptake of lactate exceeded the filtered load when GFR was reduced by partial uretral occlusion. These results suggested that some lactate must also be obtained from the peritubular capillary aspects of the cell (Barac-Nieto and Cohen, 1973). Subsequently Dies et al. (1974) observed instances of net renal lactate uptake exceeding total lactate reabsorption when dogs were in metabolic or respiratory alkalosis and during glycine infusion. These investigators calculated an equilibrium potential of -39.5 mV for lactate or approximately 50% of the known electrical gradient (-70 mV) between renal cortical tubular cells and extracellular fluid. Therefore, Dies and co-workers (1974) concluded that peritubular entry of lactate was not mediated by a simple diffusion process. However, probenecid infusion had no effect on the renal uptake of lactate, thus, obscuring any direct relationship between lactate uptake and the PAH transport mechanism (Dies et al., 1974).

In the dog, uptake and utilization of α-ketoglutarate (α-KG) and citrate is limitied to the liver and kidney (Mårtensson, 1940; Selleck and Cohen, 1965). This organ specificity parallels that of PAH secretion and led Selleck and Cohen (1965) to postulate that the major function of the PAH transport system was to deliver these and other organic acids into the intracellular fluid. Although filtration and reabsorption appear to be the primary mechanisms for tubular handling

of these acids, evidence for peritubular entry of citrate and α-ketoglutarate exist.

Net renal uptake of citrate was proportional to the arterial plasma concentration (Herndon and Freeman, 1958; Baruch et al., 1973, 1975). If the plasma citrate concentration was elevated without altering extracellular fluid pH, the renal citrate uptake rate exceeded the rate at which citrate was filtered, indicating that a considerable fraction of citrate entered renal tubule cells across the peritubular membrane, probably by the organic acid transport system (Baruch et al., 1973, 1975; Cohen and Kamm, 1976). Baruch et al. (1973, 1975) estimated that peritubular citrate transport was approximately 13% of the peritubular load at endogenous concentrations but only 6% of the peritubular load at high plasma citrate levels. Net renal citrate uptake increased seven-fold at high plasma citrate concentration and the fraction of citrate entering the kidney by peritubular transport did not vary with plasma citrate concentration. Baruch et al. (1973) concluded that the limited rate of citrate uptake at high plasma concentrations could be due to approaching saturation of membrane transport systems for citrate.

Net secretion of α-KG by dog kidney has been observed during citrate infusion and following induction of metabolic alkalosis (Balagura and Stone, 1967). Cohen and Wittman (1963) reported that probenecid administration inhibited net utilization of α-KG by the dog kidney in vivo. Therefore, these authors postulated that α-KG entered proximal tubule cells by the PAH transport system and that the rate of α-KG utilization was limited by the rate of α-KG transport

(Cohen and Wittman, 1963). However, Cohen et al. (1969) later demonstrated that α-KG was not accumulated against a concentration gradient (S/M ratio = 0.65) by dog kidney slices. Although probenecid inhibited α-KG utilization by dog kidney slices, no significant decrease in the α-KG S/M ratio was produced. Thus, probenecid did not inhibit α-KG utilization by depressing entry of α-KG into the slice but apparently altered α-KG metabolism at some intracellular locus (Cohen et al., 1969). In contrast, Pakarinen and Runeberg (1969) demonstrated that guinea pig kidney cortical slices accumulated α-KG from the incubation medium against a concentration gradient. Accumulation of α-KG was inhibited by decreased temperature (0°C) and probenecid. These results were consistent with accumulation of α-KG by the PAH transport system. In addition α-KG can depress the activity of the organic acid transport system. Balagura-Baruch and Stone (1969) demonstrated that infusion of α-KG depressed PAH T_m to 50% of control, results which were similar to those of Knoefel and Huang (1959). Cross and Taggart (1950) and Koishi (1959) demonstrated that α-KG inhibited uptake of PAH by renal cortical slices. Thus, both the in vivo and in vitro observations are consistent with an interaction (possibly competitive) between peritubular transport of PAH and α-KG.

Nonesterified fatty acids (NEFA) are transported in plasma as tightly-bound, noncovalent complexes with albumin and lipoproteins. At normal physiological concentrations only 1-6% of plasma NEFA remains unbound, presumably in the form of acyl carboxylate anions (Nikkila, 1971; Spector, 1971). Due to the tight binding of NEFA to plasma proteins, virtually none of the plasma NEFA is filtered at the

glomerulus (Cohen and Kamm, 1976). Therefore, all of the NEFA utilized by the kidney must enter the renal tubule cell across the peritubular cell membrane (Cohen and Kamm, 1976).

The mechanism involved in the transfer of NEFA into the renal tubule has not as yet been elucidated. However, the preferential extraction of palmitate by the kidney over other long-chain NEFA suggested that palmitate penetrates the cells by a mechanism other than by simple diffusion (Pakarinen, 1970). Several investigators have provided evidence to suggest that palmitate is transported into proximal tubule cells by a mechanism similar to that for PAH. Cross and Taggart (1950) demonstrated that addition of medium chain fatty acids (e.g., octanoate) inhibited accumulation of PAH by rabbit renal cortical slices. Barac-Nieto and Cohen (1968) demonstrated that the *in vivo* uptake of NEFA by dog kidney could be blocked by substrates of the PAH transport system. Probenecid produced a reversible blockade of net renal NEFA uptake while chlorothiazide inhibited renal NEFA uptake irreversibly (Barac-Nieto and Cohen, 1968). Similarily, Dies *et al.* (1970) found that infusion of furosemide depressed the uptake of NEFA by dog kidney. Cohen (1964) also demonstrated that α-KG, which inhibits PAH transport *in vitro* (Cross and Taggart, 1950; Koishi, 1959) and *in vivo* (Balagura-Baruch and Stone, 1968) depressed net uptake of NEFA by dog kidney. Interpretation of these investigations was hampered by possible intracellular effects of probenecid, chlorothiazide and furosemide. However, Barac-Nieto and Cohen (1968) concluded that NEFA entry into renal tubular cells occurred via a discrete transport system inhibitable by substrates of the PAH transport system or that substrates of the PAH system inhibited the

intracellular utilization of NEFA. Barac-Nieto (1971) subsequently demonstrated that palmitate competitively inhibited PAH accumulation by rat renal cortical slices. In addition, PAH accumulation by rabbit cortical slices was depressed when intracellular NEFA concentration was increased following blockade of NEFA oxidation by (+)-palmitylcarnitine or 2-bromopalmitate (Maxild, 1971). These results supported the earlier suggestion that the PAH transport system participates in the uptake of palmitate by the renal proximal tubule (Barac-Nieto, 1971).

However, several investigators have presented what appear to be conflicting data. Pakarinen (1970) investigated the effects of probenecid, PAH and PSP on the uptake and oxidation of palmitate by guinea pig kidney cortical slices. Low concentrations of probenecid (3-6 mM) inhibited slice oxygen consumption without affecting the uptake and oxidation of exogenous palmitate. Increased probenecid concentration (12 mM) decreased oxygen consumption further and inhibited palmitate uptake and oxidation. Addition of PAH (1-9 mM) stimulated palmitate oxidation by cortical slices whereas added PSP (0.4-3.6 mM) had no significant effect on palmitate oxidation or cellular respiration although 3.6 mM PSP produced a slight, but significant decrease in palmitate uptake. Pakarinen (1970) concluded that probenecid inhibited the intracellular metabolism of palmitate, not its entry into renal cells. Heineman *et al.* (1975) supported these observations, demonstrating that probenecid limited the oxidation and esterification of palmitate without altering palmitate uptake by rat renal cortical slices. Various experimental maneuvers that depress slice PAH accumulation (*i.e.*, addition of ethacrynate, meralluride, ouabain, oligomycin and reduction of medium sodium concentration)

routinely altered the degree of oxidation and/or esterification of palmitate without altering total slice palmitate uptake (Heinemann et al., 1975).

Barac-Nieto (1971) demonstrated that inclusion of carnitine in incubation medium stimulated PAH accumulation by rat kidney cortex slices. Carnitine enhancement of PAH accumulation was eliminated when exogenous palmitate was added, suggesting that enhanced PAH accumulation was secondary to reduction of intracellular NEFA concentration (Barac-Nieto, 1971). Based on these results Cohen and Kamm (1976) concluded that a component(s) of the renal organic acid (PAH) transport mechanism participated either in the cellular entry or intracellular binding of NEFA, resulting in delivery of NEFA to their intracellular sites of dissimilation (rather than secretion into the urine).

Development and Substrate Stimulation of Renal Organic Anion Transport

The kidneys of newborn humans and animals are markedly undeveloped in their ability to excrete foreign organic anions such as penicillin (Barnett et al., 1949) and phenolsulfonphthalein (Williamson and Hiatt, 1947). Undoubtedly the anatomic and hemodynamic immaturity of the neonatal kidney is at least partially responsible for this phenomenon. However, the reduced extraction of organic anions by the kidney suggests that the transport capacity (e.g., secretion) of proximal tubule cells is immature as well (Sereni and Principi, 1968).

The renal clearance and extraction of PAH by the newborn has been reported to be low in human subjects (Calcagno and Rubin, 1963), puppies (Horster and Valtin, 1971; Hook et al., 1970), rats (Horster and Lewy, 1970), rabbits (Lewy and Grosser, 1974) and sheep (Phelps et al., 1976). In addition, slices of renal cortex from newborn animals

of several species exhibited a reduced transport capacity for PAH when compared to adult or fetal tissue (New et al., 1959; Rennick et al., 1961; McIsaac, 1965; Hirsch and Hook, 1969a, 1970b). Maturation of tissue transport capacity proceeds in a characteristic pattern. Accumulation of PAH by renal tissue increases from birth to two weeks of age. Accumulation then increases rapidly to a peak at four weeks and declines to an intermediate value (Hook, 1974). Since kidney weight develops more linearly, these data suggest that increased accumulation of PAH represents more than simple organ growth and development. That is, there appears to be specific maturation of the transport system.

A question arises as to the triggering stimuli responsible for initiating the maturation of PAH transport capacity. The decreased transport capacity observed in renal tissue from newborn as compared to fetal rabbits could be interpreted as an indication that the functional load presented to the kidney had increased (Hirsch and Hook, 1970b). At birth, the sudden change in supply of energy yielding substrates from maternal blood glucose to a milk diet high in lipid (including medium and long chain NEFA) would increase the circulating concentration of organic anion substrates (Smith and Abraham, 1975; Aprille and Rulfs, 1976; Aprille, 1976). In addition, renal vascular resistance falls and renal blood flow increases during the immediate newborn period (Edelmann and Spitzer, 1969). The combination of these factors would serve, presumably, to increase the functional load imposed upon the transport system (Hook, 1974).

The functional capacity of many developing enzyme systems increase in response to an increased functional load (Yaffe and Juchau, 1974).

Similarly, when Hirsch and Hook (1969a,b; 1970a,b) treated newborn rabbits prior to maturation with large exogenous loads of penicillin, the ability of renal tissue to accumulate PAH was more than doubled. However, upon reaching maturation (i.e., four weeks), exogenous substrate loads produced no effect (Hirsch and Hook, 1969a). Substrate stimulation of PAH transport has been produced with a variety of secreted organic anions. In addition to penicillin, transport was stimulated with PAH and triiodothyronine (Hirsch and Hook, 1969b,c; 1970a). This phenomenon has been observed in species other than rabbit, including rat and dog (Hirsch and Hook, 1970; Johnson et al., 1974). Substrate stimulation also altered the excretion of drugs in the intact animal. Lewy and Grosser (1974) used modified micropuncture techniques to document the low renal clearance and extraction of PAH by newborn rabbits. Following penicillin treatment, PAH extraction was significantly increased. Noordewier and Withrow (1976) demonstrated that the low clearance and transport maximum of PAH in newborn rats was increased after penicillin. Extraction of PAH across the newborn dog kidney was also increased by substrate stimulation (Bond et al., 1976). A recent report of Schwartz et al. (1976) suggested that the phenomenon of substrate stimulation of anion transport occurs in human neonates. These investigators were treating a human neonate with oxacillin and phenobarbital. Subsequently, they were unable to obtain therapeutic concentrations of dicloxacillin even with supratherapeutic doses. Intestinal absorption appeared to be high, but plasma levels were low and were correlated with a high urinary excretion rate. Therefore, these investigators postulated that the renal tubular transport of dicloxacillin was stimulated by the administration of oxacillin (and/or phenobarbital).

The enhanced PAH transport capacity following administration of penicillin occurred without any change in organic cation transport (Hirsch and Hook, 1970) and did not result from an alteration in the morphologic (Hirsch et al., 1971) or ultrastructural (Pegg et al., 1976), characteristics of the proximal tubule cell. Although substrate stimulation enhanced the ability of renal tissue to accumulate PAH, it did not alter the oxygen consumption of proximal tubules (Ecker and Hook, 1974b). In addition, penicillin and acetate stimulated PAH accumulation via separate mechanisms, since the acetate-induced stimulation of PAH accumulation was not blunted in renal tissue from treated neonates (Hirsch and Hook, 1970b; Pegg and Hook, 1975b).

The degree of stimulation produced by penicillin pretreatment was found to be dose-dependent (Pegg and Hook, 1975b). Maximal stimulation of PAH transport capacity was observed 24 hours after the last of four injections of 90,000 I.U. of procaine penicillin G were administered to young rabbits at 12 hour intervals (Pegg and Hook, 1975b). Penicillin excretion was essentially completed during the intervening 24 hour period (Pegg and Hook, 1975b). Therefore, the enhanced PAH transport capacity could not be attributed to a mechanism (e.g., counter-transport) dependent upon the immediate presence of penicillin in kidney tissue during estimation of PAH accumulation. Indeed, when penicillin was added directly to incubation beakers containing PAH and renal cortical slices, PAH accumulation was markedly depressed (Hirsch and Hook, 1970b). Interestingly, if estimation of transport capacity was delayed more than 36 hours after the final penicillin injection, no stimulation of PAH accumulation could be detected (Hirsch and Hook, 1970b; Pegg and Hook, 1975b). The transitory nature of this effect suggested that the proximal tubule cells responded to the increased

functional load produced through penicillin injection by increasing the number of specific transport proteins available for anion transport (Hirsch and Hook, 1970c; Pegg and Hook, 1975b). This hypothesis appeared reasonable since both normal (Ecker and Hook, 1974a) and penicillin-induced (Pegg et al., 1976) maturation of transport capacity were associated with an increase in the apparent maximum velocity (V_{max}) for PAH accumulation. In addition, Hirsch and Hook (1970c) and Pegg and Hook (1975b) demonstrated that an intact protein synthetic mechanism was required for substrate stimulation since concomitant administration of cycloheximide prevented penicillin enhancement of PAH transport capacity. The effect of penicillin administration on renal protein biochemistry was further analyzed by Hirsch and Hook (1970c). Substrate stimulation was associated with increased uptake of ^{14}C-L-leucine and ^{14}C-L-glutamine into the trichloroacetic acid insoluble fractions of rat renal cortical homogenates. However, no effect was observed on amino acid uptake into the corresponding fraction from rat medulla. In addition, a non-specific stimulus (chronic NH_4Cl acidosis) of renal protein synthesis increased labelled amino acid uptake but had no stimulating effect on PAH transport (Hirsch and Hook, 1970c). Therefore, the effect of penicillin was probably not the result of nonspecific increases in protein content. From these observations, Hirsch and Hook (1970c) concluded that the stimulatory effect of penicillin on PAH transport was the result of increased synthesis of specific transport proteins.

Objectives

The unifying purpose of this investigation was to further define the specific biochemical sequences and the physiological functions involved in the translocation of organic anions across the proximal tubule cell. Three primary objectives were identified: 1) to demonstrate the existence of multiple transport systems for the secretion of organic acids within the kidney; 2) to provide additional evidence that palmitate is an endogenous substrate of the PAH transport system; 3) to provide evidence that an intracellular modulator of the PAH transport system exists and to elucidate the role of this modulator in acetate-induced stimulation of PAH accumulation by renal cortical slices. The maturing kidney was used as an experimental model during several segments of this investigation because of its selective physiological response to exogenously administered functional loads.

METHODS

GENERAL

1) Experimental Animals

Litters of New Zealand white rabbits were bred and raised in departmental animal quarters or purchased from a local breeder. Young animals remained with their mothers during treatment or until the time of experimentation. Rabbits used for determination of developmental patterns of anion transport and metabolism were weaned at 4 weeks of age. Adult female New Zealand white rabbits (2.5-4.0 kg), male Sprague-Dawley rats (150-250 gm) and mongrel dogs of either sex were purchased from local suppliers and used after a suitable period of acclimation. Male bullfrogs (Rana Catesbiana, 500-750 gm) were purchased from Southwestern Scientific Co., Tucson, Arizona.

2) Determination of Slice Anion Accumulation

Animals were killed by a blow to the head, the kidneys removed and placed in ice-cold saline (0.13 M NaCl - 0.02 M KCl). Thin renal cortical slices were prepared freehand and incubated in 3.0 ml of the phosphate buffered medium[1] described by Cross and Taggart (1950) which contained the anion under study. Metabolic substrates (i.e., acetate, lactate, citrate, α-ketoglutarate, succinate

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[1] 97 mM NaCl, 40 mM KCl, 0.7 mM $CaCl_2$, 10 mM NaH_2PO_4-Na_2HPO_4.

glucose) were added to the media as sodium salts and equimolar amounts of NaCl were added to control media. Incubations were performed in duplicate (150-200 mg slices/beaker) in a Dubnoff metabolic shaking incubator at 25°C under an atmosphere of 100% O_2. Duration of incubation varied with the study undertaken. After incubation slices were removed from the medium, blotted and weighed. Tissue and a 2.0 ml aliquot of medium were extracted in 3.0 ml of 10% trichloroacetic acid (TCA). Water was added to a final volume of 10.0 ml and the samples were centrifuged. Aliquots of tissue and medium extracts were taken for assay and results expressed as slice-to-medium (S/M) ratios where S = mg (DPM) of anion/gm wet weight of tissue and M = mg (DPM of anion/ml of medium.

3) Preparation of Suspensions of Isolated Proximal Tubules

The technique of preparing separated renal proximal tubules was similar to the method of Brendel and Meezan (1975) and Meezan et al. (1973) with modifications drawn from the work of Burg and Orloff (1962) and Huang and Lin (1965). Rabbits were stunned by a blow to the head and rapidly exsanguinated. In young (2 week) rabbits, the abdominal aorta was clamped superior to the branching of the renal arteries. A cannula (blunted, 20 ga. stainless steel needle) was inserted inferior to the kidneys, both renal branches of the vena cava slit, and the kidneys perfused simultaneously with chilled, oxygenated, Ringer's solution[2] until clear of blood. The infusion was continued with a suspension of magnetic iron oxide (MIO, 5 mg/ml) in

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[2]120 mM NaCl, 16.2 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 10 mM NaAcetate, 10 mM KH_2PO_4-Na_2HPO_4, pH 7.40.

Ringer's solution until the kidneys were a uniform gray-black in color (25-30 mls were usually sufficient). The kidneys were removed and the cortex rapidly dissected and finely minced in 40-50 ml (≃1 gram cortex/5 ml Ringer's) cold Ringer's solution. The cortical mince was gently homogenized with 6-8 vertical strokes in a hand held Potter-Elvehjem homogenizer with teflon pestle. The resulting homogenate was poured over a 253 μ nylon sieve and thoroughly washed with a large volume of chilled, oxygenated Ringer's solution (1-1.5 liters). The resulting filtrate was poured through an 83 μ nylon sieve and washed with buffer. The material remaining on this sieve (tubules and glomeruli) was suspended in Ringer's solution and placed on top of a powerful, permanent magnet. The iron-containing glomeruli were attracted to the magnet allowing the supernatant, containing the proximal tubules, to be decanted. This procedure was repeated three times to ensure removal of iron-containing glomeruli. The tubule suspension was centrifuged at room temperature for 60 seconds at 1000 rpm and the supernatant decanted. The pellet was resuspended and washed three times with 50 ml of oxygenated 5% calf serum-Ringer's solution. The tubules were again pelleted and resuspended in the appropriate incubation medium at a concentration of 90-120 mg tubule/ml medium. The procedure was similar when proximal tubules from older rabbits (4 week-adult) were prepared except that each renal artery was cannulated and the kidneys perfused separately.

4) Determination of Proximal Tubule PAH Accumulation

Proximal tubules were suspended (≃100 mg tubule/ml) in Ringer's medium containing 10 mg/ml defatted (Chen, 1967) bovine serum

albumin (BSA) and 10 mM Na acetate. Six to 8 ml samples of the tubule suspension were added to 25 ml Erlenmeyer flasks in a New Brunswick Scientific Gyrotory Shaker and preincubated for 15 minutes at 37°C under a gas phase of 100% oxygen. Following preincubation, PAH was added to attain a final medium concentration of 7.4×10^{-5}M and incubation continued for a further 30 minutes. Two samples (3-4 ml/sample) were taken at the end of incubation and rapidly centrifuged for 10 minutes, 10,000 x g at 0°C in the special centrifuge tubes designed by Burg and Orloff (1962). Inulin was added to a concentration of 0.09% prior to centrifugation to estimate trapped medium in the tubule pellets. Tissue water content was determined by drying the pellets to a constant weight. Pellets were homogenized in 5.0 ml of 3% trichloroacetic acid and precipitated protein removed by centrifugation. Duplicate 2 ml aliquots of the medium were deproteinized with 3.0 ml of 10% trichloroacetic acid, centrifuged and brought to a final volume of 10.0 ml with water. Aliquots of the trichloroacetic acid extracts of tubule pellets and media samples were taken for assay of PAH (Smith et al., 1945) and inulin (Schreiner, 1950). PAH accumulation was expressed as the tubule-to-medium (T/M) ratio where T equals milligrams of PAH per milliliter of intracellular water and M equals milligrams of PAH per milliliter of medium.

5) Determination of Proximal Tubule Palmitate Oxidation

Proximal tubules were suspended in Ringer's medium containing 10 mg/ml defatted (Chen, 1967) bovine serum albumin and 10 mM Na acetate. An aliquot of the tubule suspension was transferred to 25 ml erlenmeyer flask and pre-incubated for 15 minutes at 37°C under a gas phase of 100% oxygen. Following pre-incubation, the tubules were pelleted by centrifugation (1,000 rpm, 1 minute, room temperature) and

resuspended in an equivalent volume of temperature and gas equilibrated Ringers medium containing 1 mM palmitate-1-^{14}C (0.4 μCi/μmole) complexed to 10 mg/ml defatted bovine serum albumin ([NEFA]/[BSA] ≃ 6) and 10 mM Na acetate. The tubule suspension was transferred to a 25 ml erlenmeyer flask (Kontes Glass Co. #K-882300), gassed for 1 minute with 100% O_2 and the flask sealed with a serum rubber stopper (Kontes Glass Co. #K882310) to which an empty plastic cup for CO_2 collection was attached (Kontes Glass Co. #K882320). The tubules were incubated for a further 30 minutes. Incubation was terminated by addition of 0.2N H_2SO_4 (0.2 ml/ml suspension) to the incubation mixture (Heinemann et al., 1975). Liberated CO_2 was trapped in 0.4 ml of 1 M hyamine hydroxide (New England Nuclear Co.). Hyamine was added with a syringe and needle to the plastic cup suspended from the stopper of each flask, immediately after the addition of H_2SO_4. Incubation was continued for an additional hour at 37°C with constant shaking to achieve complete trapping of CO_2 in the hyamine hydroxide. The cup containing hyamine carbonate was then transferred to a scintillation vial containing 10 ml of Omniflour (New England Nuclear Co.) in toluene. The radioactivity in $^{14}CO_2$ was determined by liquid scintillation spectrometry using a Beckman LS-100 liquid scintillation counter. Counting efficiency was determined by internal standardization with ^{14}C-toluene. Aliquots of the incubation medium containing palmitate-1-^{14}C, equivalent to the volume of the tubule suspension were handled in a similar fashion in each experiment to correct for nonspecific evolution of $^{14}CO_2$. Liberation of $^{14}CO_2$ by medium blanks represented less than 1% of the $^{14}CO_2$ produced by tubule suspensions in each experiment.

6) Determination of Palmitate Esterification by Suspensions of Proximal Tubules

Incubation of proximal tubules was essentially the same as that outlined for the palmitate oxidation studies. Incubation was terminated by centrifugation (3,500 rpm, 1 minute, room temperature). The medium supernatant was decanted and the tubule pellet suspended in 2.0 ml of 0.2N H_2SO_4. Following centrifugation palmitate loosely attached to the tissue pellet was removed by resuspending the pellet in 10.0 ml cold Ringer's medium containing 10 mg/ml defatted BSA (Heinemann et al., 1975). The suspension was centrifuged and the pellet weighed. All calculations were based on the wet weight of this pellet.

Esterification of palmitate was established by homogenization and extraction of the tissue pellet according to the method of Folch et al. (1957). Homogenization was performed on ice with 2:1 $CHCl_3$: CH_3OH (20:1 volume:pellet wet weight) and left overnight for extraction. Insoluble material was pelleted by centrifugation following addition of 0.2 volumes CH_3OH. The resulting supernatant was returned to the correct solvent ratio and the extract partitioned with 0.2 volumes of 0.05 M KCl and washed with equilibrated upper phase to remove water soluble products (Heinemann et al., 1975; Barac-Nieto, 1976; Folch et al., 1957). The lower phase was taken to dryness under 100% N_2, a known volume of $CHCl_3$:CH_3OH (2:1) added and the sample stored at -20°C until analyzed. An aliquot of the pooled upper phase and washings was added to scintillation vials containing 10 ml of Omniflour in toluene for determination of ^{14}C-radioactivity in water soluble compounds. Insoluble material contained in the original homogenate was solubilized in a known volume of SHT (8% sodium hydroxide, 1% Triton X405, 3%

methanol) by heating at 80°C for 2 hrs with continuous shaking (J. Dent, personal communication). An aliquot was neutralized with 4.4 M nitric acid and added to scintillation vials containing 15 ml of Aquasol (New England Nuclear Co.). Duplicate aliquots of the lower phase of the Folch extract were added to scintillation vials containing 10 ml of Omniflour in toluene for determination of the ^{14}C-radioactivity present in the total tissue lipids (TL). Individual lipid classes were separated by thin layer chromatography on Silica Gel G, using as solvent, n-hexane, diethylether, glacial acetic acid (80:20:1, v/v) (Blank and Snyder, 1975). Duplicate aliquots of the lower phase were spotted on heat activated (30 min, 110-120°C), 250 μ Silica Gel G TLC plates (Analtech Inc., Wilmington, Del.) and developed in closed glass chambers lined with filter paper. Component spots were identified by comparison with known standard mixtures (TLC Mix A and C, Supelco Inc.; Mixture TLC-8, Applied Science Laboratories, Inc.) included on each plate. Spots were visualized with iodine vapor, circumscribed, and when the iodine had disappeared, scraped and transferred to scintillation vials. Carbon-14 activity was determined by suspending the scrapings in 4% Cab-O-Sil (Eastman Kodak Co.) in Omniflour-toluene. Five fractions were obtained (in descending order from the solvent front): 1) cholesterol esters (CHE), 2) triglycerides (TG), 3) nonesterified fatty acids (NEFA), 4) cholesterol (CH; di- and mono-glycerides migrated with this fraction), and 5) phospholipids (PL). Carbon-14 activity was determined by liquid scintillation spectrometry using a Beckman LS-100 liquid scintillation counter. Counting efficiency was determined by internal standardization with ^{14}C-toluene. Carbon-14 activity in each fraction was converted to

palmitate equivalents by comparing it with the specific activity of the palmitate initially placed in the incubation medium. Results were expressed as μmoles of palmitate incorporated per gram wet weight of tubule.

The NEFA concentration (Ho, 1970) and the concentration of radioactivity in the incubation medium before and after incubation with proximal tubule suspensions was determined in several experiments to verify constancy of medium NEFA specific activity (Barac-Nieto, 1976). The ratio of initial-to-final medium NEFA specific activities approximated 1 in each case, suggesting that medium NEFA concentration was not diluted by endogenous tubule NEFA. These results were similar to those of Barac-Nieto (1976).

In certain experiments, total nonesterified fatty acids present in the lower phase of the Folch extract was determined by a modification of the method of Ho (1970; Bieber et al., 1975). The plasma NEFA concentration was also determined in several experiments.

7) Preparation and Incubation of Suspensions of Renal Cortical Mitochondria

Adult, female New Zeland white rabbits were stunned by a blow to the head, exsanguinated and the kidneys removed and rinsed in an ice cold saline (0.13 N NaCl-0.02 N KCl). The cortex was removed by scissor dissection, minced and homogenized (1.0 gm cortex/5.0 ml solution) in 0.3 M sucrose, 5 mM Hepes[3], 1 mM EDTA, pH 7.4 with six strokes of a motorized (600 rpm) Potter-Elvehjem homogenizer with Teflon pestle (Simpson and Angielski, 1973; Adam and Simpson, 1974, 1975; Simpson and Adam, 1975). The homogenate was centrifuged for 5

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[3]Hepes: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid.

min, 700 x g at 2°C in a Beckman J-21 centrifuge. The supernatant was decanted and saved, and the pellet rehomogenized in an equivalent volume of the sucrose-Hepes-EDTA solution. Following centrifugation, the two supernatants were combined and centrifuged for 10 min, 8,000 x g (max) at 2°C. The supernatant was discarded and the mitochondrial pellet resuspended and washed twice in 0.3 M sucrose, 5 mM Hepes, pH 7.4 and centrifuged in the same manner. The final pellet was suspended in sufficient 0.3 M sucrose to yield a mitochondrial protein concentration of 30-60 mg/ml. Mitochondrial protein concentration was determined by the Biuret method (Layne, 1957).

Mitochondrial oxygen consumption and respiratory control ratios were determined by a modification of the methods of Simpson and Adam (1975) and Sordahl et al. (1971). Aliquots (0.2 ml/4-6 mg protein) of the mitochondrial suspension were added to 2.8 ml of incubation medium[4] and oxygen consumption determined at 25°C using a Yellow Springs Instrument model 53 oxygen monitor employing a Clark-type oxygen electrode. State 3 oxygen consumption was determined following addition of sufficient adenosine-5'-diphosphate to achieve a concentration of 0.3 mM. State 4 oxygen consumption was determined after the state 3 burst had subsided.

Mitochondrial PAH accumulation was estimated by a modification of the methods of Simpson and Angielski (1973) and Adam and Simpson (1974, 1975). For measurement of PAH uptake, incubations were performed in 25 ml Erlenmeyer flasks containing 0.5 ml of the mitochondrial suspension and 4.5 ml of incubation medium[5] containing either 1

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[4] 60 mM KCl, 10 mM potassium phosphate, 5 mM $MgSO_4$, 142 mM sucrose, 5 mg/ml bovine serum albumin, 10 mM malate and 10 mM pyruvate, pH 7.4.

[5] 85 mM KCl, 25 mM $KHCO_3$, 5 mM Hepes, 5 mM $MgSO_4$, 2.5 mM KH_2PO_4, 10 mM acetate, pH 7.4.

mM (sp.ac. = 0.5 μCi/μmole) or 10 mM (sp.ac. = 0.05 μCi/μmole) ^{14}C-PAH. Mannitol ^{3}H (0.5 μCi/ml) was added to the medium to determine concurrently the outer space of the mitochondria. The relationship of the outer mitochondrial space to total mitochondrial water was determined in parallel flasks containing ^{14}C-sucrose (0.5 μCi/ml) and ^{3}H-H_2O (0.5 μCi/ml). Incubations were performed at 25°C under a gas phase of 95% O_2-5% CO_2. Duration of incubation varied from 5-20 minutes. Mitochondria and medium were separated by the method of Harris and Van Dam (1968) as adapted by Simpson and Angielski (1973) and Simpson and Adam (1975). Perchloric acid (500 μl, 1 N) was placed in a 1.5 ml polypropylene microcentrifuge tube (Arthur H. Thomas Co.) and a layer of silicone oil (0.5 ml of Versilube F-50, General Electric Co.) was introduced on top. At each time point, duplicate 200 μl aliquots of the incubation mixture were layered on top of silicone oil and the tubes immediately centrifuged in an Eppendorf 5412 microcentrifuge (12,000 rpm, 8,000 x g) for 1.0 minute. A second pair of 200 μl aliquots were added directly to 500 μl of 1 N HCl)$_4$ for determination of medium radioactivity. Neutralized samples were counted in 10.0 ml Aquasol (New England Nuclear) in a Packard Tri-Carb liquid scintillation spectrometer. Samples were corrected for quench by the channel ratio technique. The volume of a space was determined by dividing the total dpm of a labelled compound present in the mitochondrial pellet by the dpm/μl of that substance in the medium. The matrix space was calculated by subtracting the volume of the outer (sucrose-impermeable) space from the total mitochondrial water space (^{3}H-H_2O-permeable) (Simpson and Adam, 1975). The outer space was also

calculated in the presence of ^{14}C-PAH by the distribution of ^{3}H-mannitol and the matrix space in these samples calculated from the ratio of matrix to outer space in those samples containing $^{3}H\text{-}H_2O$ and ^{14}C-sucrose (Adam and Simpson, 1974). The ^{14}C-PAH dpm in the outer space was calculated from the PAH concentration (dpm/µl) in the medium and the measured outer space (^{3}H-mannitol) in each sample. This quantity was subtracted from the total ^{14}C-PAH dpm in the mitochondrial extract to give the total ^{14}C-PAH dpm in the matrix space. A ratio of matrix ^{14}C-PAH dpm per µl of matrix space to the ^{14}C-PAH concentration (dpm/µl) in the medium greater than 1.0 was defined as mitochondrial PAH accumulation. Mitochondrial PAH uptake was also calculated as nmole matrix PAH per mg mitochondrial protein following conversion of ^{14}C-PAH dpm by comparison to the PAH specific activity initially placed in the medium.

USE OF TRANSPORT ONTOGENY AND SELECTIVE SUBSTRATE STIMULATION AS MODELS FOR IDENTIFICATION OF MULTIPLE RENAL ORGANIC ANION TRANSPORT SYSTEMS

1) Effect of Age

The ability of renal cortical slices to accumulate PAH and several other organic anions was quantified using rabbit pups randomly selected from litters at the designated ages. Rabbit pups ranging from 1-8 weeks of age were used. The values obtained were compared to those obtained from unrelated adult rabbits.

2) Effect of Substrate Stimulation

Within each litter animals were proportioned equally among the several dosages (treatments) employed with the appropriate control being included for each treatment. Procaine penicillin G was administered

subcutaneously as a suspension while suspensions of sulfisoxazole and uric acid were prepared in 1% methylcellulose and administered intraperitoneally. The sodium salts of iodipamide, probenecid, acetylsalicylic acid and para-aminobenzoic acid were dissolved in 0.9% saline brought to pH 7.4 and injected intraperitoneally. In each treatment group control animals received a volume of the appropriate vehicle equivalent to that required to deliver the maximum dose of the corresponding drug. Treatment was begun on day 10 or 11 of age. Procaine penicillin G was administered either in a dose of 30,000 I.U. twice daily (60,000 I.U./day) for 3 days or in a dose of 90,000 I.U. twice daily (180,000 I.U./day) for 2 days. All other drugs were administered twice daily for 3 days beginning on day 10 of age. Animals were sacrificed 24 hours after the final drug injection, on day 14 of age.

3) Slice Anion Accumulation

Renal cortical slices from rabbits in each treatment group (control or drug) were pooled and then randomly distributed among beakers containing the anions under study. Each pair of duplicate beakers contained a single organic anion substrate in 3.0 ml of the standard incubation medium. In this manner, accumulation of 3 anions (PAH and 2 others) could be determined in a single litter of rabbits. Alternatively, accumulation of a single anion (PAH) could be determined at four drug dosage levels. Anionic transport substrates added to the various incubation media included: p-aminohippurate (PAH), 7.4×10^{-5}M; phenolsulfonphthalein (PSP), 7.4×10^{-5}M; sulfisoxazole (SULFI), 7.4×10^{-5}M; benzyl (^{14}C) penicillin G (PEN), 7.4×10^{-5}M; acetyl (carboxyl-^{14}C) salicylic acid (ASA), 7.4×10^{-5}M; uric acid-2-^{14}C (UA),

5.0×10^{-4}M; chenodeoxycholate (carboxyl-^{14}C) (CDC), 1.0×10^{-6}M. In some instances 10 mM sodium acetate was also included in the incubation medium.

Duration of incubation was 90 min. After incubation the slices were removed from the beakers, blotted and weighed. Trichloroacetic acid extracts of slices and media were prepared as outlined for assays of PAH, PSP, PEN, UA and SULFI. ASA accumulation was determined following extraction of slice and medium samples in 0.1 N NaOH (Putney and Borzelleca, 1973). Slices incubated with CDC were digested by incubating (37°C) with Soluene-100 (Packard) Bárány, 1975). Aliquots of medium containing CDC were treated with 10% TCA.

PAH and SULFI were estimated by the method of Bratton and Marshall (1939) as modified by Smith et al. (1945). PSP was determined with minor modification of the method of Forster and Copenhaver (1956). Carbon-^{14} activity was determined by adding aliquots of tissue and medium digest to scintillation vials containing 10 ml of modified Bray's solution (Hirsch and Hook, 1970) or 10 ml of Omniflour (New England Nuclear) in toluene and counted in a Beckman LS-100 liquid scintillation counter. Counting efficiency was determined by internal standardization with toluene-^{14}C.

INVESTIGATIONS ON THE METABOLIC MODULATION OF p-AMINOHIPPURATE ACCUMULATION BY RABBIT RENAL CORTICAL SLICES

1) Effect of Acetate Washout

Rabbit renal cortical slices were prepared as outlined. Three groups of slices were incubated for a total of 90 minutes, the final 30 minute period in the presence of PAH. The control group was pre-incubated for 60 minutes in the normal Cross and Taggart buffer

with PAH omitted. The slices were then transferred to beakers containing medium + PAH for an additional 30 minutes. Acetate-incubated slices were handled in a similar fashion except sodium acetate (10 mM) was included in the final incubation. Acetate-washed slices were preincubated for 30 minutes with acetate (10 mM) and washed without acetate in 3 separate 10 minute washings. The final incubation was conducted as in the control group.

2) Onset of Acetate Stimulation

Slices from the cortices of kidneys of two rabbits were pooled. Approximately one half of the slices were incubated in 7.4×10^{-5}M PAH for intervals from 0 to 90 minutes. The second group of slices was incubated in PAH for 60 minutes. Sodium acetate (10 mM) was then added and PAH accumulation measured at 0.5, 1, 2, 3, 5, 10, 15, 20 and 30 minutes. PAH accumulation was expressed as µg PAH/g wet weight of tissue.

3) Effect of 100% N_2 on PAH Accumulation

Rabbit renal cortical slices were pooled. Approximately one half of the slices were preincubated under 100% O_2 for 30 minutes while the remaining slices were preincubated under 100% N_2. PAH was added at the end of the pre-incubation period to a concentration of 7.4×10^{-5}M and incubation continued for durations of 15-60 minutes.

4) Effect of Acetate on the Rate of PAH Uptake

Renal cortical slices from the kidneys of two rabbits were pooled. Half the slices were pre-incubated under 100% O_2 and half under 100% N_2 for 30 minutes. Within each group Na acetate (10 mM) was present in one-half of the beakers. PAH was added at the end of

the pre-incubation period to attain final medium concentrations of 1, 2, 4, 8, 16, 32, 64, 128 and 256×10^{-4}M. Slices were removed from the incubation beakers after 15 minutes and handled as before. PAH uptake under 100% N_2 was subtracted from uptake under 100% O_2 to estimate the rate of uptake of the oxygen-requiring transport of PAH.

5) Effect of Acetate on Medium pH

Cortical slices were pre-incubated in medium (initial pH adjusted to 7.40) containing either 10 mM Na Acetate or 10 mM NaCl. PAH was added at the end of pre-incubation to a final concentration of 7.4×10^{-5}M. Slices were incubated for 15, 30, 60 or 90 minute intervals. After incubation, the final pH of the medium was determined. Final pH was measured within one (1.0) minute of removal from the shaker with an Instrumentation Laboratory, Inc. pH meter (Model 205) and an H^+-sensitive glass electrode (I.L. Cat. No. 14043). Hydrogen ion concentration was calculated from the measured pH under the assumption that the activity for hydrogen ion is 1.0. Mean hydrogen ion concentration ± S.E. was calculated from replicated experiments. For illustrative purposes, the data were then converted back to pH.

6) Effect of Acetate (10 mM) on Slice PAH Accumulation and Citrate Concentration

The kidneys from two rabbits were pooled for each experiment. One-quarter of the cortex from each kidney was removed for determination of the endogenous renal cortical concentration of citrate. The cortical samples were placed immediately in pre-weighed beakers containing 2.0 ml of ice-cold 10% TCA (w/v). The remaining cortex was placed in ice-cold saline and sliced. 250-500 mg of slices were incubated for 90 min in 3.0 ml of media containing either 10 mM Na acetate or 10 mM

NaCl. PAH was not present in the incubation media. The slices from two beakers (total sample wt = 500-1,000 mg) were combined at the end of incubation and placed immediately in pre-weighed beakers containing 2.0 ml of ice-cold 10% TCA. Both the cortical samples and the slice samples were weighed and homogenized in cold TCA using a Polytron homogenizer. The samples were centrifuged and the proteinfree filtrates were analyzed for citrate content by the pentabromacetone method of McArdle (1955). The results from duplicate samples were expressed as μmoles citrate/gram wet weight tissue. PAH accumulation in control and acetate media was determined in each experiment during a 60 minute incubation period after an initial 30 minute pre-incubation without PAH.

7) Citrate Dose-response Curve

Renal cortical slices were pre-incubated for 30 minutes in Cross and Taggart buffer (pH 7.40) containing 0.3, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 mM Na_3 citrate (Citric Acid, A Grade, Calbiochem.; adjusted to pH 7.40 with NaOH). Control slices were pre-incubated in media containing the appropriate concentrations of NaCl ([NaCl] = 3[Na_3 Citrate]). PAH (pH 7.40) was added at the end of pre-incubation to a final concentration of 7.4×10^{-5}M. Slices were removed from the incubation beakers at the end of a 60 minute incubation period and handled as before. Final medium pH of the incubation beakers was obtained as described above.

8) Effect of Altered Potassium Concentration

Rat renal cortical slices were incubated in 3.0 ml of normal Cross and Taggart buffer ($[K^+]$ = 40 mM) or in 3.0 ml of a reduced potassium medium ($[K^+]$ = 5 mM). In some beakers, Na acetate (10 mM)

or Na lactate (10 mM) were added as metabolic substrates. PAH accumulation and slice citrate concentration were estimated as outlined previously.

9) Effect of Chronic Metabolic Acidosis

Metabolic acidosis was induced in groups of rats by ingestion of 0.28 M ammonium chloride as sole drinking fluid for 7 days (Adam and Simpson, 1974). Control rats had free access to tap water. The pH and pCO_2 of arterial blood (drawn from the abdominal aorta of ether-anesthetized rats) were measured at 37°C with an Instrumentation Laboratory Model 27 pH/gas analyzer. The bicarbonate concentration of blood was calculated from the Henderson-Hasselbalch equation. A pK of 6.1 was used for blood. The solubility coefficient used to correct CO_2 tensions to H_2CO_3 was 0.0306 (Alguire et al., 1974). The endogenous renal cortical citrate concentration and slice PAH accumulation were determined as before.

10) Effect of Pre-incubation in Citrate on Stimulation of PAH Accumulation (S/M Ratio) by Acetate

Renal cortical slices were pre-incubated in medium containing either 10.0 mM Na_3 citrate or 30.0 mM NaCl for 30 minutes. After pre-incubation the slices were transferred to beakers containing Na acetate (0.5 mM or 10 mM) or NaCl in phosphate buffer and incubated with 7.4×10^{-5} M PAH for 60 minutes. All media were adjusted to pH 7.40 before the addition of tissue. PAH S/M ratios and final media pH were determined as described above.

11) Effect of Acetate (1 mM) on Slice PAH Accumulation, Citrate and α-Ketoglutarate Concentration

Preparation of slices, rabbit kidney cortex samples and slice incubation studies were performed essentially as outlined above. The concentration of Na acetate in the incubation medium was reduced to 1 mM. Samples taken for α-ketoglutarate analysis were placed in preweighed beakers containing 3.0 ml cold 3% (w/w) $HClO_4$, weighed and homogenized with a motor driven Potter-Elvehjem homogenizer with Teflon pestle. The homogenates were centrifuged at 11,000 x g for 30 minutes at 2°C. Supernatants were brought to pH 7.4 with 1 N NaOH (final volume = 15.0 ml) and assayed by the glutamic dehydrogenase method of Selleck et al. (1964). The results of duplicate samples were expressed as μmoles α-ketoglutarate/gm wet weight of tissue.

EFFECT OF AGE AND SUBSTRATE STIMULATION ON RABBIT PROXIMAL TUBULE PAH ACCUMULATION AND PALMITATE METABOLISM

1) Effect of Ages

The ability of renal proximal tubule suspensions to accumulate PAH and to oxidize and esterify palmitate-1-^{14}C was quantified using rabbit pups randomly selected from litters at the designated ages. Rabbit pups ranging from 1-6 weeks of age were used. In some instances, a mixed arterial and venous blood sample was collected during exsanguination in heparinized tubes. The samples were centrifuged and the plasma stored at -20°C until analysis. Plasma concentration of NEFA was estimated by a modification of the method of Ho (1970). Total NEFA present in the lower phase of the Folch extracts was also determined in several experiments. The values obtained were compared to those obtained from unrelated adult rabbits.

2) Effect of Penicillin Pretreatment of 2-Week-Old Rabbits

Procaine penicillin G was administered to rabbits in a dose of 90,000 I.U. twice daily for 2 days beginning on the 11th day of life. Animals were sacrificed 24 hours following the final injection. Suspension of proximal tubules prepared from control and penicillin treated littermates were incubated in a paired fashion for determination of PAH accumulation and oxidation and esterification of palmitate-1-^{14}C. The NEFA concentration of plasma and Folch extracts was determined in several experiments as discussed previously. Results were expressed as the mean difference ± standard error for the difference between functions measured in control and treated tubule suspensions.

3) Effect of Iodipamide Pretreatment of 2-Week-Old Rabbits

The sodium salt of iodipamide was administered intraperitoneally in a dose of 100 mg/kg body weight. Rabbits were treated twice daily for 3 days beginning on the 10th day of life. Animals were sacrificed 24 hours following the final injection. Proximal tubular PAH accumulation and palmitate oxidation and esterification were determined as before. Results were expressed as the mean difference ± standard error for the difference between functions measured in control and treated tubule suspensions.

4) Effect of Pencillin Pretreatment of Adult Rabbits

Pairs of adult, female New Zealand white rabbits were matched by body weight for use in these experiments. Rabbit body weight ranged from 2.5 kg to 4.0 kg. Procaine penicillin G was administered in a dose of 450,000 I.U./kg body weight twice daily for 2 days. Animals were sacrificed 24 hours after the last injection. Proximal tubular PAH accumulation, palmitate oxidation and palmitate

esterification were determined as described. Results were expressed as the mean difference ± standard error for the difference between functions measured in control and treated tubule suspensions.

CHEMICAL AND ENZYMATIC ASSAYS

1) Citrate Assay

Tissue citrate concentration was determined spectrophotometrically following oxidation and bromination of citrate in an acid medium to form pentabromoacetone (McArdle, 1955). Aliquots of the trichloroacetic acid tissue extracts (containing 10-50 µg citrate) were added to tubes containing 5.0 ml of 33 N H_2SO_4 and thoroughly mixed. After 10 minutes, 5.0 ml of a 2% potassium bromide-0.5% potassium bromate-1.2% sodium vanadate solution was added, the tubes capped and mixed and incubated for 20 minutes at 30°C. Following incubation, 3.0 ml of a 22% ferrous sulphate solution was added, the contents mixed and the tubes left for at least 5 minutes to collect excess bromine generated. The pentabromoacetone formed was extracted with 7.0 ml of petroleum ether and the aqueous layer removed. The petroleum ether extract was washed by shaking with 20.0 ml water and the aqueous layer removed. A small amount of anhydrous Na_2SO_4 was added to remove the last traces of water. An aliquot (5.0 ml) of the petroleum ether extract was transferred to a second tube and mixed with 4.0 ml of a 0.2% sodium sulfide (Ventron Alfa Products, Beverly, Mass.); 4% thiourea, 2% borate solution by gentle bubbling with 100% N_2. A colored, water-soluble complex of pentabromoacetone in thiourea-borate-sulfide was formed which had a maximum absorption at 435 nm. The absorption of unknown samples was compared to that of known standards of anhydrous citric acid treated in a similar fashion. The citrate concentration

of tissue samples was calculated assuming a molecular weight of 192.1 for citric acid. The standard curve was linear over a range of 5-25 μg citrate/ml. The metabolic substrates used in the slice incubation sudies did not interfere with the assay procedure. Recovery of known amounts of citrate added to trichloroacetic acid tissue extracts ranges from 95-101%.

2) α-Ketoglutarate Assay

Tissue α-ketoglutarate (αKG) concentration was determined by measuring the decrease in absorbance at 340 nm produced by the oxidation of NADH coupled to the enzymatic reduction of α-KG by glutamic dehydrogenase (Selleck et al., 1964). Approximately 0.05-0.10 ml of an $NADH_2$ (Calbiochem, A grade) solution (30 mg/ml in 1% $NaHCO_3$) was added to 15 ml of a neutralized perchloric acid tissue extract ([α-KG] = 0.02-0.10 mM). Duplicate aliquots (5.0 ml) were added to test tubes. One aliquot (blank) received 0.05 ml of water while 0.05 ml of glutamic dehydrogenase (Calbiochem, A grade; 2 mg protein/ml in 2 M $(NH_4)_2SO_4$) was added to the second aliquot. The change in absorbance between the tubes was measured after 20 minutes at 340 nm using a Hitachi-Perkin Elmer spectrophotometer. The change in absorbance of unknown samples was compared to that produced by known amounts of α-KG. The standard curve was linear over a range of 0.02-0.10 mM α-KG. Recovery of added α-KG varied from 93-102%.

3) Determination of Nonesterified Fatty Acids (NEFA) Using ^{63}Ni

Nonesterified fatty acids were determined by liquid scintillation spectrometry following formation of a chloroform soluble ^{63}Ni-NEFA complex as described by Ho (1970). Duplicate plasma samples (50-200 μl) were brought to a known volume (200 μl) with 0.9% NaCl, mixed

with 1.0 ml of Dole extraction mixture[6] and placed on ice. After 10 minutes, 200 µl of heptane and 200 µl of distilled water were added, the tubes mixed and placed on ice. After the phases had separated (5-10 minutes) 200 µl of the heptane layer (upper phase) was withdrawn and placed in polypropylene microcentrifuge tubes. Chloroform (800 µl) and 100 µl of a ^{63}Ni-solution (20 µCi/ml; 1 mg/ml $NiCl_2$ in 1 M triethanolamine) were added to each tube, the tubes thoroughly mixed and centrifuged for 10 minutes at 500 x g. The remaining ^{63}Ni-triethanolamine was aspirated and discarded. A 500 µl aliquot of the chloroform layer containing the ^{63}Ni-NEFA complex was added to a scintillation vial and the chloroform evaporated. Omniflour-toluene (10.0 mls) was added to each scintillation vial and the samples counted in a Beckman LS-100 liquid scintillation counter using a channel optimized for ^{63}Ni. The NEFA content of unknowns was determined by comparison to a standard curve following correction for ^{63}Ni-cpm in blank samples. Palmitic acid was used as standard. The standard curve was linear over a range of 10-100 nmole palmitic acid. Recovery of known amounts of palmitic acid was essentially complete.

Total nonesterified fatty acids present in the lower phase of Folch extracts of proximal tubules was determined by a modification of the method of Ho (1970), similar to that described by Bieber et al. (1975). Quadruplicate aliquots of the Folch extract were added to tubes containing approximately 50 mg silicic acid. The chloroform: methanol was evaporated and NEFA extracted as outlined above. This procedure did not alter the recovery of known amounts of palmitic acid.

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[6]Dole extraction mixture - isopropanol:heptane:1 N H_2SO_4 (40:40:1 v/v).

In several experiments an aliquot of the upper heptane layer was subjected to thin layer chromatography on silica Gel G with hexane: diethyl ether:acetic acid (80:30:1 v/v) as solvent. All of the ^{14}C-label recovered from the TLC plates was associated with the NEFA fraction. No contamination by phospholipids was detected following visualization of TLC plates with iodine vapor or rhodamine-6-G.

Following extraction of NEFA, two of the aliquots were assayed with ^{63}Ni as described while the remaining pair of aliquots was assayed with solution containing an equivalent amount of unlabelled $NiCl_2$. The ^{14}C-activity appearing in the ^{63}Ni-channel from these samples was subtracted from the total counts appearing in those samples assayed with ^{63}Ni. This served to correct for the counts added by ^{14}C-NEFA.

MATERIALS

1) Magnetic iron oxide was prepared as described by Cook and Pickering (1958), except that it was stored as a suspension in 0.9% NaCl and diluted before use with Ringers medium. Sodium hydroxide (2.6 gm) and potassium nitrate (20 gm) were dissolved in 100 ml of oxygen saturated water and added to a solution of ferrous sulphate (9 gm) dissolved in 100 ml of oxygen saturated water. The mixture was boiled for approximately 20 min. The precipitate of magnetic iron oxide was washed by resuspending in water and attraction to a permanent magnet. The wash water containing any nonmagnetic substance was decanted. This procedure was repeated 5-10 times. The iron oxide was dried, weighed and suspended in 0.9% NaCl at a concentration of 50 mg/ml.

2) Contaminating NEFA were removed from bovine serum albumin (Fraction V, Sigma Chemical Co.) by the method of Chen (1967). Norit A charcoal (3.0 gm) was added to a solution of 7.0 gm BSA in 70 ml distilled water. The pH of the solution was adjusted to 3.0 by addition of HCl and stirred continuously for 1 hr at 0°C. The solution was centrifuged at 20,200 x g, 20 min at 2°C. The supernatant was brought to pH 7.4 with NaOH assayed for protein content and stored at -20°C.

3) Probenecid was the kind gift of Dr. John Baer, Merck, Sharpe and Dohme. Dr. Michael Cohen, Hoffman LaRoche, Inc. generously provided the sulfisoxazole used in these experiments. Iodipamide was the gracious gift of Dr. S.J. Lucania, E.R. Squibb and Sons, Inc. Procaine penicillin G (Crystacyllin) was purchased from E.R. Squibb and Sons.

STATISTICAL ANALYSES

Where appropriate, data were analyzed using Student's "t" test, paired or group comparison, or analysis of variance, completely random or randomized complete block design. Treatment means were tested using Tukey's-w-procedure (Sokal and Rohlf, 1969). The 0.05 level of probability was used as the criterion of significance.

RESULTS

USE OF TRANSPORT ONTOGENY AND SELECTIVE SUBSTRATE STIMULATION AS MODELS FOR IDENTIFICATION OF MULTIPLE RENAL ORGANIC ANION TRANSPORT SYSTEMS

1) Effect of Age

Three distinct developmental patterns of organic anion accumulation by rabbit renal cortical slices were observed (Figures 1,2,3,4). Active accumulation of UA into slices (defined as S/M ratio greater than 1.0) was not evident until two weeks of age (one week S/M = 1.21±0.06) (Figure 1)[1]. Transport capacity for UA in adult tissue (S/M = 3.38±0.24) was observed by 4 weeks of age (S/M = 3.08±0.30) (Figure 1). In contrast, active accumulation of PAH occurred in slices from one week rabbits (PAH S/M = 3.54±0.01) (Figure 1). PAH accumulation increased rapidly with age reaching a peak at 4 weeks (S/M = 15.27±1.40) and then declining to adult values (S/M = 10.07±1.52) (Figure 1). Accumulation of PSP followed a developmental pattern similar to that of PAH (Figure 1).

Accumulation of CDC produced a third pattern of development (Figure 2). CDC accumulation at two weeks (S/M = 37.58±1.78) was approximately 5-fold greater than that of PAH and continued to increase throughout development, being greatest in slices from adults (CDC S/M = 127.17± 12.79). When renal cortical slices were incubated at 0°C accumulation

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[1]Identity of the radiolabelled carbon present in several tissue digests was confirmed as uric acid by thin layer chromatography (Orsulak et al., 1968).

Figure 1. Relationship between age and accumulation (S/M ratio) of PAH, PSP, and UA by rabbit renal cortical slices. Acetate was absent from the incubation media. Each point represents the mean ± standard error (S.E.) determined in 3 to 9 rabbits from 5 litters. Where no vertical line is shown the S.E. is less than the radius of the point.

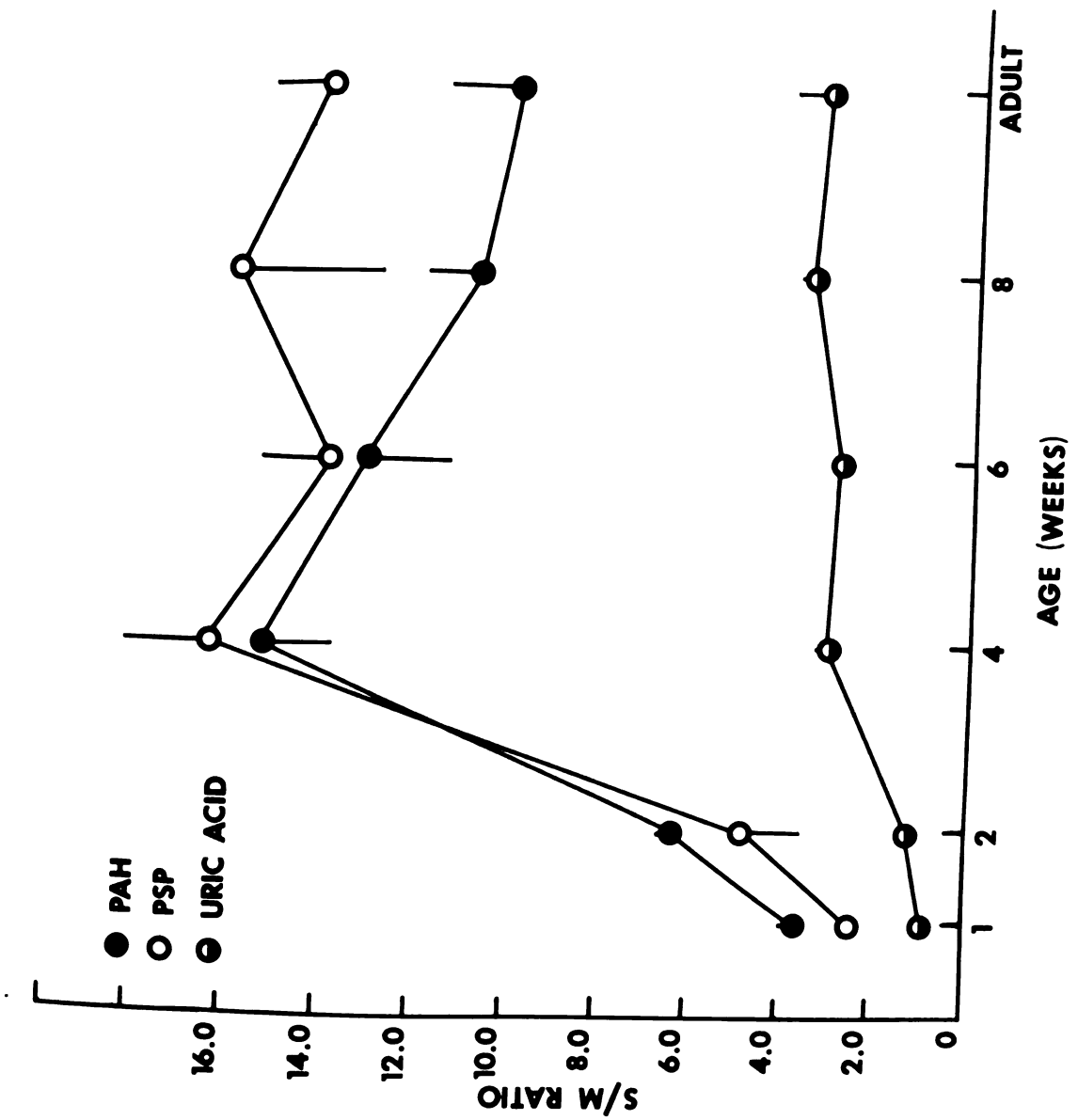
PAH
PSP
URIC ACID
S/M RATIO
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
1
2
4
6
8
ADULT
AGE (WEEKS)

Figure 1

Figure 2. Relationship between age and accumulation (S/M ratio) of PAH, CDC and SULFI by rabbit renal cortical slices. Acetate (10 mM) was present in the incubation media. Each point represents the mean ± S.E. determined in 3-5 rabbits from 4 litters. Where no vertical line is shown the S.E. is less than the radius of the point.

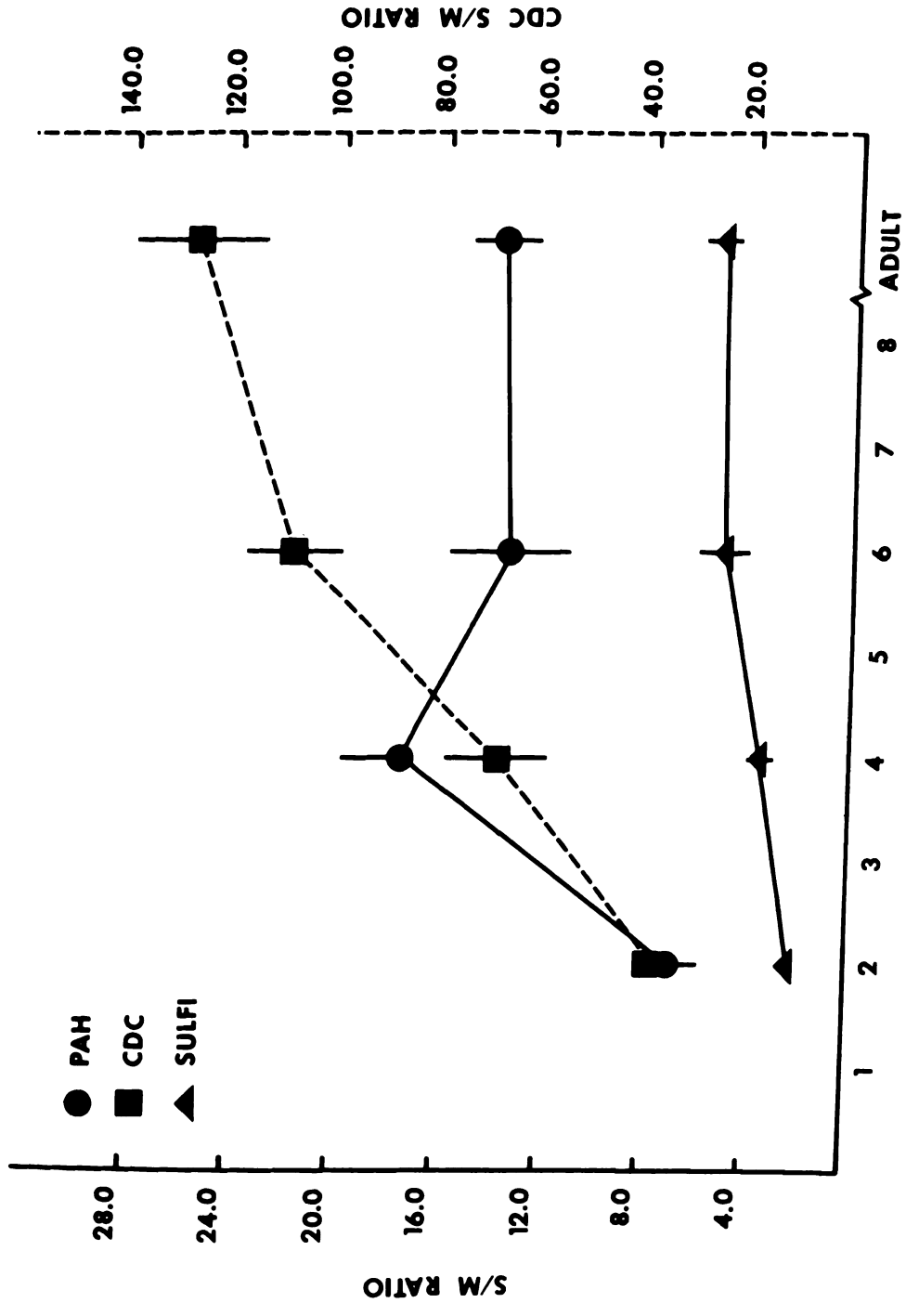
CDC S/M RATIO
140.0
120.0
100.0
80.0
60.0
40.0
20.0
PAH
CDC
SULFI
28.0
24.0
20.0
16.0
12.0
8.0
4.0
S/M RATIO
1
2
3
4
5
6
7
8
ADULT
RABBIT AGE (WEEKS)

Figure 2

of CDC was not related to age (2 wk CDC S/M = 23.15±6.53; 4 wk CDC S/M = 34.32; adult CDC S/M = 31.93±3.74) (Figure 3). Accumulation of SULFI (Figure 2) appeared to follow a pattern similar to that of uric acid (Figure 1) in that the S/M was less than 1.0 at 2 weeks of age and gradually increased with development.

Whereas PEN S/M ratios increased rapidly from two weeks to 4 weeks and then declined during development, accumulation of ASA did not change appreciably with age (Figure 4).

2) Effect of Substrate Stimulation

For convenience in subsequent studies anions were tentatively placed in 3 groups based on similarities in patterns of development of slice accumulation. Group I contained PAH, PSP and PEN; group II contained UA, ASA and SULFI; and CDC was placed alone in group III. The effect of administering various agents to newborn rabbits was evaluated according to this tentative classification.

Administration of PEN to neonatal rabbits selectively enhanced the ability of renal cortical slices to accumulate PAH and similar group I anions (PSP, PEN) (Figure 5 and 6) without markedly altering accumulation of the group II anions, UA (Figure 5) or ASA (Figure 6). Following PEN treatment slice accumulation of PAH was increased 49% and PSP was enhanced 35%. Similarly, probenecid significantly increased PAH and PSP uptake without altering the accumulation of UA (Figure 5).

Increasing the dose of PEN to 180,000 units/day increased stimulation of slice accumulation of PAH to 90% above control (Figure 6) while increasing PEN accumulation by 54%. This dose of PEN had no effect on accumulation of ASA (Figure 6).

Figure 3. Effect of reduced incubation temperature (0°C) on CDC acumulation by renal cortical slices prepared from rabbits of various ages. The curve representing CDC accumulation at 25°C was taken from Figure 2. Each point represents the mean ± S.E. determined in 2-5 rabbits from 2-4 litters.

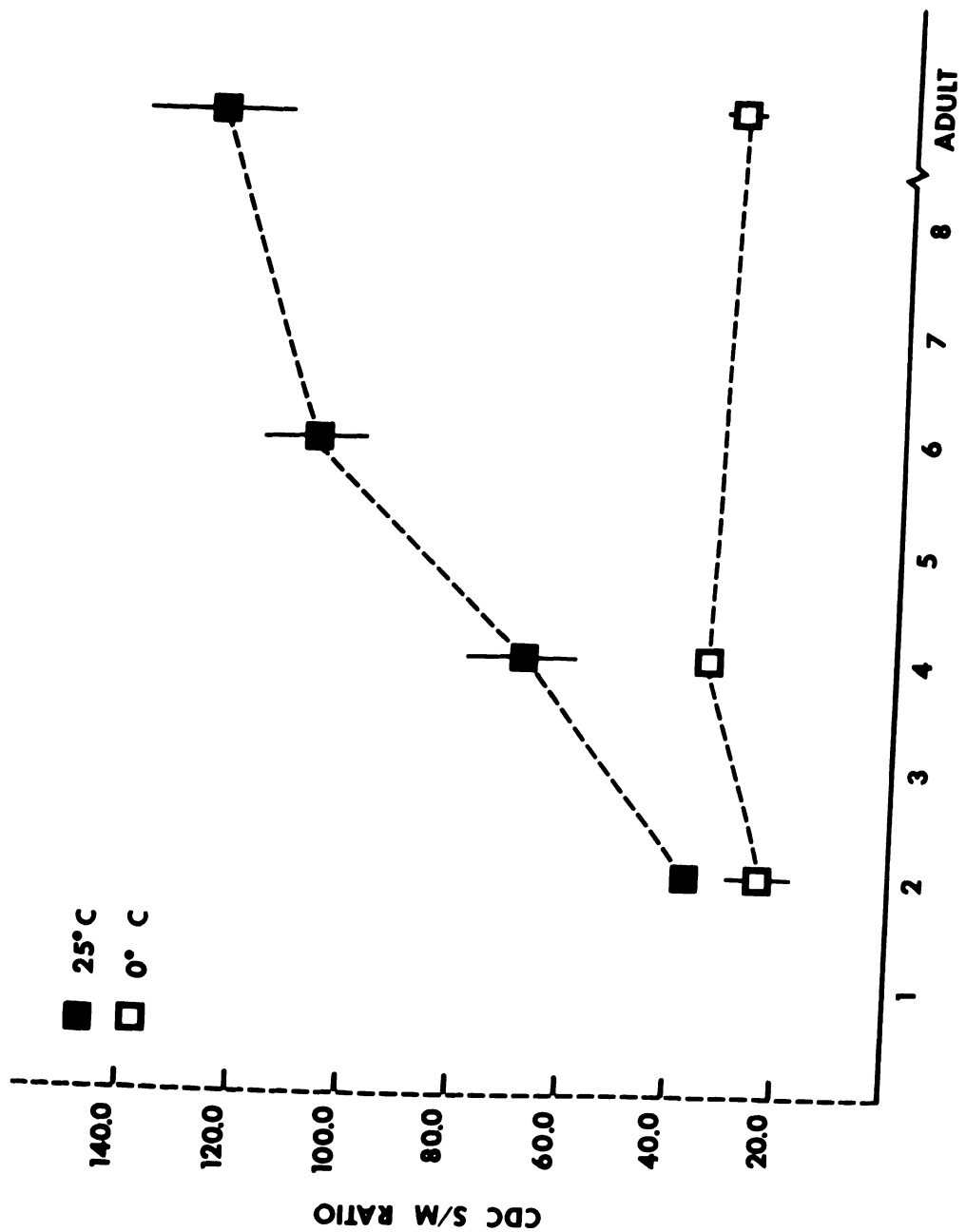
25°C
0° C
140.0
120.0
100.0
80.0
60.0
40.0
20.0
CDC S/M RATIO
1
2
3
4
5
6
7
8
ADULT
RABBIT AGE (WEEKS)

Figure 3

Figure 4. Relationship between age and accumulation (S/M ratio) of PAH, PEN, and ASA. Acetate (10 mM) was present in the incubation media. Each point represents the mean ± S.E. determined in 3-4 rabbits from 4 litters. Where no vertical line is shown the S.E. is less than the radius of the point.

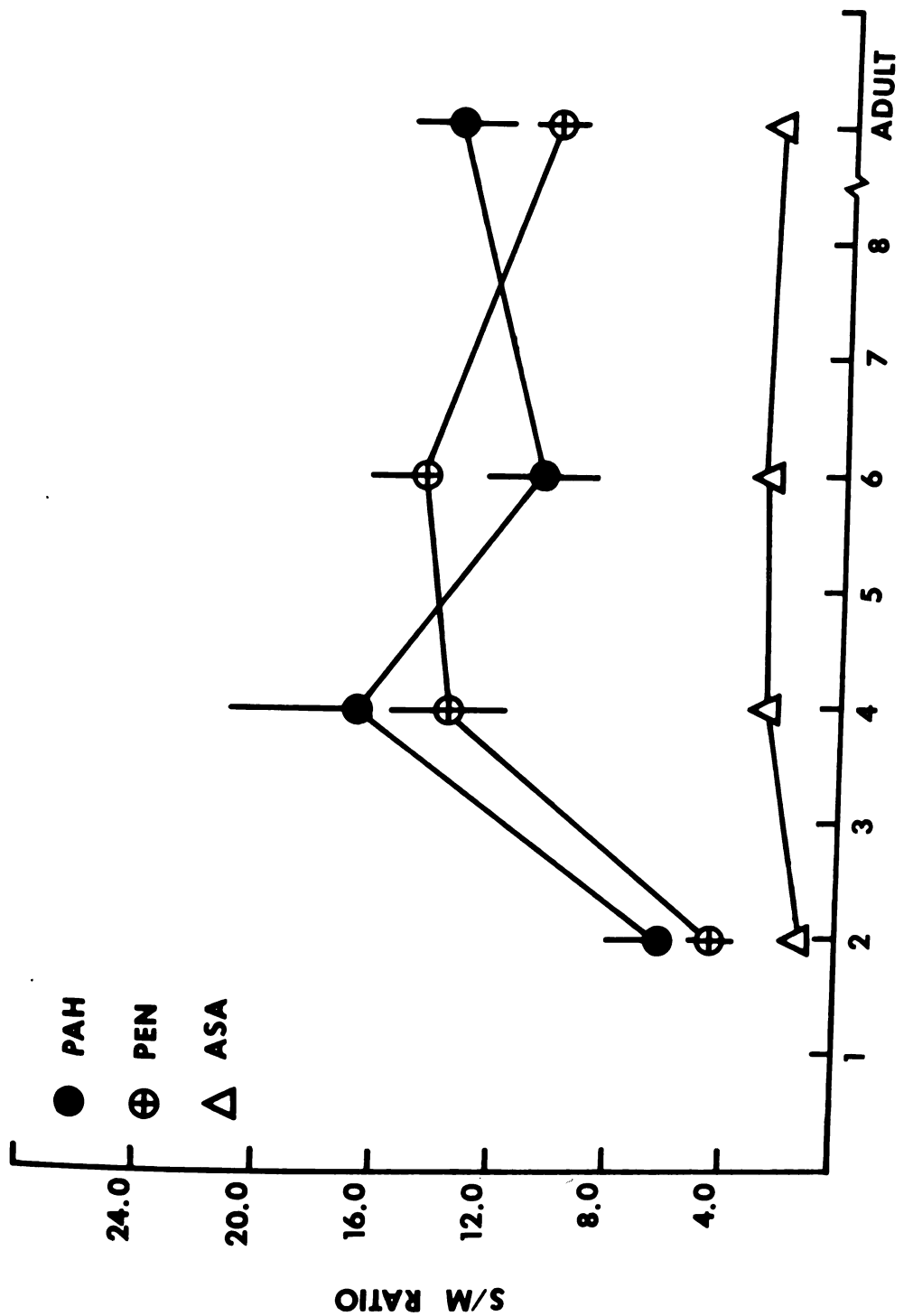
PAH
PEN
ASA
24.0
20.0
16.0
12.0
8.0
4.0
S/M RATIO
1
2
3
4
5
6
7
8
ADULT
RABBIT AGE (WEEKS)

Figure 4

Figure 5. Effect of penicillin and probenecid administration on accumulation of PAH, PSP and UA by rabbit renal cortical slices. Acetate was not included in the incubation media. Procaine penicillin G (30,000 I.U.) and probenecid (100 mg/kg) were administered twice daily for three days. Control rabbits received isovolumic amounts of saline. Rabbits were 10 days old at the beginning of treatment and were killed 24 hrs after the last injection. Each bar represents the mean ± S.E. obtained from 4 litters.

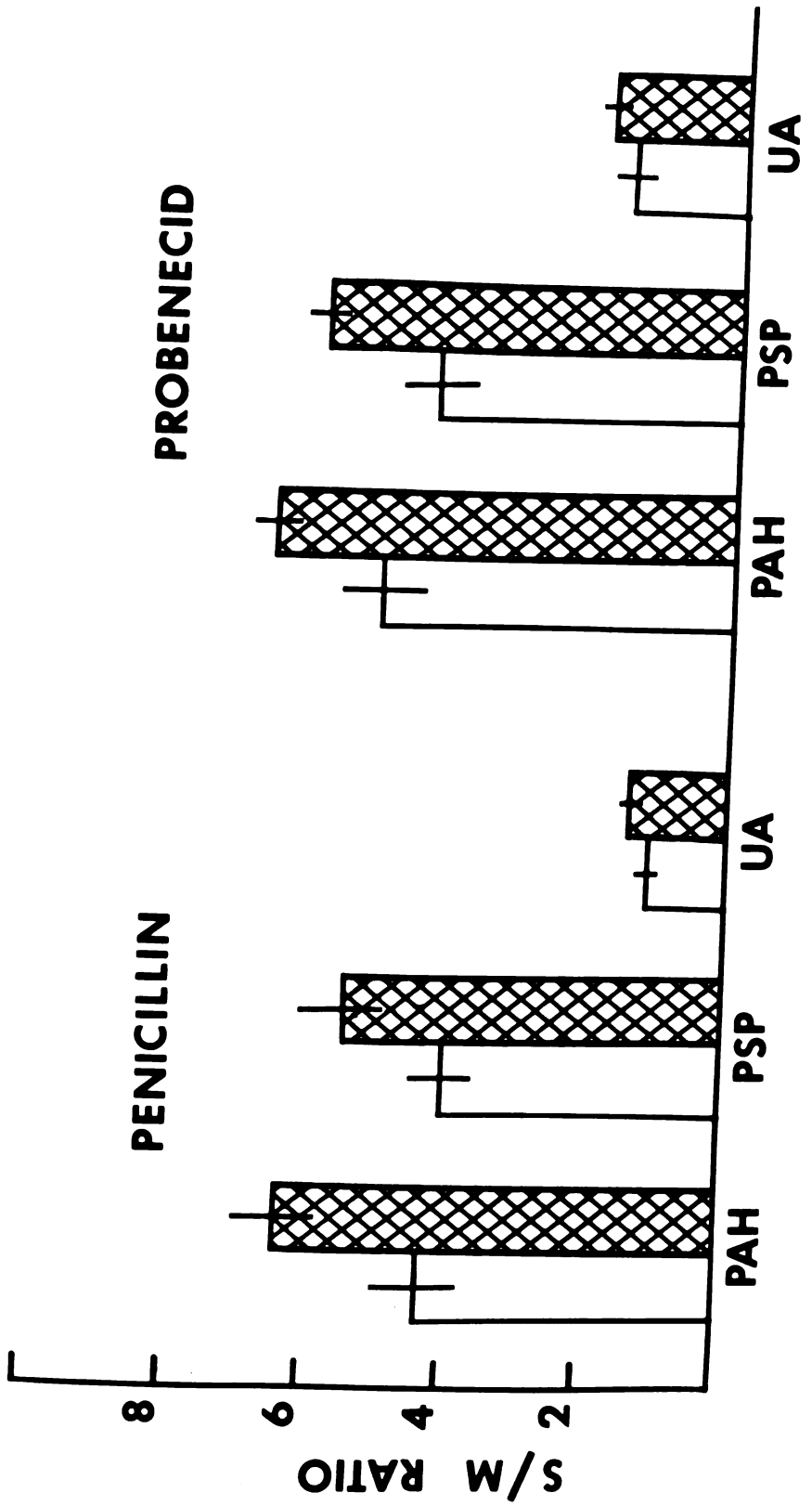

Figure 5

Figure 6. Effect of penicillin administration on slice accumulation of PAH, PEN, and ASA. Acetate (10 mM) was included in the incubation media. Procaine penicillin G (90,000 I.U.) was administered twice daily for 2 days. Control rabbits received isovolumic amounts of saline. Rabbits were killed at day 14 of age 24 hours after the last injection. Each bar represents the mean ± S.E. obtained from 4 litters.

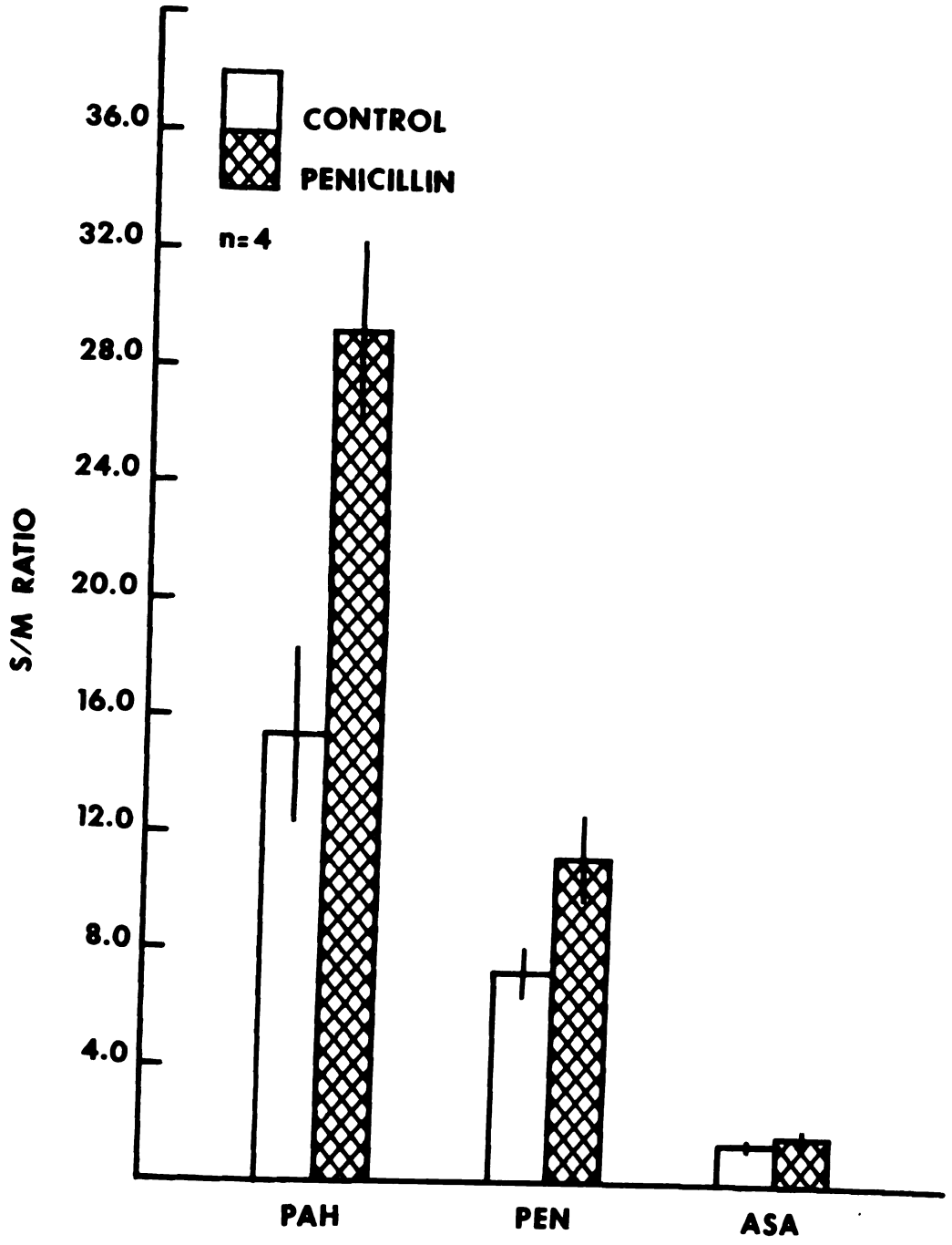
CONTROL
PENICILLIN
n=4
S/M RATIO
36.0
32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
PAH
PEN
ASA

Figure 6

Whereas pretreatment of neonatal rabbits with PEN did not alter slice uptake of the group II anions, UA and ASA, this treatment did significantly enhance SULFI accumulation by 47% (Figure 7).

Accumulation of CDC (group III) was not affected by prior treatment of neonatal rabbits with penicillin (Figure 8). In these tissues the stimulation of PAH accumulation induced by penicillin was shown to occur only when slices were incubated at 25° (not at 0°).

In contrast to penicillin pretreatment, administration of SULFI (Figure 9) and UA (Figure 10) to neonatal rabbits did not stimulate group I anion accumulation. SULFI in doses from 50-200 mg/kg did not alter PAH accumulation nor kidney weight to body weight ratios (Figure 9). UA at a dose of 100 mg/kg affected neither PAH, nor PSP accumulation. In addition, pretreatment with UA had no effect on accumulation of urate-2-^{14}C (Figure 10).

Although SULFI and UA had no effect on PAH accumulation, pretreatment with ASA produced a dose related increase in slice accumulation of PAH (Figure 11). Stimulation increased from 74% at 25 mg/kg to 219% at 150 mg/kg (Figure 11). Higher doses of ASA produced less than maximal stimulation. No significant effect of ASA on kidney weight/body weight ratios were observed (Figure 11). However, significant mortality was observed with doses of ASA greater than 50 mg/kg (100 mg/kg - 38%; 150 mg/kg - 50%; 200 mg/kg - 56%).

Iodipamide (group III) had no significant effect on slice accumulation of either PAH (group I) or chenodeoxycholate (group III) (Figure 12). No mortality was associated with the use of iodipamide.

Figure 7. Effect of penicillin administration on slice accumulation of PAH and SULFI. Experiments were performed as before (Figure 6). Each bar represents the mean ± S.E. obtained from 3 litters.

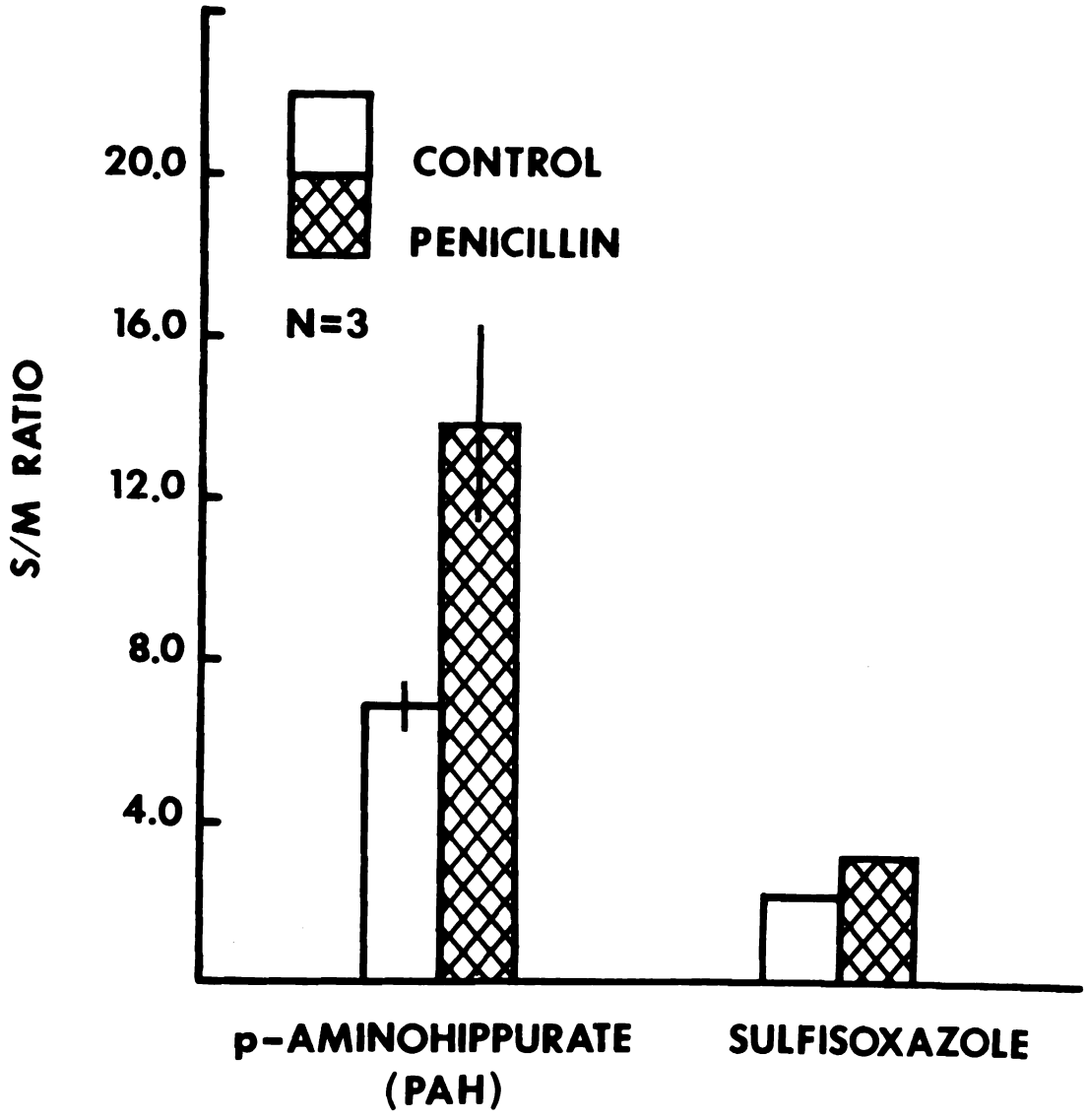
S/M RATIO
20.0
16.0
12.0
8.0
4.0
CONTROL
PENICILLIN
N=3
p-AMINOHIPPURATE
(PAH)
SULFISOXAZOLE

Figure 7

Figure 8. Effect of penicillin administration and incubation temperature on accumulation of PAH and CDC by rabbit renal cortical slices. Experiments performed as before (Figure 6) with the exception that one-half of each tissue pool (control, penicillin treated) was incubated at 0°C rather than 25°C. Bars represent mean ± S.E. obtained from 4 litters.

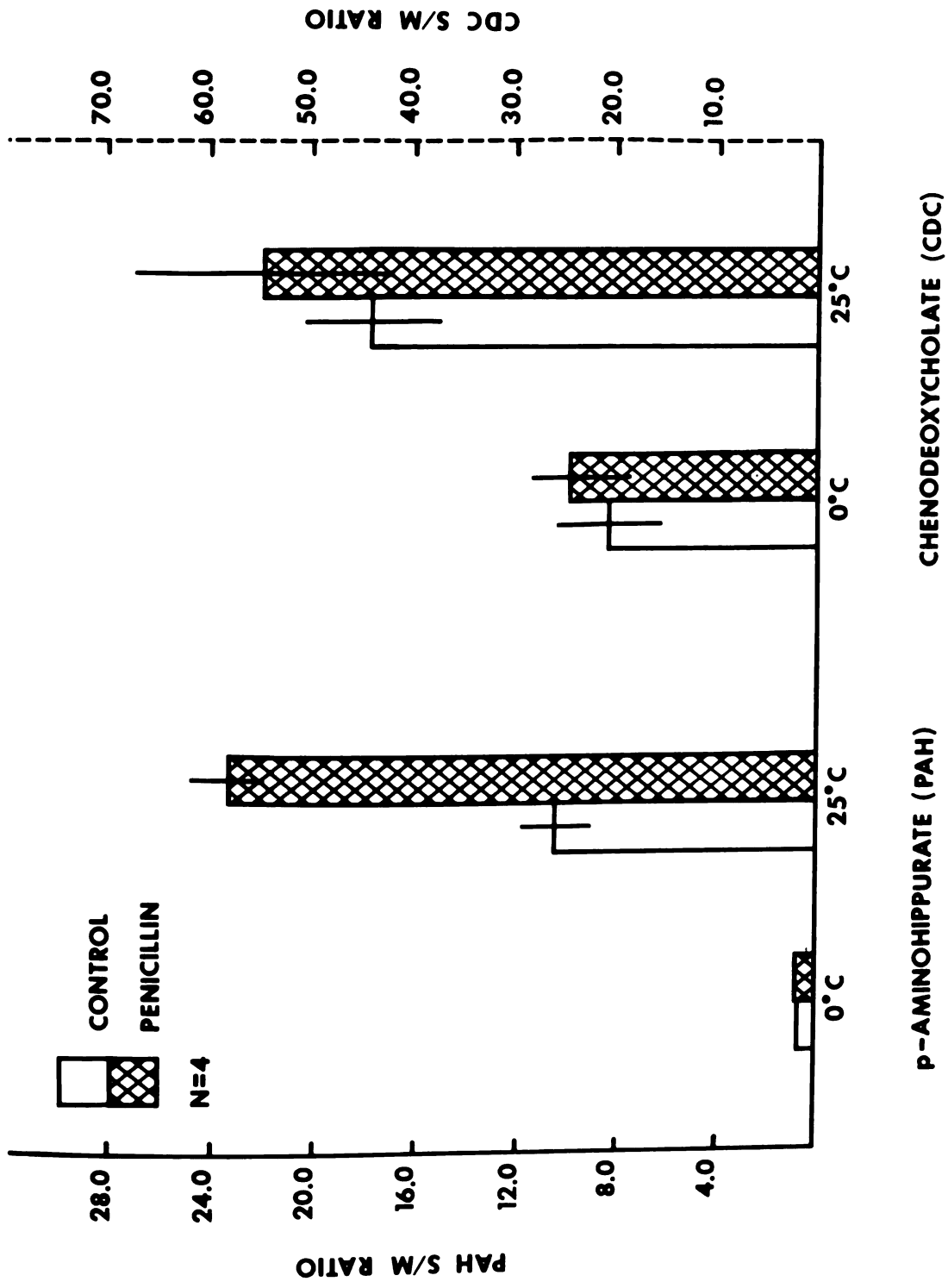
CDC S/M RATIO
70.0
60.0
50.0
40.0
30.0
20.0
10.0
25°C
0°C
CHENODEOXYCHOLATE (CDC)
p-AMINOHIPPURATE (PAH)
25°C
0°C
CONTROL
PENICILLIN
N=4
28.0
24.0
20.0
16.0
12.0
8.0
4.0
PAH S/M RATIO

Figure 8

Figure 9. Effect of sulfisoxazole administration on PAH accumulation and kidney weight/ body weight ratio. Sulfisoxazole was administered in the indicated doses twice daily for 3 days. Controls received isovolumic amounts of vehicle. Rabbits were killed 24 hours after the last injection on day 14 of age. Acetate (10 mM) was present in the incubation medium. Bars represent mean ± S.E. obtained from the indicated number of litters (n).

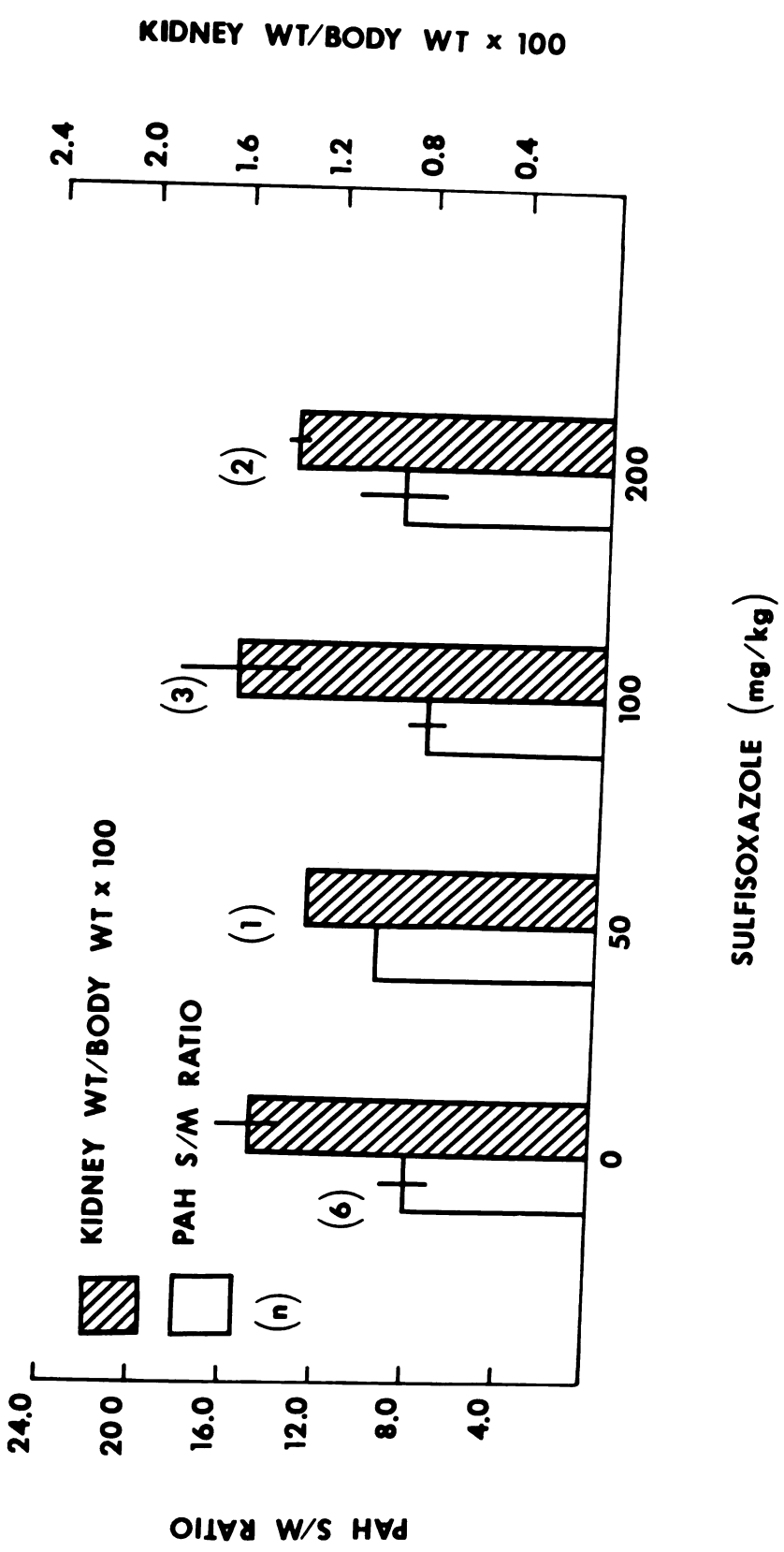
KIDNEY WT/BODY WT x 100
2.4
2.0
1.6
1.2
0.8
0.4
(2)
(3)
(1)
(6)
200
100
50
0
KIDNEY WT/BODY WT x 100
PAH S/M RATIO
(n)
24.0
20.0
16.0
12.0
8.0
4.0
PAH S/M RATIO
SULFISOXAZOLE (mg/kg)

Figure 9

Figure 10. Effect of uric acid administration on slice accumulation of PAH, PSP and UA. Urate (100 mg/kg) was administered twice daily for 3 days. Controls received isovolumic amounts of vehicle. Rabbits were killed 24 hours after the last injection on day 14 of age. Acetate was not included in the incubation media. Bars represent mean ± S.E. determined in 5 litters.

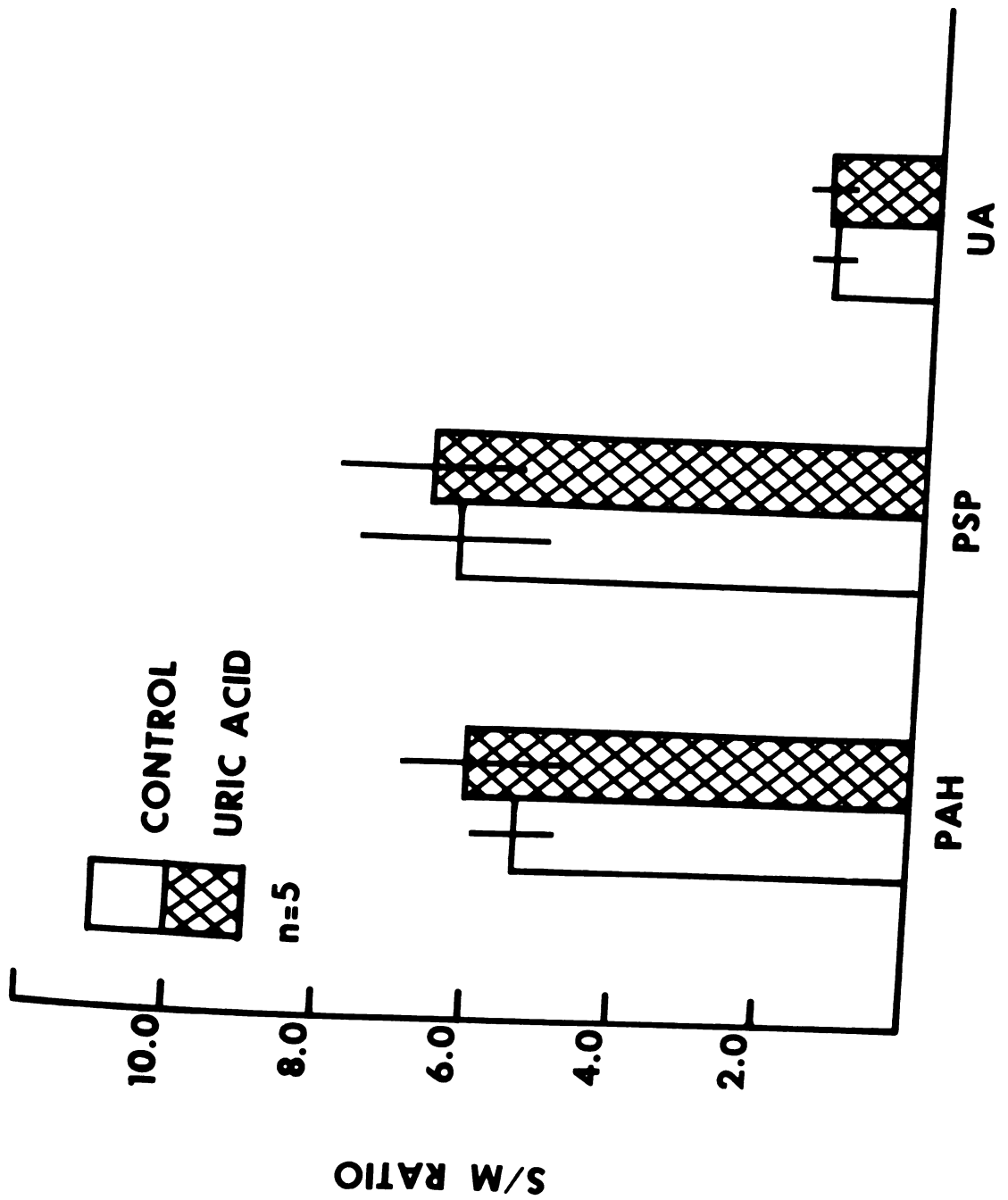

Figure 10

Figure 11. Effect of acetylsalicylic acid administration on slice PAH accumulation and kidney weight/body weight ratio. Experiments were performed as before (Figure 8). Bars represent the mean ± S.E. obtained from the indicated number of litters (n).

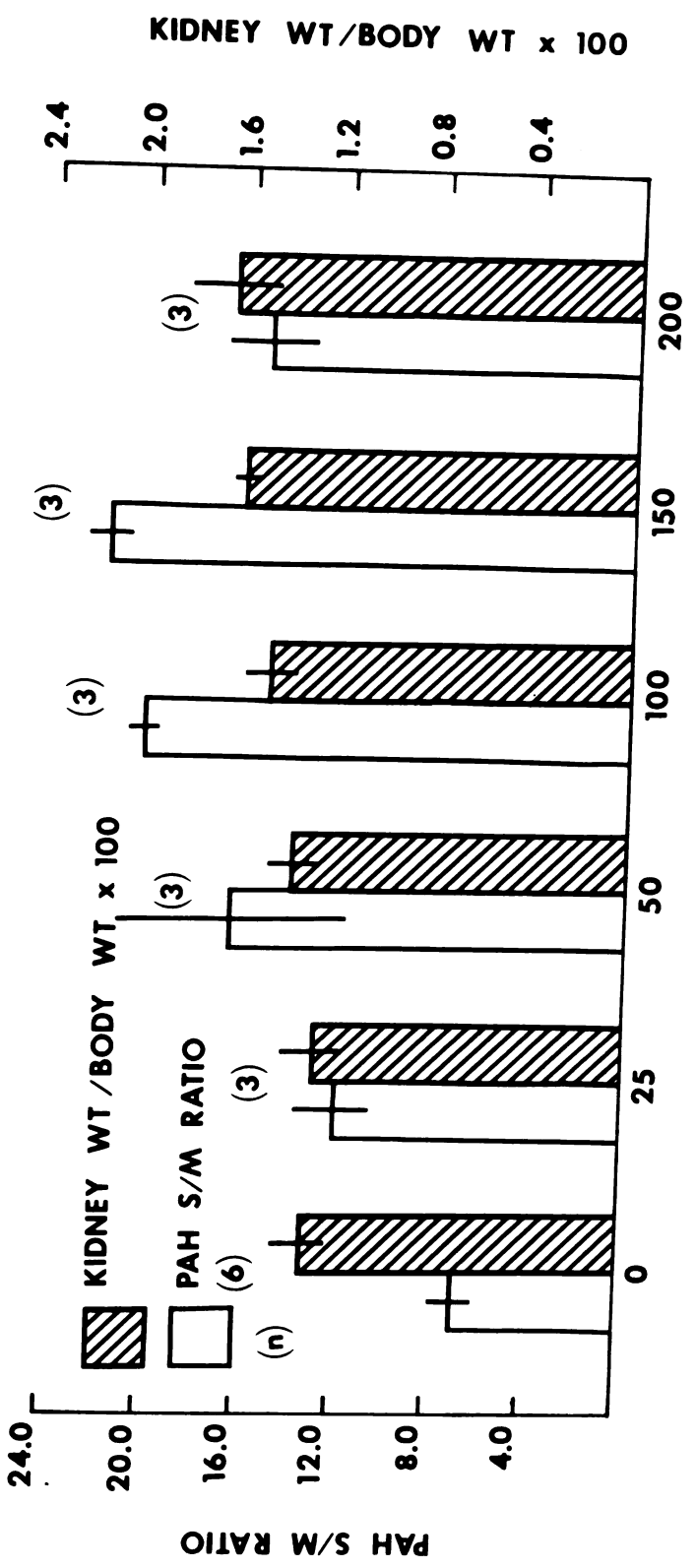
KIDNEY WT/BODY WT x 100
2.4
2.0
1.6
1.2
0.8
0.4
(3)
200
(3)
150
(3)
100
(3)
50
(3)
25
(6)
0
(n)
KIDNEY WT /BODY WT x 100
PAH S/M RATIO
24.0
20.0
16.0
12.0
8.0
4.0
PAH S/M RATIO
ACETYLSALICYLIC ACID (mg/kg)

Figure 11

Figure 12. Effect of iodipamide administration on accumulation of PAH and CDC by rabbit renal cortical slices. Experiments were performed as before (Figure 8). Bars represent the mean ± S.E. obtained from the indicated number of litters (n).

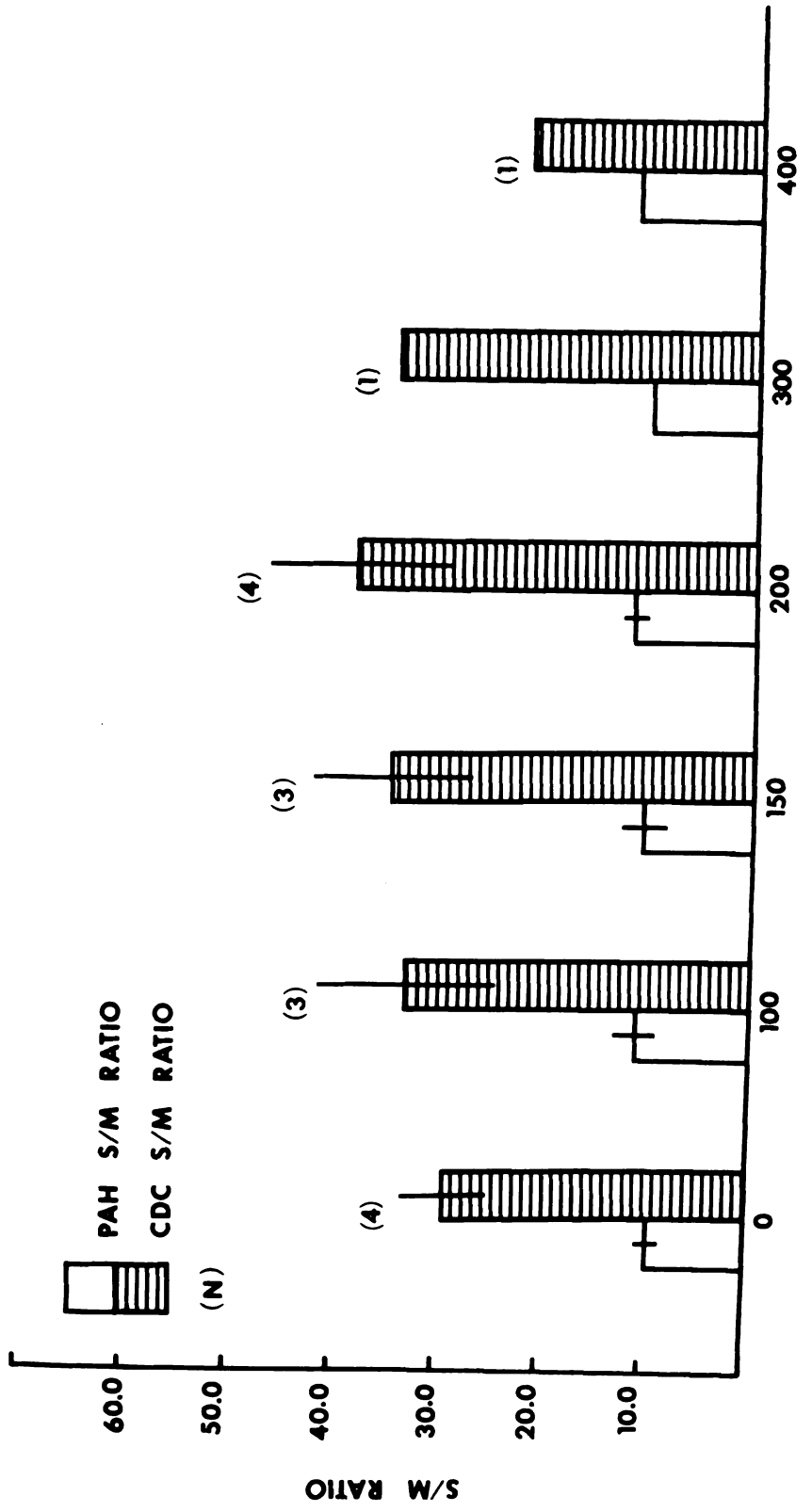
S/M RATIO
60.0
50.0
40.0
30.0
20.0
10.0
PAH S/M RATIO
CDC S/M RATIO
(N)
(4)
(3)
(3)
(4)
(1)
(1)
0
100
150
200
300
400
[IODIPAMIDE] (mg/kg)

Figure 12

Slice accumulation of PAH was not enhanced after low doses of the nontransported organic anion para-aminobenzoic acid (Figure 13). However, after 200 mg/kg of PABA, PAH accumulation was significantly increased. At no dose did PABA change kidney weight/body weight ratio (Figure 13).

INVESTIGATIONS ON THE METABOLIC MODULATION OF p-AMINOHIPPURATE ACCUMULATION BY RABBIT RENAL CORTICAL SLICES

1) Effect of Acetate Washout

The immediate presence of acetate was required for stimulation of PAH accumulation. No stimulation of PAH accumulation was observed when the slices were pre-incubated in a medium containing acetate, washed and finally incubated with PAH (PAH S/M = 3.23±0.36) (Table 1). However, incubation of slices in 10 mM acetate during the final 30 minute incubation period significantly increased the PAH S/M ratio from 3.54±0.48 to 6.22± 0.60.

2) Onset of Acetate Stimulation

In control experiments, tissue concentration of PAH increased with duration of incubation. However, the rate of rise in tissue PAH concentration decreased after approximately 30 minutes of incubation (Figure 14). Addition of acetate after 60 minutes immediately increased the rate of PAH accumulation, producing concentrations 40-50% higher than control within 10 minutes (Figure 14).

3) Effect of 100% N_2 on PAH Accumulation

To determine the effect of acetate on the oxygen-requiring component of PAH uptake the optimal duration of incubation was first established by incubating slices under 100% O_2 or 100% N_2 (Figure 15).

Figure 13. Effect of p-aminobenzoic acid administration on slice PAH accumulation and kidney weight/body weight ratios. Experiments were performed as before (Figure 8). Bars represent the mean ± S.E. determined in 3 litters.

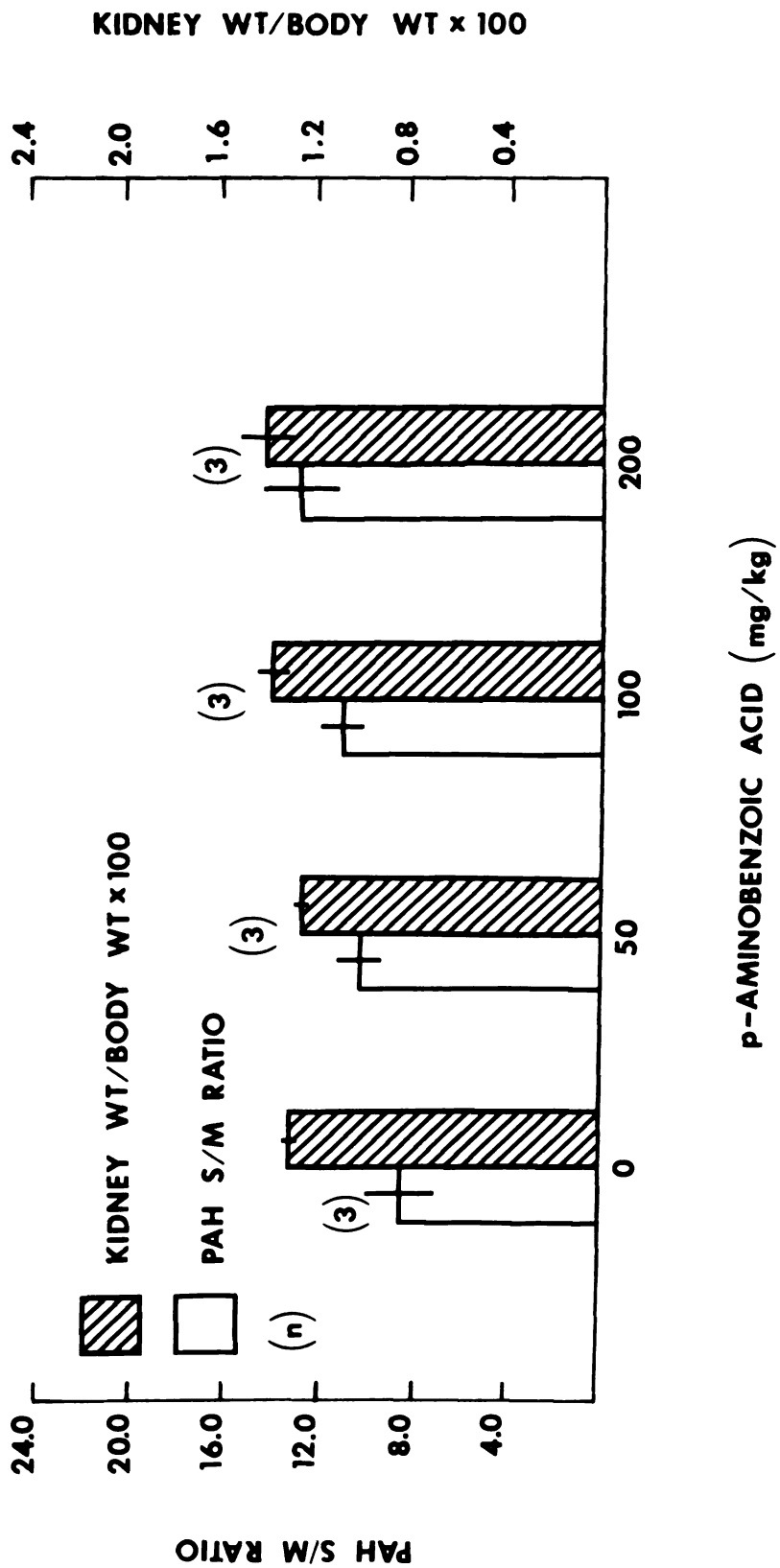
KIDNEY WT/BODY WT x 100
2.4
2.0
1.6
1.2
0.8
0.4
(3)
(3)
(3)
(3)
0
50
100
200
p-AMINOBENZOIC ACID (mg/kg)
KIDNEY WT/BODY WT x 100
PAH S/M RATIO
(n)
24.0
20.0
16.0
12.0
8.0
4.0
PAH S/M RATIO

Figure 13

TABLE 1

Effect of Acetate Washout on PAH S/M Ratio*

Conrrol	Acetate Incubated	Acetate Washed
3.54±0.48	6.22±0.60+	3.23±0.36

*Each number is mean ± S.E. from 3 experiments. +Significantly different than control ($p<0.01$). Control: 60 min incubation without Na Acetate, without PAH; 30 min incubation with 7.4×10^{-5}M PAH. Acetate Incubated: 60 min incubation without Na Acetate, without PAH; 30 min incubation with 10.0 mM Na Acetate, with 7.4×10^{-5}M PAH. Acetate Washed: 30 min incubation with 10.0 mM Na Acetate, 3x10 min incubation without Na Acetate, without PAH; 30 min incubation with 7.4×10^{-5}M PAH.

Figure 14. Onset of effect of acetate on steady-state PAH concentration (μg PAH/g tissue). Na Acetate (10.0 mM) added to rabbit kidney slices previously incubated with 7.4×10^{-5}M PAH (60 min). PAH accumulation measured from 0.5-3.0 min after addition. Control slices incubated in 7.4×10^{-5}M PAH for 15-90 min intervals. Points represent means ± S.E. of 4 experiments. Each experiment was the average of duplicate determinations.

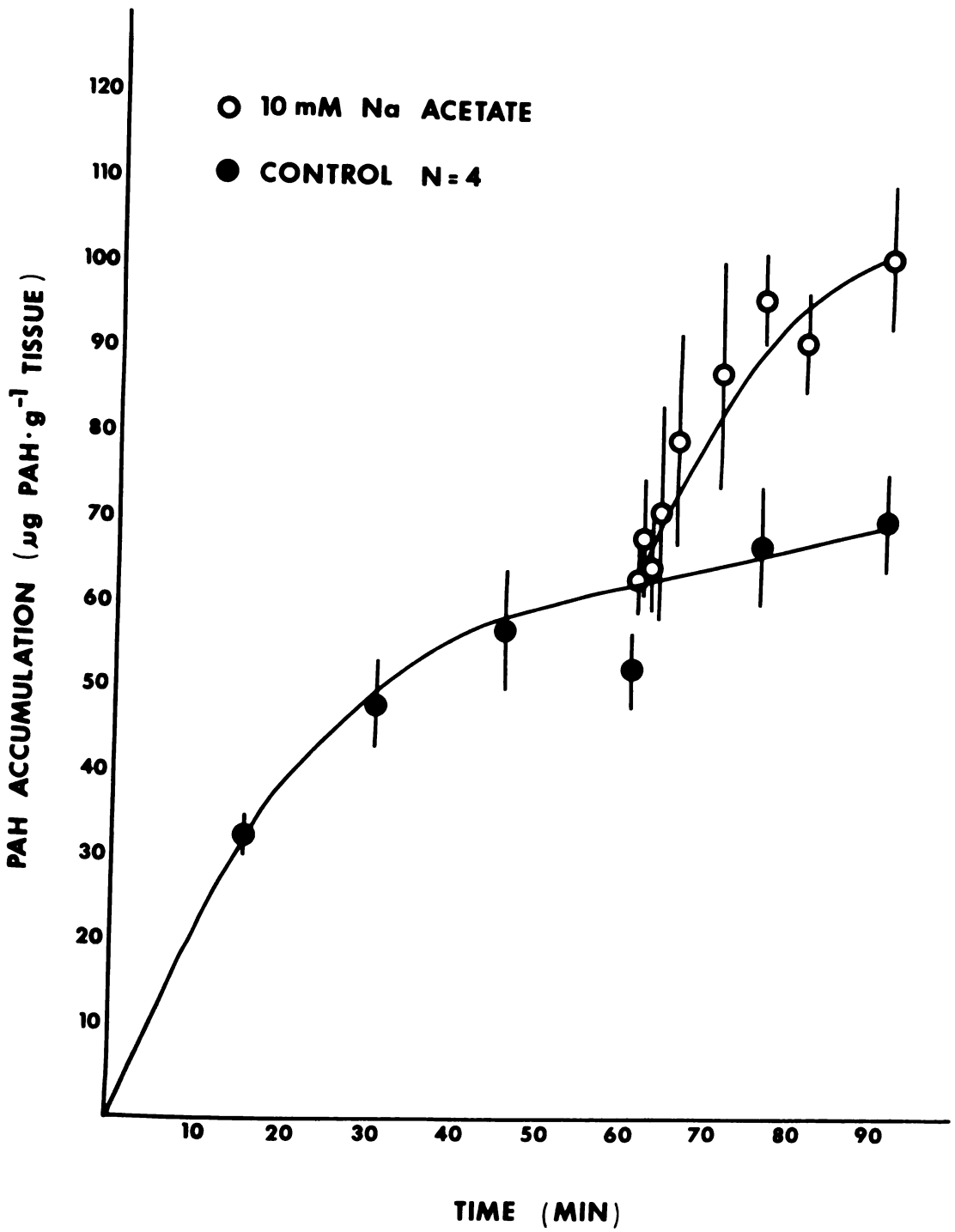
10 mM Na ACETATE
CONTROL N = 4
PAH ACCUMULATION (µg PAH·g⁻¹ TISSUE)
120
110
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
TIME (MIN)

Figure 14

Figure 15. Accumulation (S/M ratio) of PAH by rabbit renal cortical slices under oxygen or nitrogen. Slices were preincubated 30 min under O_2 or N_2. Time of incubation measured from addition of PAH (7.4×10^{-5}M). Points represent means ± S.E. of three experiments.

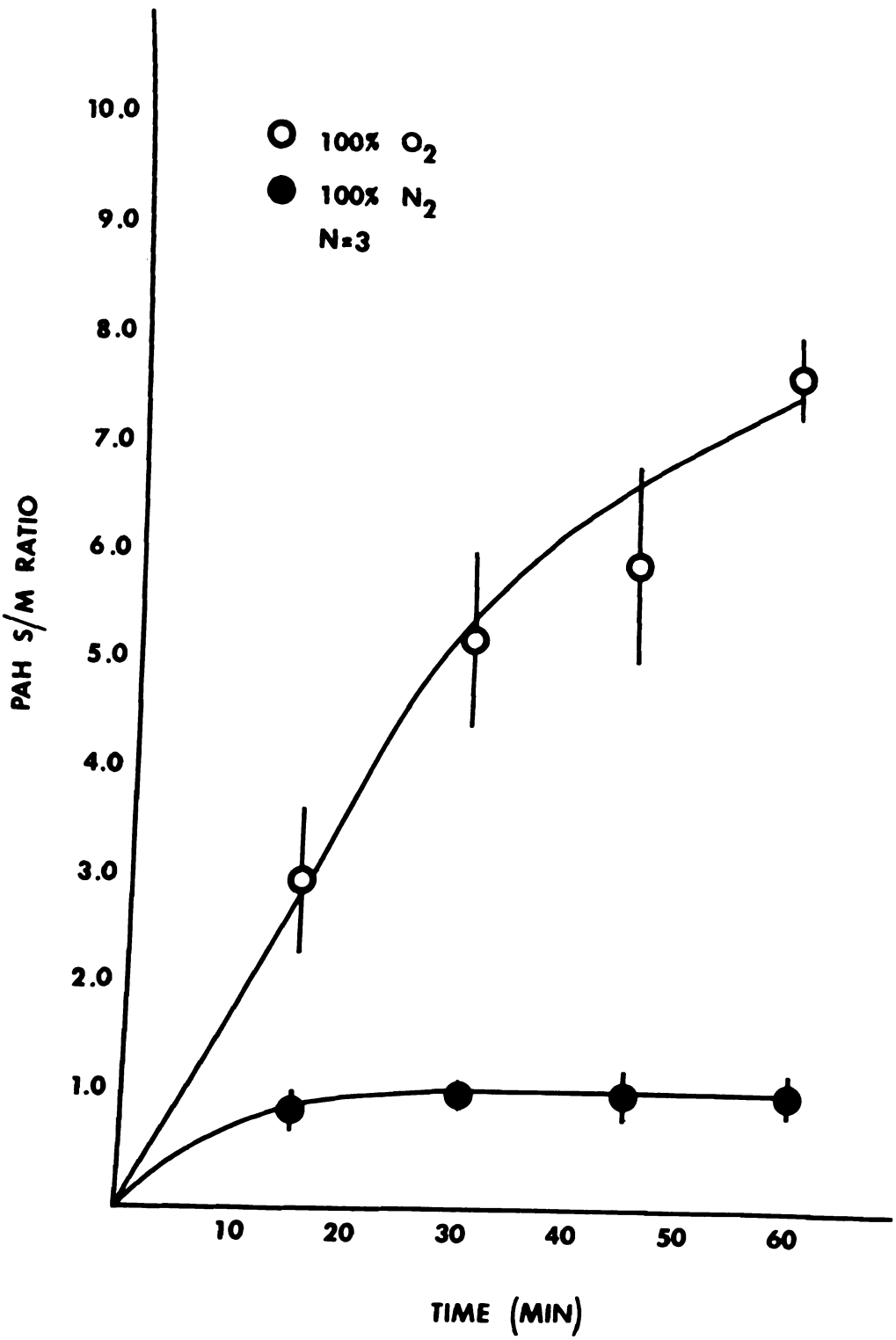
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PAH S/M RATIO
100% O_2
100% N_2
N=3
10
20
30
40
50
60
TIME (MIN)

Figure 15

PAH accumulation under 100% O_2 increased rapidly through approximately the first 30 minutes of incubation. Incubation of slices under 100% N_2 produced a PAH S/M ratio of 1.0 after 15 minutes of incubation. The S/M ratios observed under N_2 were taken to represent the passive diffusion component of PAH accumulation. The oxygen-requiring component of uptake was then calculated by subtracting the passive diffusion component obtained at 15 minutes from the total uptake observed at that time. The rate of uptake of the oxygen-requiring component of PAH accumulation was defined as micrograms of PAH accumulated under 100% O_2 per gram wet weight of tissue per minute of incubation minus that taken up under 100% N_2[2].

4) Effect of Acetate on the Rate of PAH Uptake

Rate of PAH uptake in control slices apparently saturated at a medium concentration of approximately 32×10^{-4}M PAH (Figure 16). The rate of PAH uptake in slices incubated with acetate approached saturation at approximately 60×10^{-4}M PAH. Although the rate of PAH uptake in the presence of acetate followed a saturation pattern similar to that of control, the rate of the oxygen-requiring component of PAH uptake was consistently increased (Figure 16). Acetate did not alter the uptake of PAH under nitrogen.

A kinetic analysis of the data was performed using an Eadie-Hofstee plot of v vs. v/[S] (Figure 17)[3]. Kinetic analysis suggested

---

[2] $v = [\mu g \text{ PAH} \cdot g^{-1} \text{ tissue} \cdot \text{min}^{-1}]_{O_2} - [\mu g \text{ PAH} \cdot g^{-1} \text{ tissue} \cdot \text{min}^{-1}]_{N_2}$

[3] A Hofstee plot was used to determine the kinetic parameters because it provides a superior, non-biased analysis particularly when evaluating experimental data for curvature at extreme media PAH concentrations (Gerencser *et al.*, 1973; Walter, 1974).

Figure 16. Effect of 10.0 mM NaAcetate on the rate of the oxygen-requiring component of PAH uptake in rabbit kidney slices (15 min incubation). Oxygen-requiring component represented as the differences between rate of uptake under oxygen and nitrogen. Blocked area of plot is shown in the insert. Points represent means ± S.E. of five experiments. The absence of a vertical bar indicates S.E. is within the radius of the point.

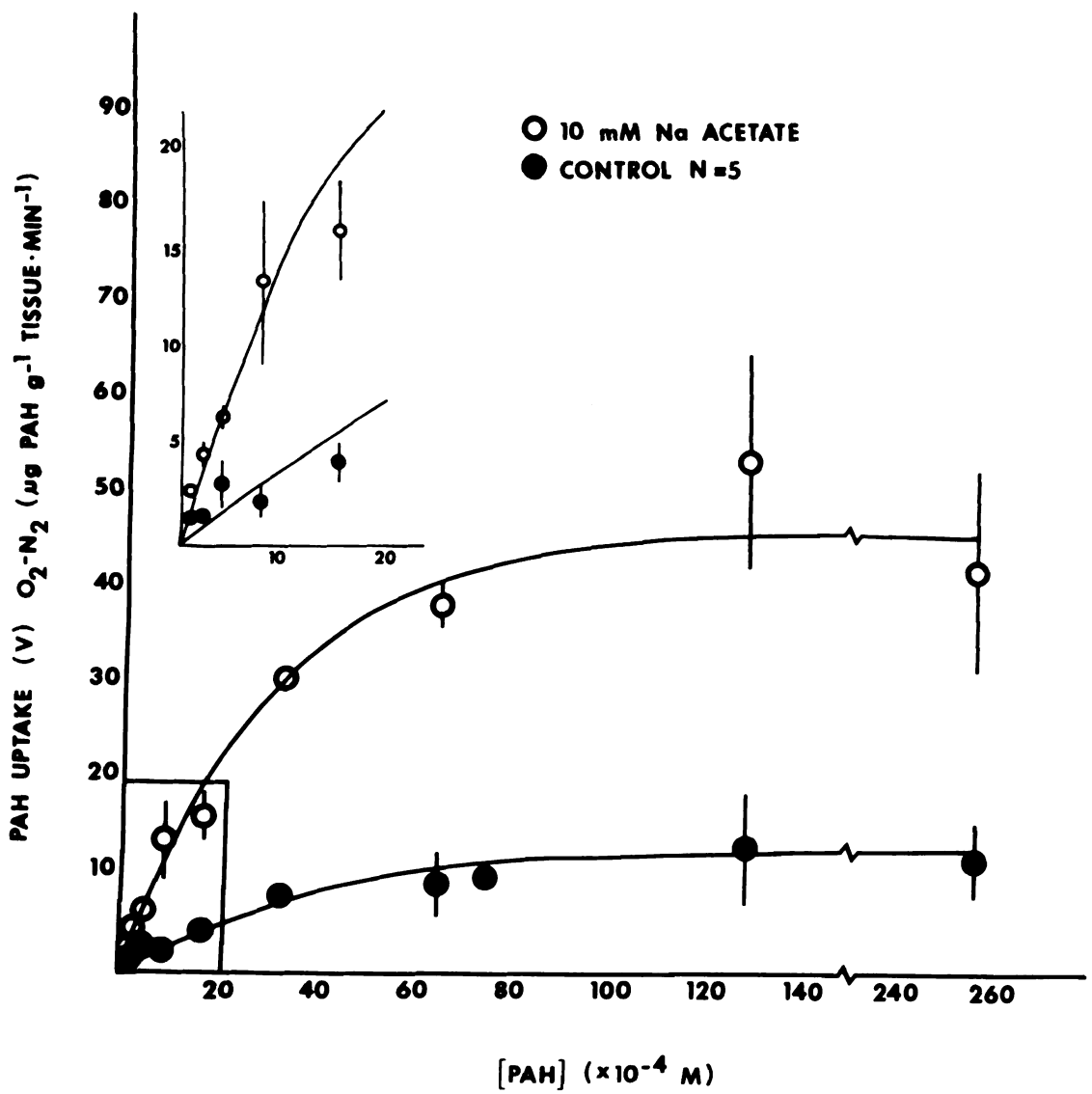
10 mM Na ACETATE
CONTROL N=5
PAH UPTAKE (V) O_2-N_2 (µg PAH g^{-1} TISSUE·MIN^{-1})
[PAH] (×10^{-4} M)
90
80
70
60
50
40
30
20
10
20
40
60
80
100
120
140
240
260
20
15
10
5
10
20

Figure 16

Figure 17. Eadie-Hofstee plot representing the effect of 10.0 mM Na Acetate on the O_2-dependent PAH uptake by rabbit kidney cortical slices. Slices from the kidneys of two rabbits were pooled, pre-incubated 30 minutes under O_2 or N_2 and incubated 15 minutes in nine concentrations of PAH. Rate of PAH uptake (v) is the difference between PAH uptake/min under 100% O_2 and 100% N_2. Points represent the mean of five experiments. Lines were drawn by inspection.

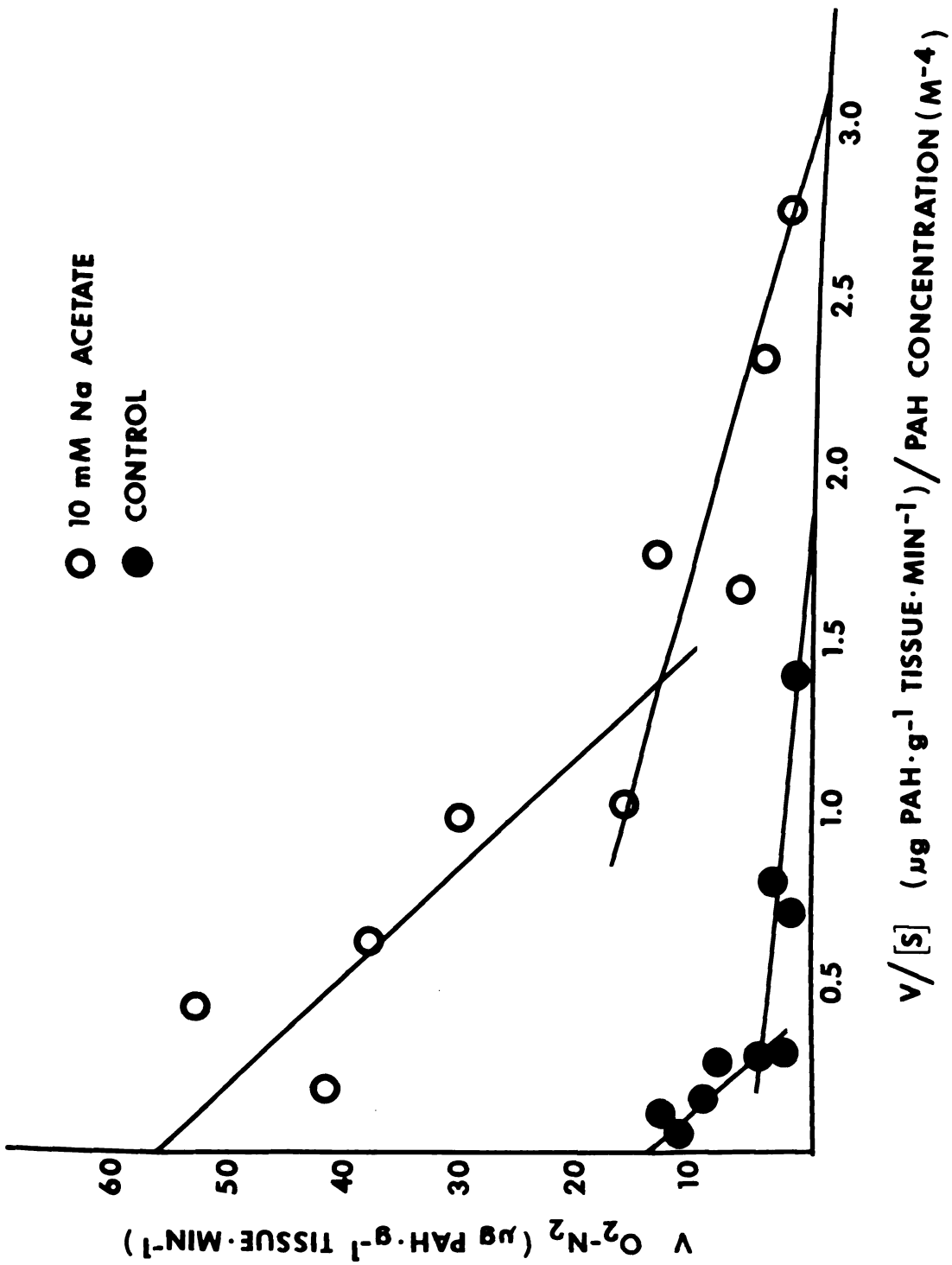
10 mM Na ACETATE
CONTROL
V O2-N2 (µg PAH·g-1 TISSUE·MIN-1)
V/[S] (µg PAH·g-1 TISSUE·MIN-1)/PAH CONCENTRATION (M-4)
10
20
30
40
50
60
0.5
1.0
1.5
2.0
2.5
3.0

Figure 17

that more than one mode of active uptake was used for PAH accumulation. One uptake mode had an apparent K_m of approximately 1.7×10^{-4}M but only a limited capacity for PAH (apparent V_{max} = 3.7 µg PAH · g^{-1} tissue · min^{-1}). The second mode of uptake had an apparent K_m of 22×10^{-4}M but possessed a relatively large capacity for PAH (apparent V_{max} = 13.6 µg PAH · g^{-1} tissue · min^{-1}). Addition of 10 mM Na acetate to the medium markedly increased the capacity of both the high (V_{max} = 51.6 µg PAH · g^{-1} tissue · min^{-1}) and low (V_{max} = 23.8 µg PAH g^{-1} tissue · min^{-1}) capacity uptake modes (Figure 17). Acetate did not appear to change the apparent K_m of either mode for PAH.

5) Effect of Acetate on Medium pH

Slices incubated in a control medium containing no acetate acidified the medium from an initial pH of 7.40 to a final pH of approximately 7.25 after 15 minutes of incubation (Figure 18). The pH of the control medium did not change significantly with further incubation. PAH S/M ratio in control slices reached 7.36±1.42 by 90 minutes. In the presence of 10 mM Na Acetate medium pH initially fell to pH 7.33 and then increased with time, reaching a final pH of approximately 7.54 after 90 minutes. In the presence of acetate the PAH S/M ratio was significantly elevated at all incubation times (Figure 18).

Addition of acetate to slices that had been incubated with PAH for 60 minutes resulted in an immediate increase in final medium pH. The rise in pH was temporally correlated with the increase in accumulation of PAH (Figure 19). The pH of medium bathing control slices was not significantly different at the times studied.

Figure 18. Effect of 10.0 mM Na Acetate on final media pH and on PAH accumulation by rabbit kidney slices. Initial medium pH adjusted to 7.40 (dashed line). Final media pH determined (methods) within one minute of termination of incubation. Hydrogen ion concentration in the media was calculated from the experimentally measured pH. Mean ± S.E. of hydrogen ion concentration was calculated from duplicate determinations of 3 experiments and reconverted to pH. PAH concentration in the media was 7.4×10^{-5}M PAH.

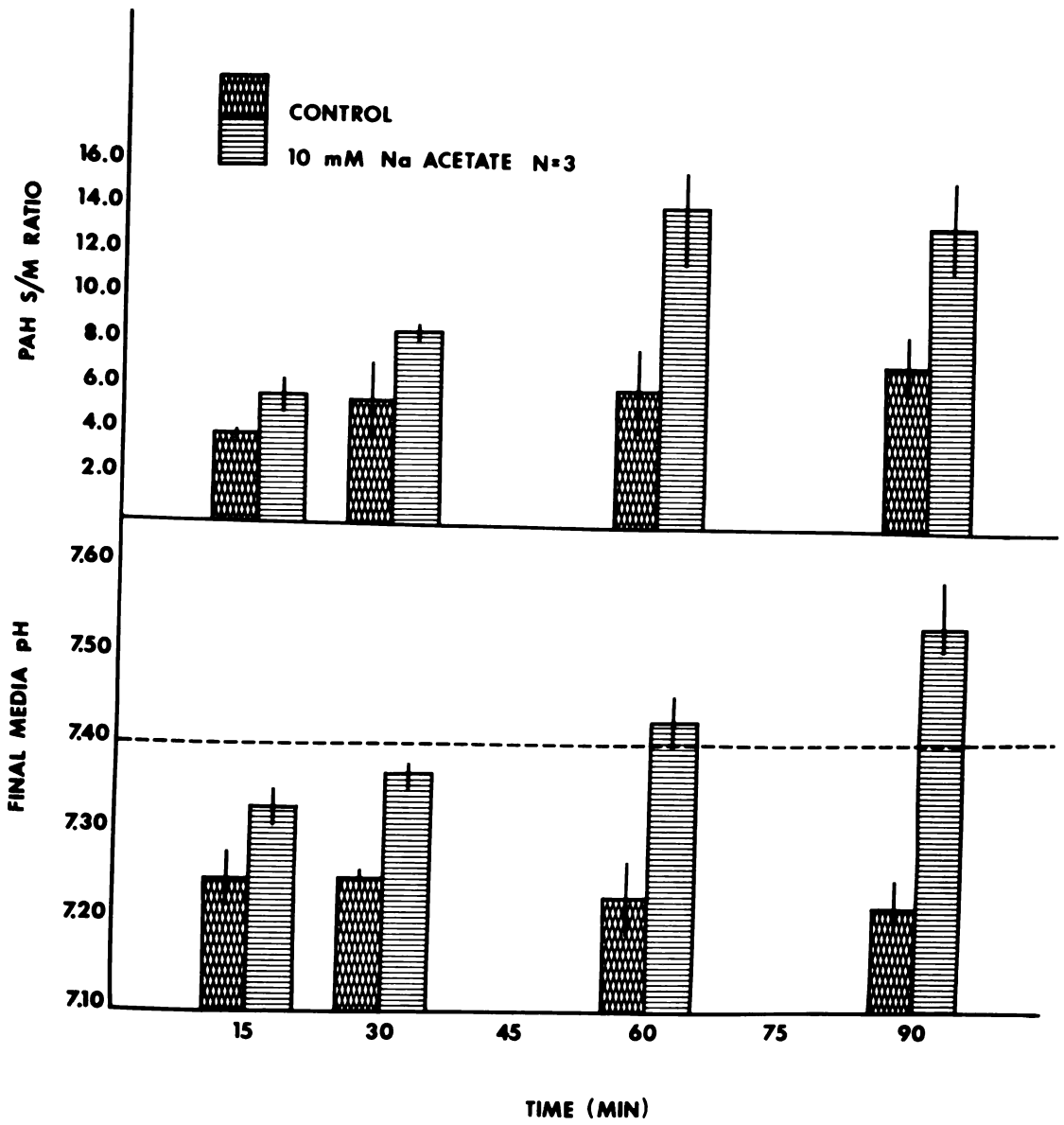
CONTROL
10 mM Na ACETATE N=3
PAH S/M RATIO
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
FINAL MEDIA pH
7.60
7.50
7.40
7.30
7.20
7.10
15
30
45
60
75
90
TIME (MIN)

Figure 18

Figure 19. Effect of 10.0 mM Na Acetate on steady state final media pH and PAH accumulation (S/M ratio) in rabbit kidney slices. Experiments performed as before (Figure 14). Points represent mean ± S.E. of four experiments.

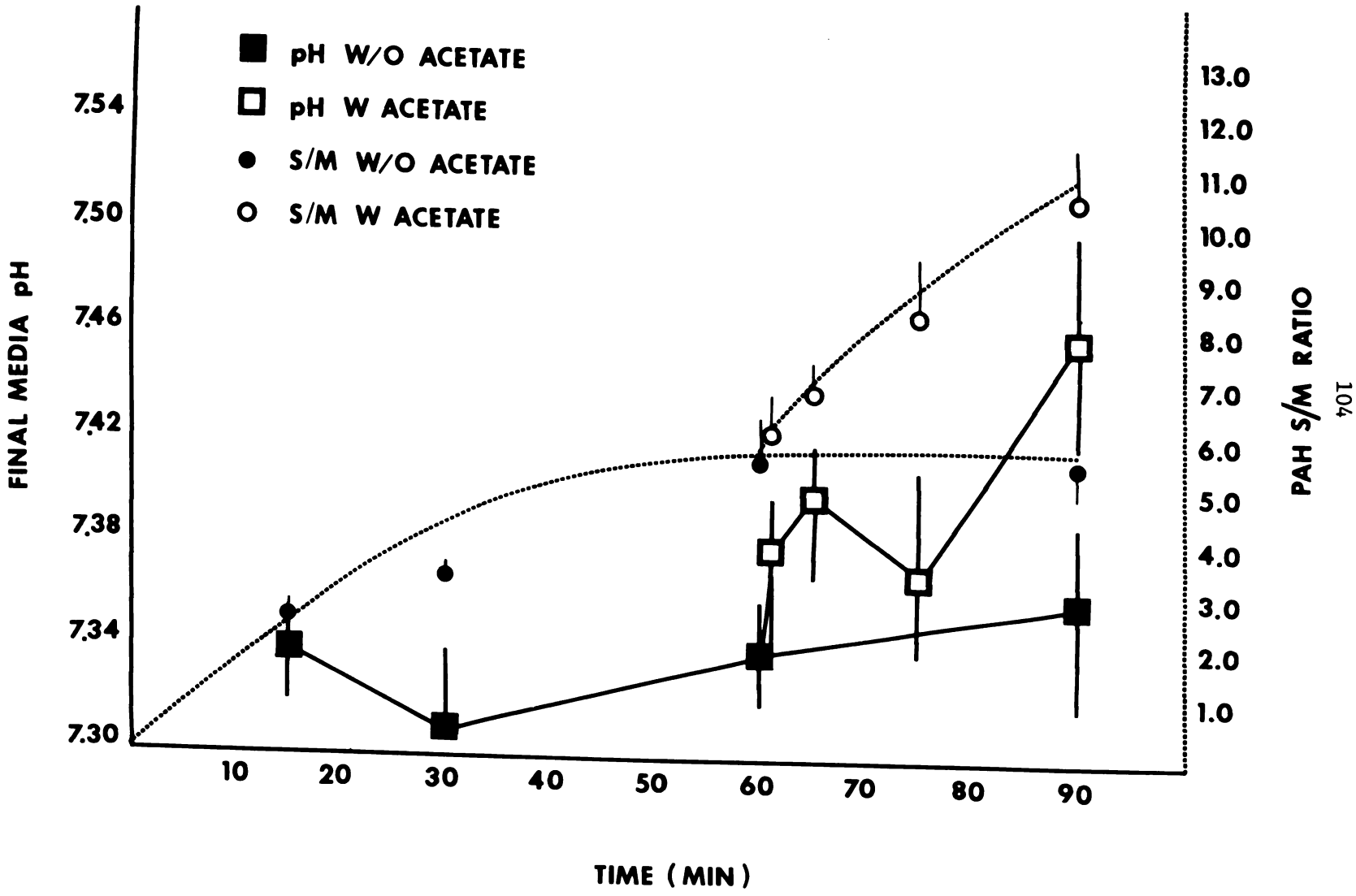
pH W/O ACETATE
pH W ACETATE
S/M W/O ACETATE
S/M W ACETATE
FINAL MEDIA pH
7.54
7.50
7.46
7.42
7.38
7.34
7.30
PAH S/M RATIO
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
70
80
90
TIME (MIN)

Figure 19

6) Effect of Acetate (10 mM) on Slice PAH Accumulation and Citrate Concentration

Citrate concentration in slices incubated with 10 mM acetate (0.405±0.032 μmoles/g tissue) was significantly higher than the endogenous concentration (0.246±0.045 μmoles/g tissue) and the concentration found in slices incubated without acetate (0.086±0.008 μmoles/g tissue) (Figure 20). The concentration of citrate in control slices after preparation and incubation was significantly less than the endogenous concentration of citrate. These relationships were not altered by the presence of PAH in the incubation medium (unpublished results). Acetate in the incubation medium significantly increased slice PAH accumulation (S/M = 11.43±0.75) when compared to control (S/M = 5.39±0.62) (Figure 20).

Addition of 0.5 mM Na_3 citrate to the incubation medium increased PAH S/M and final media pH (Figure 21). Control medium pH rose from the initial pH 7.40 but remained constant with time. PAH S/M was increased to 9.90±0.47 after 90 minutes compared to a control of 6.09±0.43. Citrate also produced an increase in final medium pH that reached 7.65 after 90 minutes of incubation.

7) Citrate Dose-response Curve

Exogenous medium citrate produced a concentration dependent biphasic effect on slice PAH accumulation (Figure 22). Low concentrations of Na_3 citrate (0.3 mM) significantly stimulated PAH accumulation from 6.90±0.40 to 9.78±0.45. Increasing citrate concentration above 1.0 mM decreased stimulation of PAH accumulation. Accumulation of PAH was significantly inhibited at 20.0 mM Na_3 Citrate (S/M = 3.79±0.27). Final medium pH increased with addition of citrate up to

Figure 20. Effect of 10.0 mM Acetate on slice concentration of citrate and PAH accumulation. Citrate concentration was measured after a 90 min incubation. PAH accumulation was determined during a 60-minute incubation period after an initial 30 minute pre-incubation without PAH. Bars represent mean ± S.E. of four experiments. *Significantly different than control ($p<0.05$). †Significantly different than endogenous renal cortical concentration of citrate.

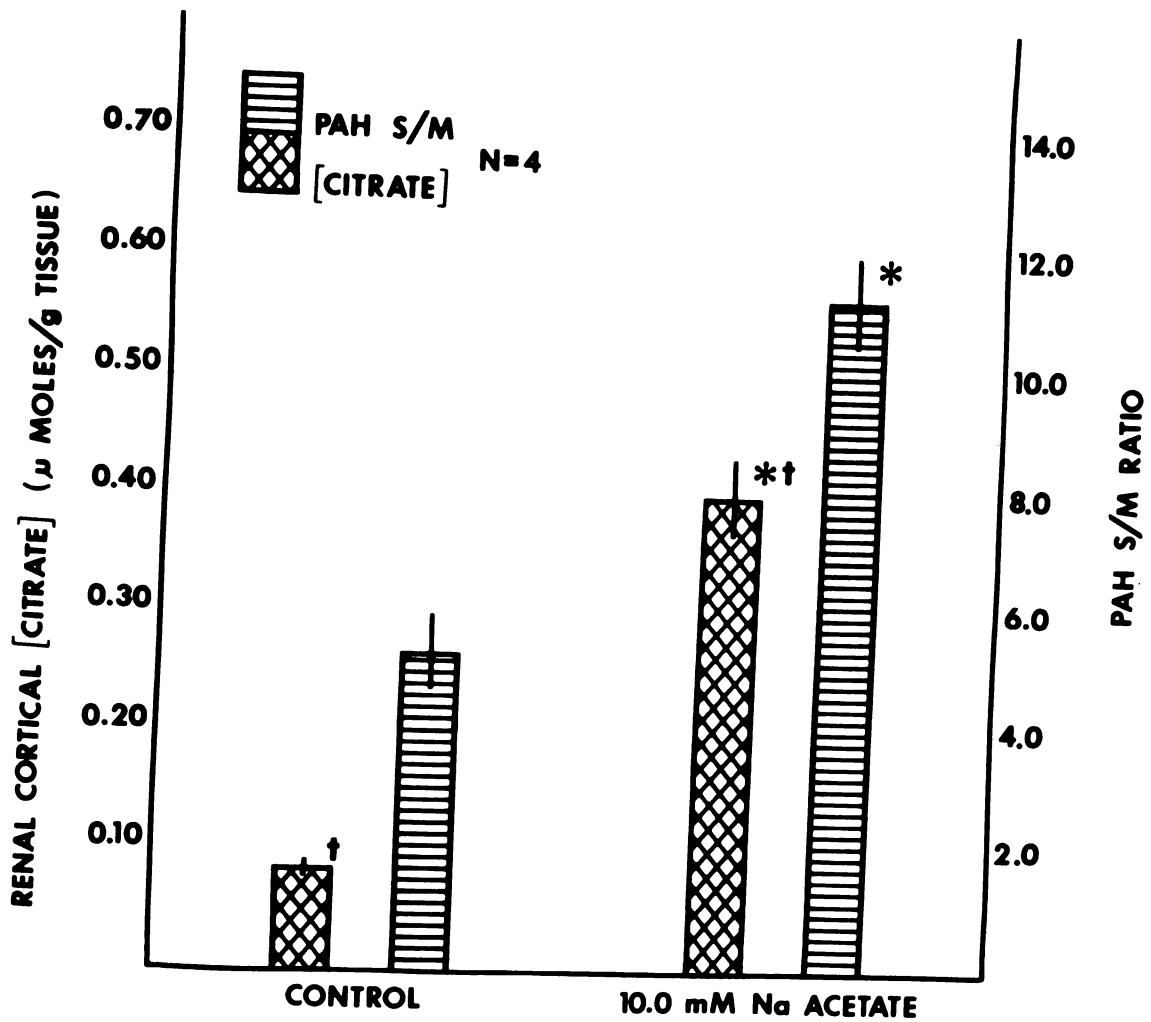
RENAL CORTICAL [CITRATE] (μ MOLES/g TISSUE)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
PAH S/M
[CITRATE]
N=4
14.0
12.0
10.0
8.0
6.0
4.0
2.0
PAH S/M RATIO
*
*†
†
CONTROL
10.0 mM Na ACETATE

Figure 20

Figure 21. Effect of citrate on final media pH and on PAH accumulation (S/M ratio) by rabbit kidney slices. Slices were pre-incubated 30 min in 0.5 mM Citrate (Control slices, 1.5 mM NaCl). PAH (7.4×10^{-5}M) added at the end of pre-incubation and final media pH and PAH accumulation determined as before. Bars represent means ± S.E. of four experiments.

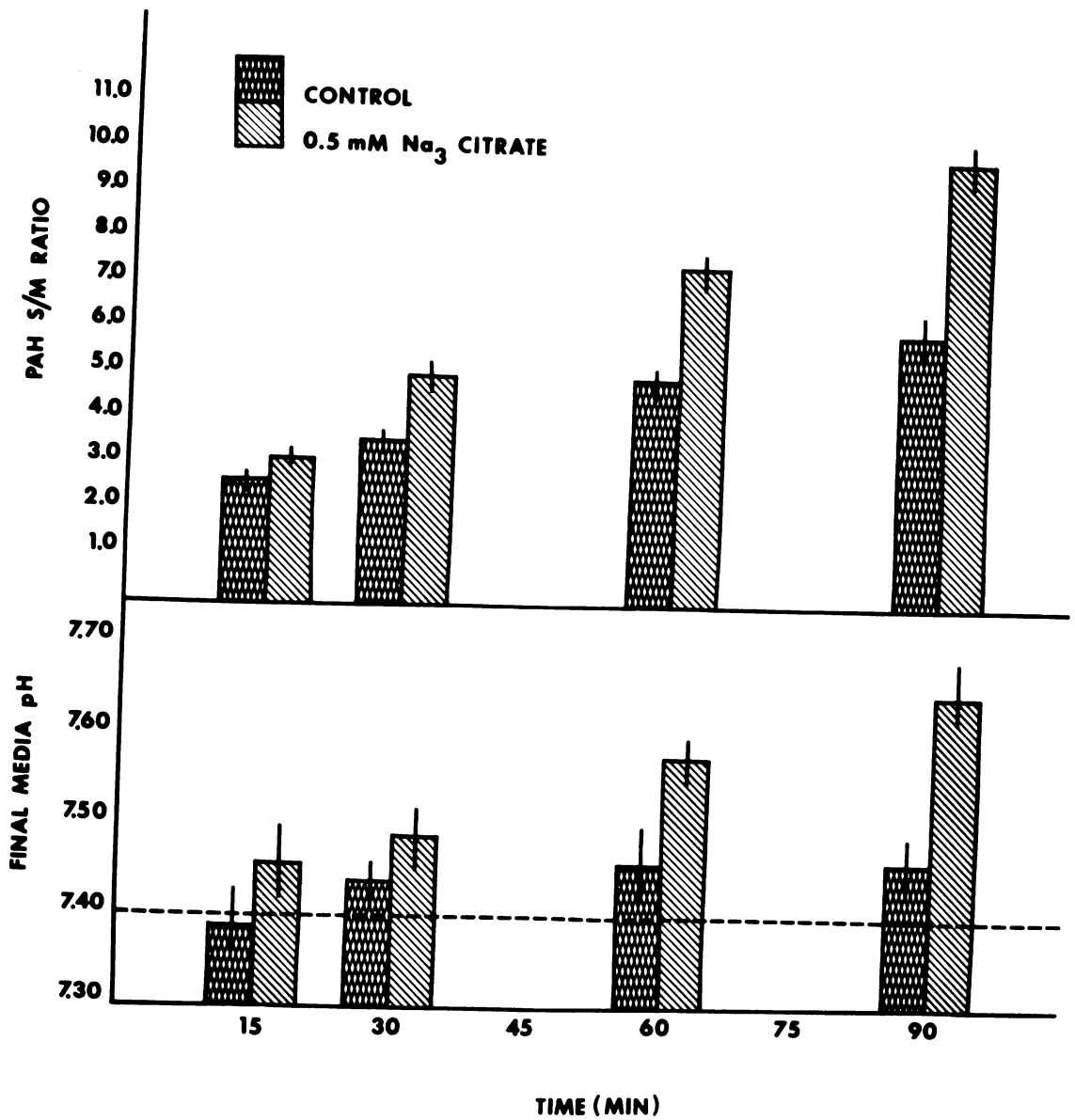
CONTROL
0.5 mM Na3 CITRATE
PAH S/M RATIO
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
FINAL MEDIA pH
7.70
7.60
7.50
7.40
7.30
15
30
45
60
75
90
TIME (MIN)

Figure 21

Figure 22. Effect of medium concentration of citrate on accumulation (S/M ratio) of PAH (○, ●) and final media pH (□, ■). Rabbit kidney slices incubated 30 min in Na_3 Citrate and incubated 60 min with 7.4×10^{-5}M PAH. Points represent means ± S.E. for the indicated number of experiments (n).

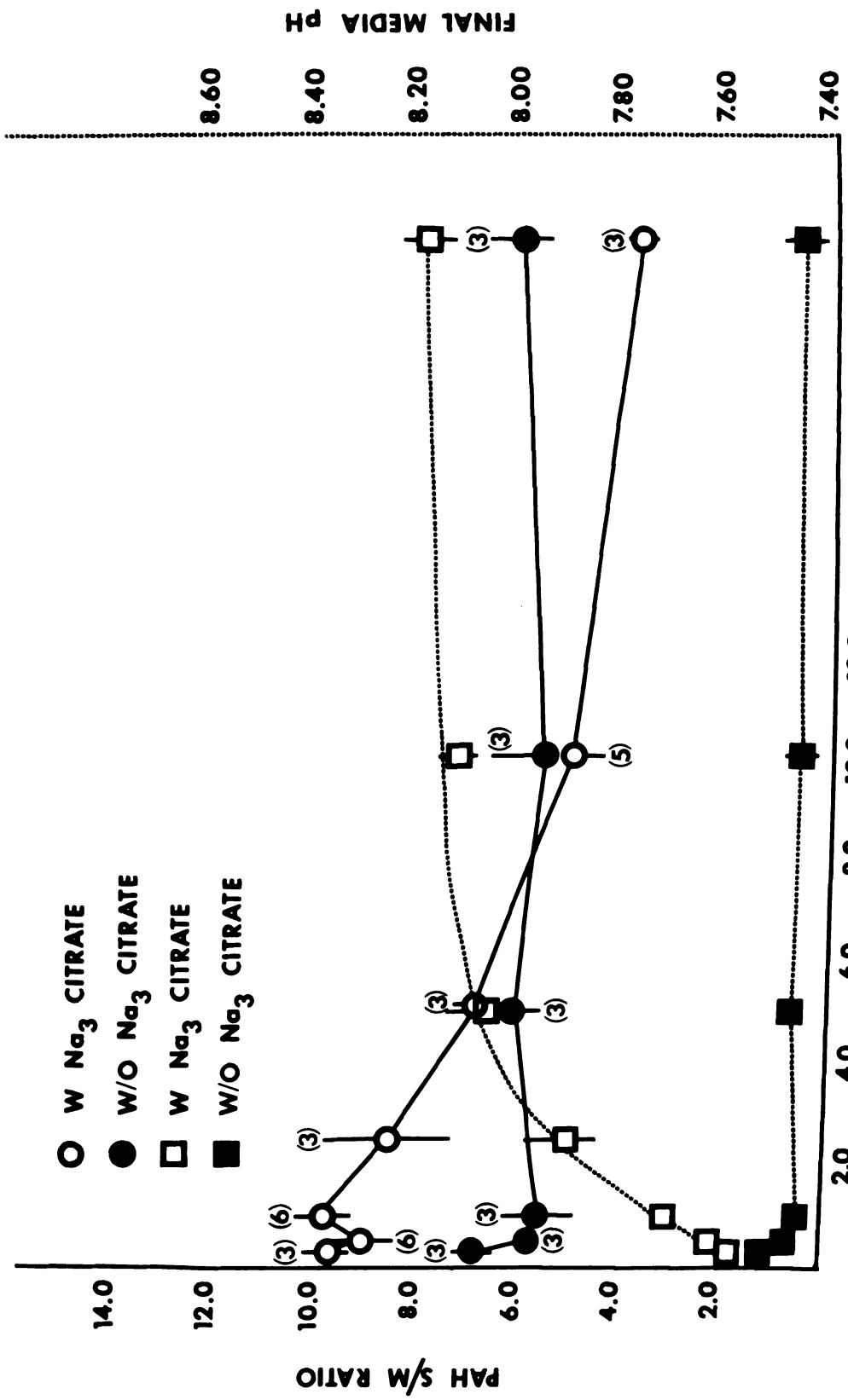
PAH S/M RATIO
FINAL MEDIA pH
[CITRATE] (mM)
W Na_3 CITRATE
W/O Na_3 CITRATE
W Na_3 CITRATE
W/O Na_3 CITRATE
14.0
12.0
10.0
8.0
6.0
4.0
2.0
8.60
8.40
8.20
8.00
7.80
7.60
7.40
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
(3)
(6)
(5)

Figure 22

5.0 mM (7.58 at 0.3 mM to 8.07 at 5.0 mM Na_3 Citrate). PAH accumulation and final medium pH in control slices was not influenced as sodium concentration was increased.

8) Interspecies Variation in Acetate-induced Stimulation of Slice PAH Accumulation and Citrate Concentration

When kidney slices from various species were incubated in a substrate-free medium (control), the ability to accumulate PAH was directly related to the endogenous renal cortical citrate concentration (Table 2). Citrate was present at the lowest concentration in dog kidney cortex (0.148±0.021 μmol/g of tissue) whereas rat cortex exhibited the greatest endogenous citrate concentration (0.393±0.036 μmol/g of tissue). Rabbit kidney cortex contained an intermediate citrate concentration (0.246±0.045 μmol/g of tissue). The endogenous citrate concentration of frog renal cortex was not determined due to inadequate amounts of tissue. Interspecies ability to accumulate PAH was highest in rat slices (S/M = 11.90±0.74) and decreased in the order; frog (PAH S/M = 6.90±0.25), rabbit (PAH S/M = 5.39±0.62) and dog (PAH S/M = 4.49±0.33) (Table 2). As observed previously (Figure 20) control slice citrate concentration decreased during preparation and incubation. Regardless of the species examined, acetate significantly increased both slice citrate concentration and PAH accumulation (Table 2). The ability of acetate to increase these parameters was inversely related to the endogenous renal cortical citrate concentration. In dog kidney slices, acetate increased PAH accumulation approximately 134% and slice citrate concentration approximately 710%. In contrast, acetate increased rat slice PAH accumulation by only 20% and increased slice citrate concentration by approximately 166% (Table

TABLE 2

Relationship Between Acetate-Induced Stimulation of PAH Accumulation and Citrate Concentration in Renal Cortex Slices from Several Species[a]

Species	PAH S/M Ratio		Citrate Concentration (μmole/g tissue)		
	Control	Acetate (10 mM)	Endogenous	Control	Acetate (10 mM)
Dog	4.49±0.33 (3)	10.52±0.52[b] (3)	0.148±0.021[b] (4)	0.081±0.005 (3)	0.656±0.062[b] (3)
Rabbit	5.39±0.62 (4)	11.43±0.75[b] (4)	0.246±0.045[b] (4)	0.086±0.008 (4)	0.405±0.032[b] (4)
Frog	6.90±0.25 (3)	12.41±0.71[b] (3)	---	0.128±0.026 (3)	0.343±0.034[b] (3)
Rat	11.90±0.74 (4)	14.22±0.95[b] (4)	0.093±0.036[b] (8)	0.080±0.006 (3)	0.213±0.005[b] (3)

[a]Slices from the kidneys of 1-6 animals of a given species were pooled in each experiment. Renal cortex for determination of endogenous citrate concentration was obtained immediately after sacrifice. In some instances, cortex from several animals was pooled to obtain sufficient tissue. Values are expressed as the mean ± standard error. (n) = number of experiments.

[b]Significantly different than control, $p<0.05$.

2). Acetate stimulation of slice PAH accumulation and citrate concentration in rabbit (PAH S/M = 112%, [citrate] = 371%) and frog (PAH S/M = 80%; [citrate] = 168%) slices was consistent with the intermediate concentrations of citrate found endogenously in renal cortex of these species (Table 2).

Addition of 0.5 mM Na_3 citrate to the incubation medium increased PAH accumulation by dog (34%), rabbit (63%), and rat (57%) renal cortical slices to approximately the same extent (Table 3). However, PAH accumulation by frog kidney slices was not altered by the addition of citrate (PAH S/M = 7.39±1.10) when compared to control (PAH S/M = 6.90±0.25) (Table 3).

9) Effect of Potassium Concentration

Reduction of medium potassium concentration from 40 mM K^+ to 5 mM K^+ significantly depressed PAH accumulation and citrate concentration in rat slices incubated either in a substrate-free medium or in media containing Na acetate or Na lactate as substrate (Table 4). Regardless of medium potassium concentration, acetate and lactate stimulated slice accumulation of PAH and increased slice citrate concentration. However, the magnitude of stimulation produced by these substrates was greater in medium containing 40 mM potassium (Table 4). Lactate was much more efficient at increasing PAH accumulation and citrate concentration than was acetate (Table 4).

10) Effect of Chronic Metabolic Acidosis

Substitution of ammonium chloride (0.28 M) for the normal drinking water of rats reduced the arterial blood bicarbonate and endogenous renal cortex citrate concentrations to approximately the

TABLE 3

Effect of Citrate on PAH Accumulation by Slices of Renal Cortex Obtained from Several Species[a]

Species	(n)[b]	PAH S/M Ratio	
		Control	Citrate (0.5 mM)
Dog	(3)	4.49±0.33	6.02±0.41[c]
Rabbit	(4)	6.09±0.43	9.90±0.47[c]
Frog	(3)	6.90±0.25	7.39±1.10
Rat	(4)	11.90±0.74	18.70±0.34[c]

[a] Slices from the kidneys of 1-6 animals of a given species were pooled in each experiment. Values are expressed as the mean ± standard error.

[b] (n) = number of experiments.

[c] Significantly different than control, $p<0.05$.

TABLE 4

Effect of Potassium Concentration on Stimulation of PAH Accumulation and Citrate Concentration in Slices of Rat Renal Cortex[a]

Substrate	PAH S/M Ratio		Citrate Concentration (μmole/g tissue)	
	5 mM K^+	40 mM K^+	5 mM K^+	40 mM K^+
Control (None)	7.25±0.69 (3)	9.83±1.05 (3)	0.040±0.010 (3)	0.080±0.006 (3)
Acetate (10 mM)	9.47±0.61 (3)	14.22±1.63 (3)	0.145±0.005 (3)	0.213±0.005 (3)
Lactate (10 mM)	---	36.14±2.86 (4)	0.478±0.060 (3)	1.492±0.173 (3)

[a]Table 4 was compiled from 3 separate studies as identified by the blocks. Slice PAH accumulation in the presence of 40 mM K^+, 10 mM Na lactate was included since PAH accumulation under control conditions (S/M = 11.90±0.74, n=4), was not statistically different from PAH accumulation in the study listed above. In each series of experiments renal cortical slices from the kidneys of 3-6 rats were pooled and subsequently distributed among the various incubation conditions listed. Values represent the mean ± standard error of the indicated number (n) of experiments.

same extent (43-45%) (Table 5). In addition chronic metabolic acidosis significantly decreased the ability of rat kidney cortex slices to accumulate PAH when compared to control (Table 5). Acetate (10 mM) significantly enhanced the ability of slices from rats in normal acid-base balance to accumulate PAH. However, acetate had no effect on PAH accumulation by slices from chronically acidotic rat kidney (Table 5).

11) Effect of Preincubation in Citrate on Stimulation of Slice PAH Accumulation by Acetate

Pre-incubation of control slices with citrate increased PAH accumulation compared to slices pre-incubated with NaCl (Figure 23). The PAH S/M ratios of control slices were 6.40±0.45 (0.5 mM NaCl) and 5.44±0.34 (10.0 mM NaCl). Citrate treatment increased PAH accumulation by control slices to 9.33±0.47 and 7.72±0.32, respectively. Transfer of slices pre-incubated with NaCl to media containing 0.5 mM acetate significantly increased PAH accumulation by 42% (S/M = 8.94± 0.54) when compared to control. Accumulation of PAH in 10.0 mM acetate was significantly greater (increased 111%) than accumulation in 0.5 mM acetate. Pre-incubation with citrate significantly reduced the effect of acetate on PAH accumulation. Accumulation of PAH by citrate treated slices was not significantly different when these slices were incubated in media containing 0.5 mM (S/M = 10.04±0.76) or 10.0 mM acetate (S/M = 10.29±0.65). PAH accumulation by citrate treated slices was not significantly increased (8%) by incubation in 0.5 mM acetate when compared to control. However, incubation of citrate treated slices in 10.0 mM acetate resulted in a significant increase (33%) in PAH accumulation when compared to control. No

TABLE 5

Effect of Chronic Ammonium Chloride Acidosis on the Endogenous Citrate Concentration of Rat Renal Cortex and PAH Accumulation by Slices of Rat Kidney Cortex[a]

Treatment	Arterial Blood [HCO_3^-] (meq/liter)	Endogenous [Citrate] (μmole/g tissue)	PAH S/M Ratio NaCl (10 mM)	PAH S/M Ratio Na Acetate (10 mM)
Control (n)[b]	27.7±0.5 (8)	0.393±0.036 (8)	11.90±0.74 (4)	14.22±0.95 (4)
Acidotic (n)	14.8±1.0[c] (8)	0.217±0.025[c] (8)	8.11±0.51[c] (5)	6.03±0.60[c] (5)

[a]Rats were maintained for 7 days on 0.28 M NH_4Cl as sole drinking fluid. Controls were allowed tap water. Blood was drawn from the abdominal aorta for 8 rats for determination of arterial bicarbonate concentration. One-half of the renal cortex of each rat was analyzed for the endogenous citrate concentration. Slices prepared from the remaining cortex of two rats were pooled with those from additional rats for determination of PAH accumulation.

[b](n) = number of experiments.

[c]Significantly different than controls; $p<0.05$.

Figure 23. Effect of pre-incubation with citrate on acetate-induced increase in final media pH and on accumulation (S/M ratio) of PAH by rabbit kidney slices. *Significantly different than appropriate control. †Significantly different than NaCl pre-incubated counterpart. ‡Significantly different than 0.5 mM Na Acetate treated slices (NaCl pre-incubated). Slices pre-incubated 30 min in Na_3 Citrate and incubated 60 min in Na Acetate + 7.4×10^{-5}M PAH. Bars represent means ± S.E. of five experiments.

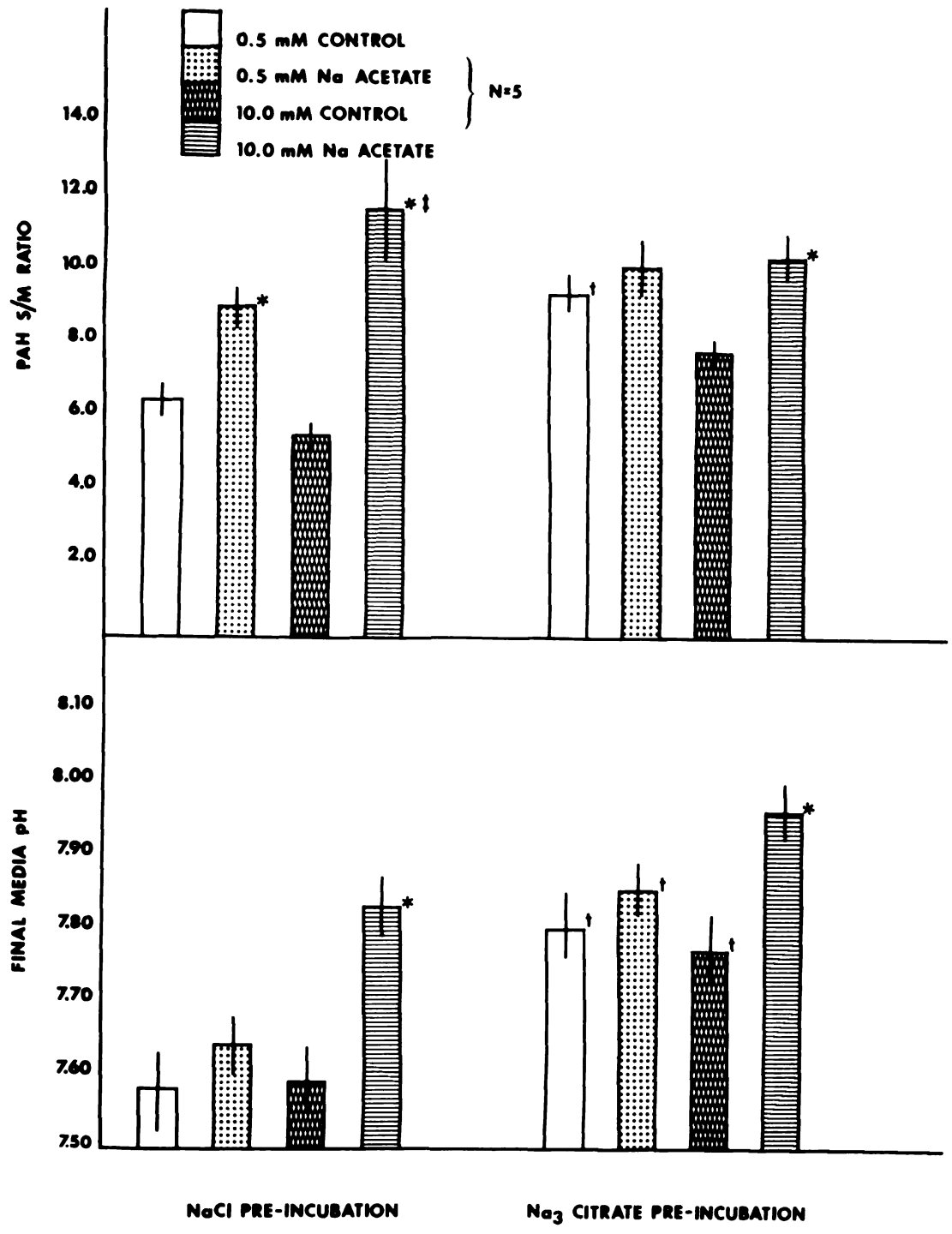
0.5 mM CONTROL
0.5 mM Na ACETATE
10.0 mM CONTROL
10.0 mM Na ACETATE
N=5
PAH S/M RATIO
14.0
12.0
10.0
8.0
6.0
4.0
2.0
FINAL MEDIA pH
8.10
8.00
7.90
7.80
7.70
7.60
7.50
NaCl PRE-INCUBATION
Na3 CITRATE PRE-INCUBATION

Figure 23

significant difference in PAH accumulation existed between incubation of NaCl treated slices in 10.0 mM acetate and incubation of citrate treated slices in either 0.5 mM or 10.0 mM acetate. Citrate preincubation produced the same pattern of results in final medium pH as observed with PAH S/M ratios.

12) α-Ketoglutarate and Succinate Dose Response Curves

p-Aminohippurate accumulation by rabbit renal cortical slices was determined at varying concentrations (0.25, 0.5, 1.0 and 5.0 mM) of α-ketoglutarate (Figure 24) or succinate (Figure 25) in the medium. Addition of α-ketoglutarate or succinate to the incubation medium produced a biphasic effect on PAH accumulation (Fisure 24, 25). α-Ketoglutarate (0.25 mM) significantly increased PAH accumulation 41%, from 5.25±0.85 to 7.40±1.05 (Figure 24). Additional increases in PAH accumulation (S/M = 9.10±0.80 and 9.40±1.30) occurred as medium α-ketoglutarate concentration was elevated to 0.5 and 1.0 mM, respectively. Accumulation of PAH was significantly inhibited at 5.0 mM α-ketoglutarate (S/M = 2.10±0.20). The same pattern was observed when succinate was added to the incubation medium. Succinate (0.25 mM) increased PAH accumulation from 7.35±0.30 to 9.83±0.72, a 34% stimulation (Figure 25). Addition of 0.5 mM or 1.0 mM succinate produced additional increases in PAH accumulation (S/M = 10.95±1.75; 11.08±1.37). At high medium concentration (5.0 mM), succinate significantly inhibited PAH accumulation (S/M = 3.52±0.40).

Final medium pH increased with addition of α-ketoglutarate from 7.42 at 0.25 mM to 7.67 at 5.0 mM α-ketoglutarate (Figure 24). The control medium pH did not vary significantly from the initial pH

Figure 24. Effect of medium concentration of α-ketoglutarate on PAH accumulation (S/M ratio) and final media pH. Experiment performed as in Figure 22. Points represent means ± S.E. of five experiments.

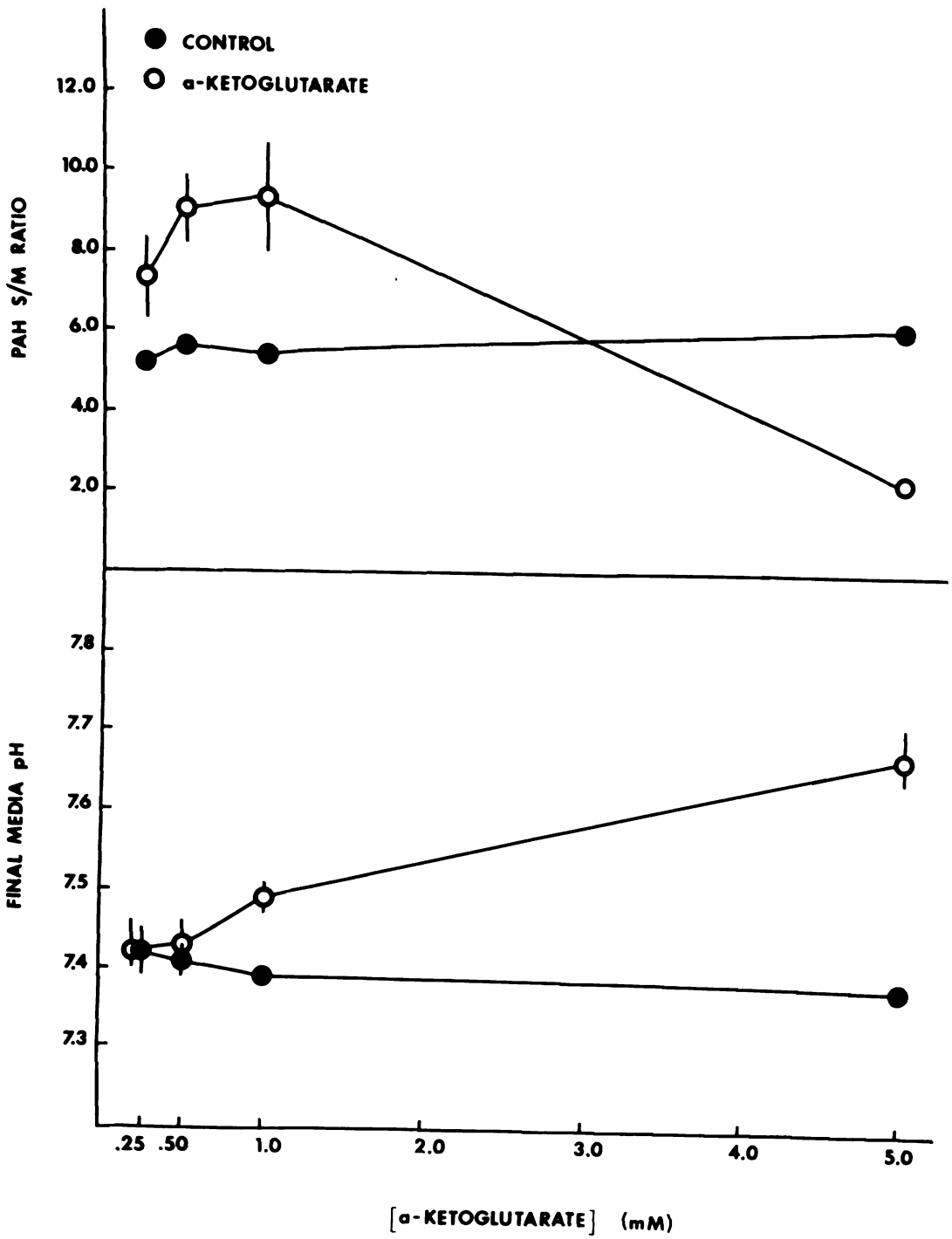
CONTROL
α-KETOGLUTARATE
PAH S/M RATIO
12.0
10.0
8.0
6.0
4.0
2.0
FINAL MEDIA pH
7.8
7.7
7.6
7.5
7.4
7.3
.25
.50
1.0
2.0
3.0
4.0
5.0
[α-KETOGLUTARATE] (mM)

Figure 24

Figure 25. Effect of medium concentration of succinate on PAH accumulation (S/M ratio) and final media pH. Experiments performed as in Figure 22. Points represent means ± S.E. of four experiments.

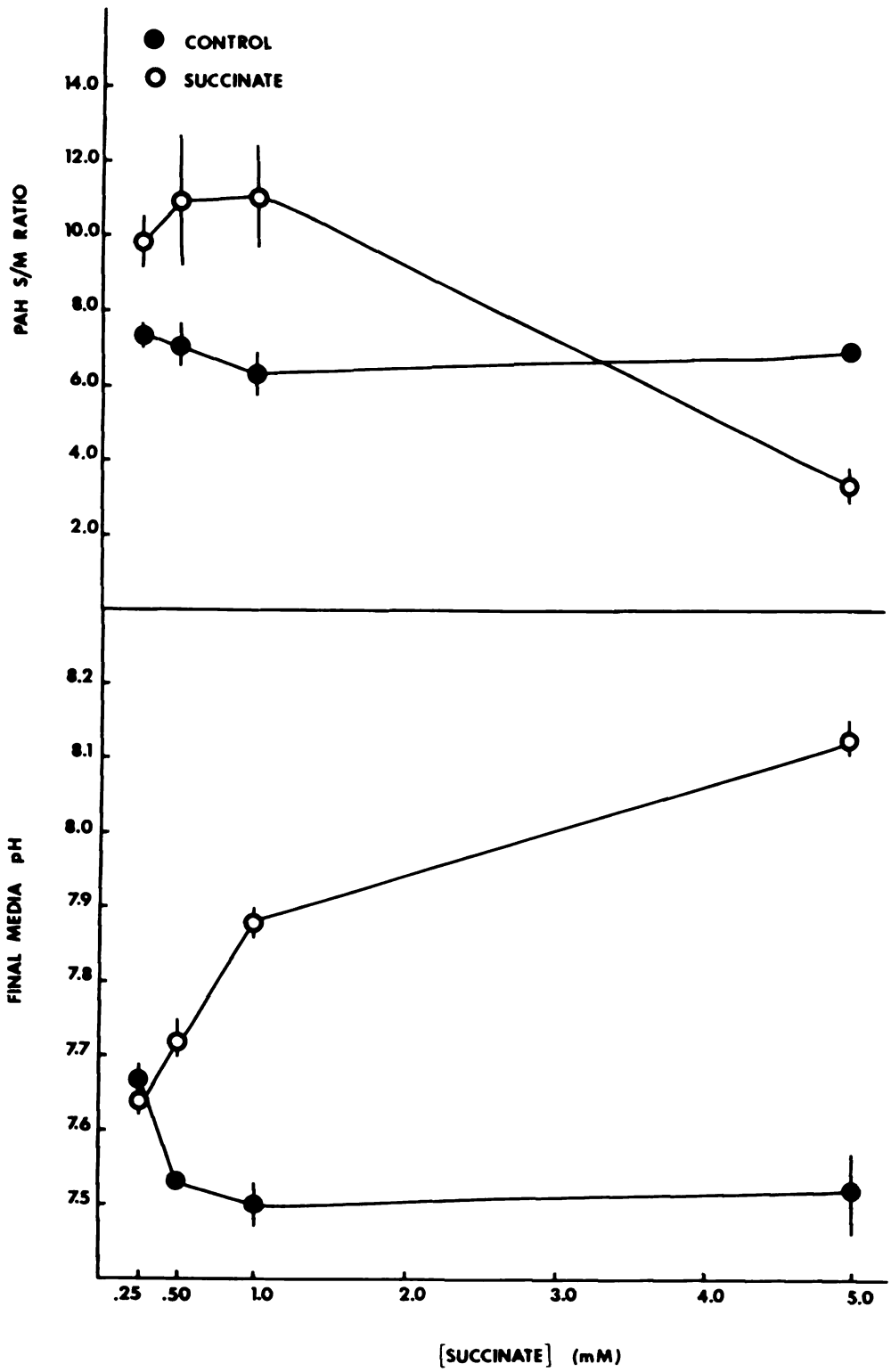
CONTROL
SUCCINATE
PAH S/M RATIO
14.0
12.0
10.0
8.0
6.0
4.0
2.0
FINAL MEDIA pH
8.2
8.1
8.0
7.9
7.8
7.7
7.6
7.5
.25
.50
1.0
2.0
3.0
4.0
5.0
[SUCCINATE] (mM)

Figure 25

of 7.40 (Figure 25). Addition of succinate to the incubation medium also increased pH from 7.64 at 0.25 mM to 8.13 at 5.0 mM succinate (Figure 25). Control medium pH rose from the initial pH (7.40) but remained relatively constant over the concentration range studied.

13) Effect of α-Ketoglutarate and Glucose on Slice PAH Accumulation and Citrate Concentrations

Stimulation of PAH accumulation by α-ketoglutarate was not correlated with restoration of depleted slice citrate concentration (Figure 26). α-Ketoglutarate (0.5 mM) significantly stimulated PAH accumulation (S/M = 10.00±0.66) when compared to slices incubated without substrate (S/M = 5.26±0.37) (Figure 26). Citrate in control slices (0.120±0.042 μmoles/g) was less than the endogenous renal cortical concentration of citrate, but this was not changed when 0.5 mM α-ketoglutarate was present as substrate (0.136±0.044 μmoles/g) (Figure 25). Glucose (10.0 mM) did not significantly alter either slice citrate concentration (0.145±0.037 μmoles/g) or PAH accumulation (S/M = 6.59±0.28) (Figure 26).

14) Effect of Acetate (1.0 mM) on Slice PAH Accumulation, Citrate and α-Ketoglutarate Concentration

Slice α-ketoglutarate concentration and PAH accumulation were significantly increased by 1.0 mM acetate in the incubation medium (Figure 27). Preparation and incubation of slices did not significantly alter the amount of α-ketoglutarate in control slices (0.063±0.009 μmoles/g) when compared to the endogenous concentration (0.050±0.011 μmoles/g). Acetate produced a 3-4 fold increase in α-ketoglutarate concentration (0.205±0.043 μmoles/g). Control slice citrate concentration was significantly less than the endogenous

Figure 26. Effect of 0.5 mM α-ketoglutarate and 10 mM glucose on slice citrate concentration and PAH accumulation. Experiments performed as in Figure 20. Bars represent means ± S.E. of four experiments.

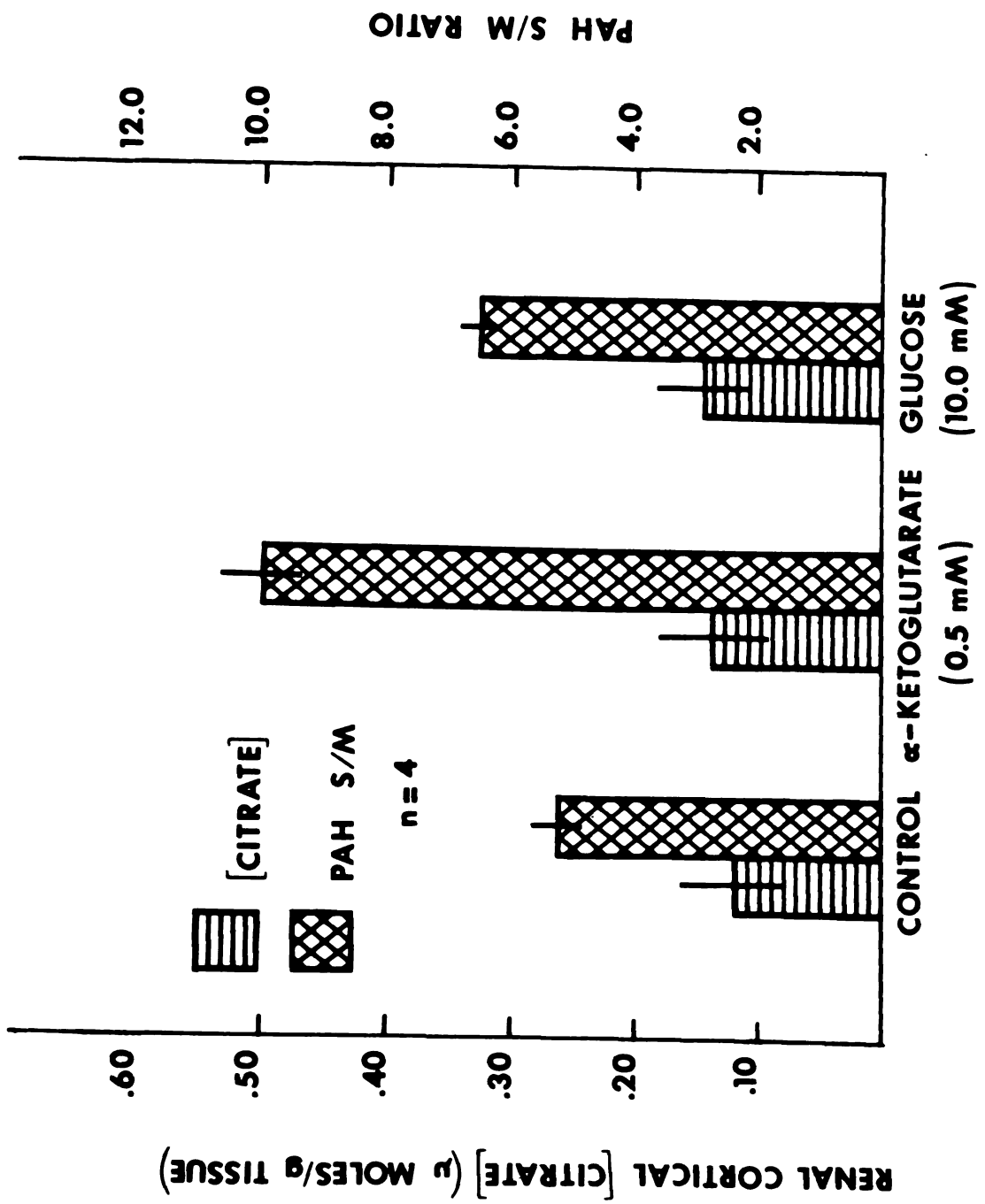
RENAL CORTICAL [CITRATE] (μ MOLES/g TISSUE)
.60
.50
.40
.30
.20
.10
PAH S/M RATIO
12.0
10.0
8.0
6.0
4.0
2.0
[CITRATE]
PAH S/M
n=4
CONTROL
α-KETOGLUTARATE (0.5 mM)
GLUCOSE (10.0 mM)

Figure 26

Figure 27. Effect of 1.0 mM acetate on slice citrate and α-ketoglutarate concentration and PAH accumulation. Citrate and α-ketoglutarate concentrations were measured after a 90 min incubation. PAH accumulation was determined during a 60 min incubation period after an initial 30 min pre-incubation without PAH. Bars represent means ± S.E. of five experiments.

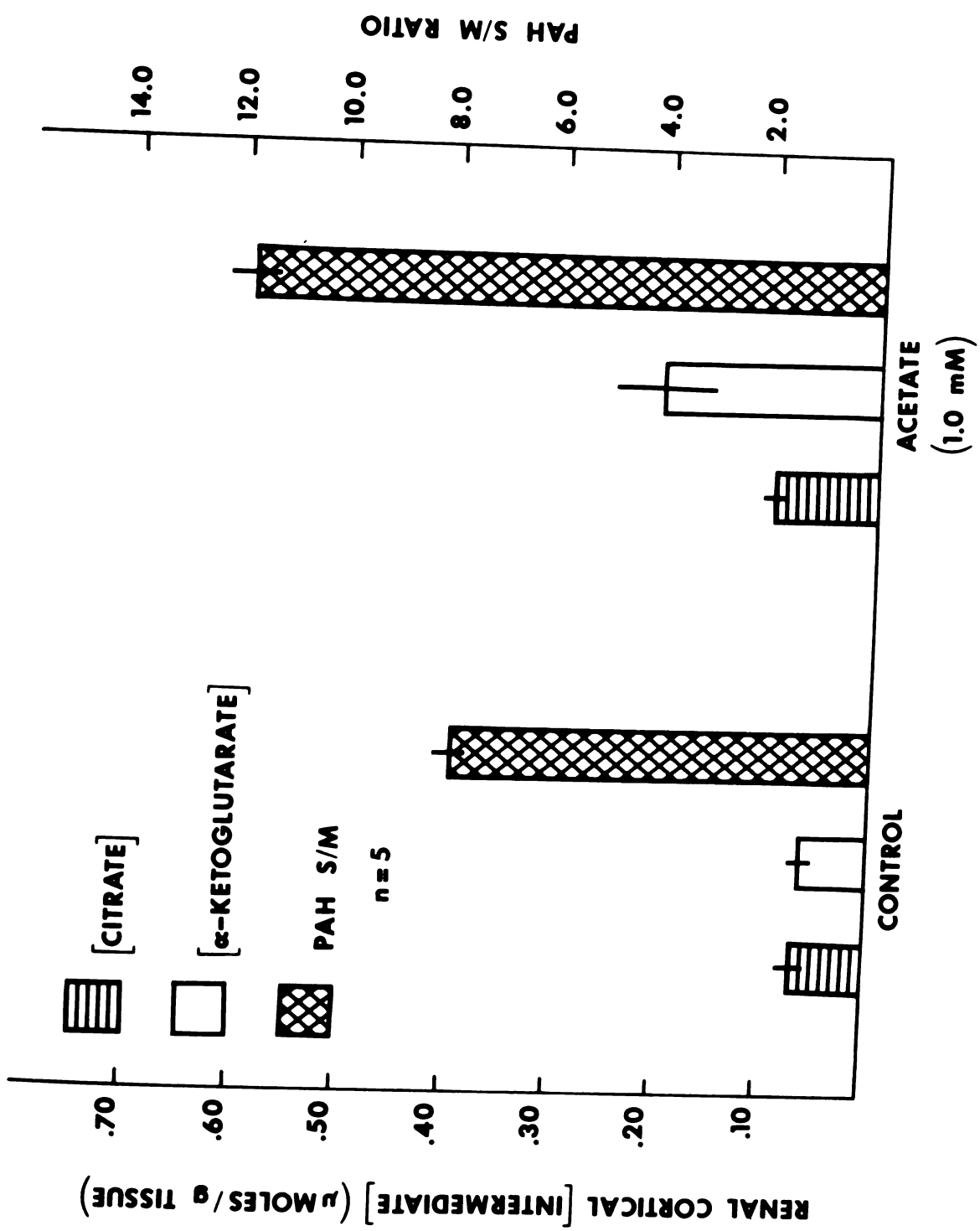
RENAL CORTICAL [INTERMEDIATE] (μMOLES/g TISSUE)
.70
.60
.50
.40
.30
.20
.10
[CITRATE]
[α-KETOGLUTARATE]
PAH S/M
n=5
CONTROL
ACETATE
(1.0 mM)
PAH S/M RATIO
14.0
12.0
10.0
8.0
6.0
4.0
2.0

Figure 27

concentration. Acetate (1.0 mM) significantly increased slice citrate concentration approximately 42% (0.096±0.013 μmoles/g) compared to control (0.069±0.009 μmoles/g). However, this was significantly less than the endogenous citrate concentration. PAH accumulation was increased 51% (S/M = 11.92±0.42) when compared to control (S/M = 7.97±0.29) by addition of 1.0 mM acetate to the incubation medium.

15) Mitochondrial Accumulation of PAH

Mitochondria prepared from rabbit kidney cortex exhibited coupled respiration (respiratory control index ranged from 7-8) and were capable of accumulating PAH against a concentration gradient (Table 6). PAH accumulated into the mitochondrial matrix space water reached concentrations 3 to 8 fold greater than those in the incubation medium when either 1.0 mM or 10.0 mM PAH was used (Table 6). Accumulation of PAH was time dependent with maximum matrix/medium PAH ratios achieved after 15 minutes of incubation. In addition mitochondria incubated with 10 mM PAH accumulated significantly more PAH than did mitochondria incubated with 1 mM PAH (Table 7).

16) Effect of Penicillin Treatment of 2-wk-old Rabbits on the Endogenous Citrate Concentration of Renal Cortex

The citrate concentration of renal cortex obtained from neonatal rabbits, was not affected by administration of penicillin in quantities sufficient to stimulate slice PAH transport capacity (Table 8).

EFFECT OF AGE AND SUBSTRATE STIMULATION ON RABBIT PROXIMAL TUBULE PAH ACCUMULATION AND PALMITATE METABOLISM

Preparation of renal proximal tubules by the magnetic iron oxide method of Brendel and Meezan (1975) produced homogeneous suspensions

TABLE 6

Accumulation of p-Aminohippurate (PAH) by Mitochondria Isolated from Rabbit Kidney Cortex[a]

[PAH]	PAH Matrix/Medium Ratio Time (min)			
	5	10	15	20
1 mM	3.0±0.4	4.6±1.1	7.6±1.7	5.6±0.8
10 mM	5.0±1.4	4.2±1.7	6.1±2.4	6.4

[a]Mitochondria were prepared from rabbit kidney cortex by differential centrifugation and incubated at 25°C in a buffered medium (25 mM HCO_3^-) containing either 1.0 or 10.0 mM PAH-^{14}C and ^{3}H-mannitol. The relationship between matrix space/outer space volume was determined by comparison to mitochondria incubated with sucrose-^{14}C and ^{3}H-H_2O. Each value represents the mean ± standard error of 3 experiments.

TABLE 7

Accumulation of p-Aminohippurate (PAH) by Mitochondria Isolated from Rabbit Kidney Cortex[a]

[PAH]	Matrix PAH (nmole/mg protein) Time (min)			
	5	10	15	20
1 mM	6.1± 0.1	6.4± 0.9	6.8±0.8	7.4±0.7
10 mM	60.2±12.6	62.6±12.2	74.3±9.2	80.4

[a]Mitochondria were prepared from rabbit kidney cortex by differential centrigugation and incubated at 25°C in a buffered medium (25 mM HCO_3^-) containing either 1.0 or 10.0 mM PAH-^{14}C and ^3H-mannitol. Matrix space PAH was determined by correcting the total mitochondrial PAH-^{14}C for that found in the outer space. Each value represents the mean ± standard error of 3 experiments.

TABLE 8

Effect of Penicillin Treatment of 2-Week-Old Rabbits on the Endogenous Citrate Concentration of Kidney Cortex[a]

Treatment	Citrate Concentration (μmole/g tissue)
Control	0.378±0.040
Penicillin	0.346±0.027

[a]Four injections of 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Animals were sacrificed 24 hours after the final injection. Renal cortex from 2 control or 2 treated rabbits were obtained for each determination. Values represent the mean ± standard error determined in rabbits from 3 litters.

of tubules (Figure 28) with intact basement membranes and brush border (Figure 29-32). Renal tubules isolated from adult and newborn rabbits accumulated PAH and were capable of oxidation and esterification of palmitic acids. Incorporation of palmitate-1-^{14}C into tubule neutral and phospholipid proceeded linearly when incubated for 15 to 45 minutes. The majority of C^{14}-label was recovered as neutral lipid (regardless of age or treatment) with smaller amounts recovered as phospholipid and carbon dioxide. The pellet and upper phase portions of the Folch extract contained relatively insignificant amounts of label.

1) Effect of Age

The maximal PAH T/M ratio obtained at 2 weeks (5.94±1.46) was approximately 36% of that obtained at 4 weeks (16.37±1.17) (Table 9). No further increases in PAH accumulation were observed in tubules from 6 week (T/M = 18.71±1.72) or adult (T/M = 14.16±2.70) rabbits although body weight increased with age (Table 9). Marked changes in tubule palmitate metabolism and plasma lipid concentration also occurred during development. Palmitate-^{14}C recovered as TG was greater in tubule suspensions prepared from 4 and 6 week rabbits as compared to 2 week rabbits (Table 8). Incorporation of label into TG fractions from adult rabbits was similar to the amount recovered from 2 week rabbits. Similar results were obtained for oxidation of palmitate-^{14}C to CO_2. Tubule suspensions from 4 and 6 week rabbits displayed an enhanced ability to oxidize palmitate when compared to tubules from 2 week rabbits (Table 9). Palmitate oxidation by adult tubule suspensions was not different than that observed at 2 weeks.

Figure 28. Light micrograph of separated proximal tubules prepared from adult rabbit kidneys (x650). Tubules were stained for succinic dehydrogenase.

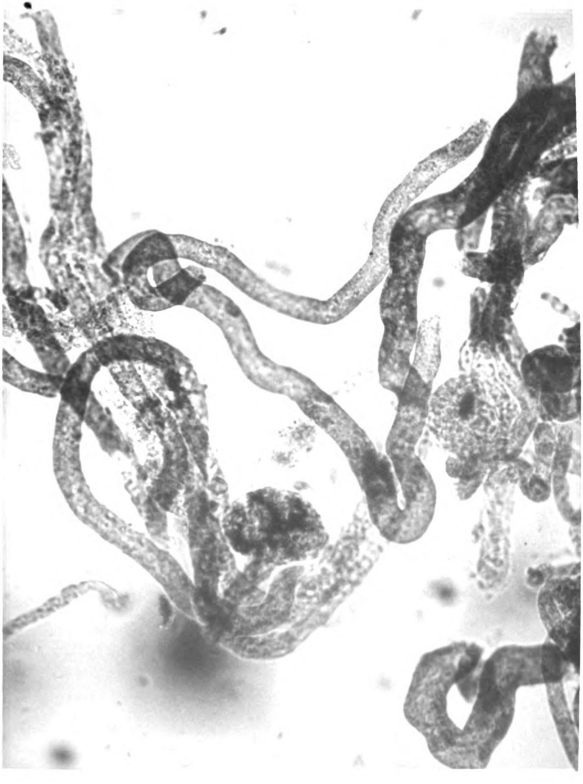

Figure 28

Figure 29. Electron micrograph of a separated proximal tubule prepared from adult rabbit kidney (x3600).

Figure 29

Figure 30. Electron micrograph of separated proximal tubules prepared from adult rabbit kidney (x6400).

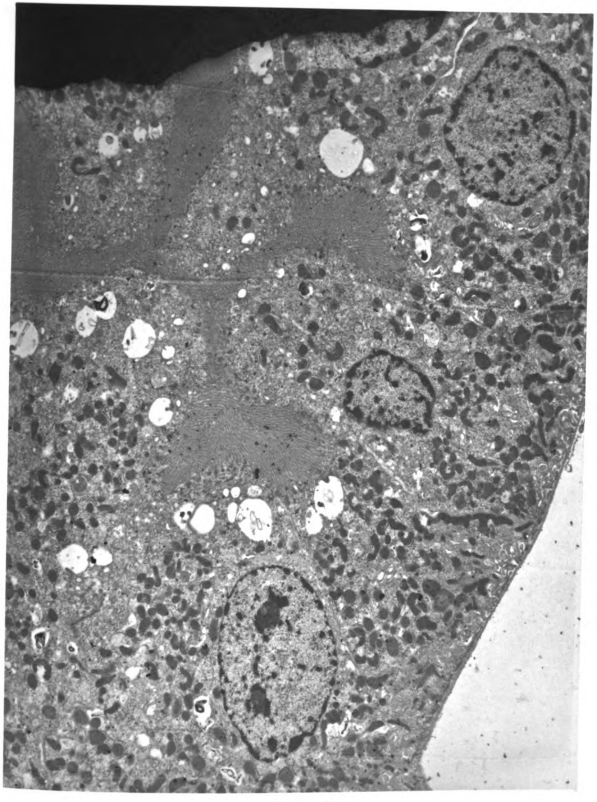

Figure 30

Figure 31. Electron micrograph of separated proximal tubules prepared from kidney of young (4 week old) rabbits (x7800).

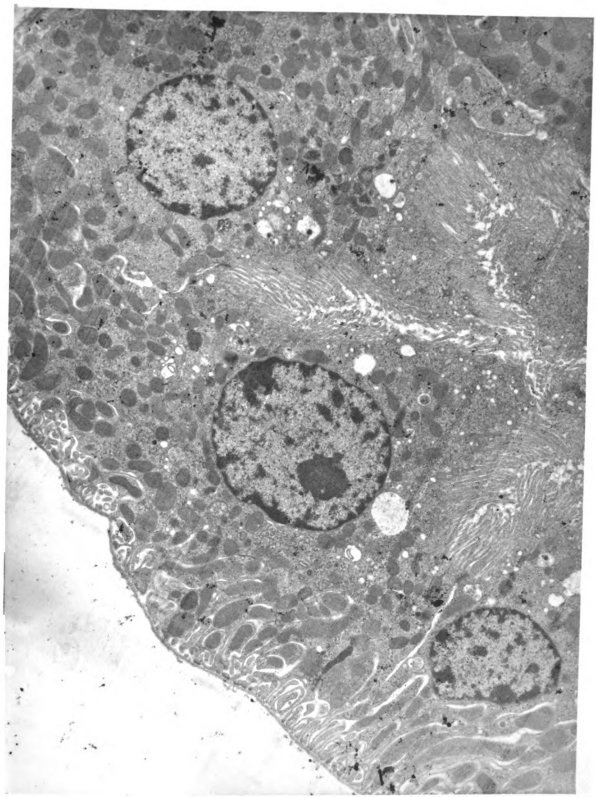

Figure 31

Figure 32. Electron micrograph of separated proximal tubules (x7800) prepared from rabbits treated with 90,000 I.U. procaine penicillin G (twice daily for 2 days). Animals were sacrificed 24 hours after the last injection.

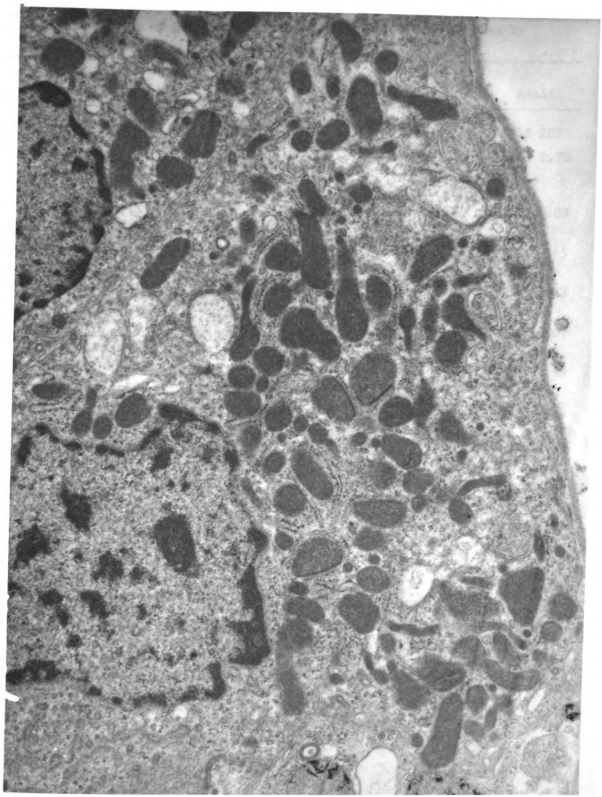

Figure 32

TABLE 9

Effect of Age on PAH Accumulation and Palmitate Metabolism by Separated Rabbit Proximal Tubules[a]

	Rabbit Age			
	2 Weeks	4 Weeks	6 Weeks	Adult
Body Wt (gm)	238 24	734 34	1285 68	2817 207
PAH T/M Ratio	5.94 1.46	16.37 1.17	18.71 1.72	14.16 2.70
Palmitate Incorporation				
CO_2 (μmole/g tubule)	0.27 0.03	0.40 0.09	0.42 0.09	0.19 0.09
TL (μmole/g tubule)	3.68 0.30	4.47 0.44	3.69 0.38	4.08 0.45
PL (μmole/g tubule)	0.66 0.08	0.71 0.07	0.63 0.06	0.46 0.12
CH (μmole/g tubule)	0.14 0.02	0.23 0.03	0.20 0.02	0.14 0.03
NEFA (μmole/g tubule)	0.63 0.08	0.68 0.12	0.36 0.05	1.02 0.39
TG (μmole/g tubule)	1.61 0.21	2.48 0.21	3.14 0.54	2.32 0.61
[NEFA]p (nmole/ml)	443 47	171 12	237 55	314 86
$[NEFA]_T$ (nmole/g tubule)	3977 224	5246 618	4303 806	4368 (n=2)

[a]Tubules were prepared from rabbits of the indicated ages and incubated in media containing either 7.4×10^{-5} M PAH or 1 mM palmitate-1-^{14}C (sp.ac. 0.4 μCi/μmole) + 10 mM acetate. Values represent the mean ± standard error of 6-9 separate determinations. Rabbits 2-6 weeks of age were drawn from 6 litters. Shown are the fractions of palmitate oxidized to carbon dioxide (CO_2) and incorporated into tissue lipids: Total lipids (TL), phospholipids (PL), cholesterol, monoglycerides and diglycerides (CH), Triglycerides (TG) and remaining as the nonesterified fatty acid (NEFA): [NEFA]p = concentration of nonesterified fatty acids in plasma. $[NEFA]_T$ = concentration of nonesterified fatty acids in Folch extracts of tubules incubated with 1 mM palmitate.

Plasma NEFA concentration displayed age-related changes (Table 9). Plasma from 2 week rabbits exhibited the highest NEFA concentration whereas plasma from 4 week rabbits contained the least NEFA.

2) Effect of Penicillin Pretreatment of 2-Week-Old Rabbits

Penicillin pretreatment significantly altered the distribution of incorporated palmitate-^{14}C within tubule lipid fractions (Figure 33, Table 10). Significantly less label was recovered as NEFA in tubules from penicillin pretreated rabbits than from paired controls. Concomitantly an increased amount of label was recovered as triglyceride esters (Figure 33, Table 10). Although penicillin pretreatment appeared to increase both palmitate oxidation to CO_2 and palmitate incorporation into TL, the differences observed were not statistically significant. Penicillin pretreatment significantly increased tubule PAH accumulation when compared to control (Figure 33, Table 10). Similar results were obtained in nine additional experiments (Figure 34). Palmitate-^{14}C incorporated into the NEFA and TG fractions of paired control and penicillin treated tubule suspensions varied widely. However, penicillin pretreatment significantly decreased the amount of palmitate present in the NEFA fraction (0.48±0.09 μmole/g tubule) when compared to control (0.76±0.07 μmole/g tubule). Palmitate-^{14}C recovered in the TG fraction was significantly greater in treated tubule suspensions (2.09±0.28 μmole/g tubule) than in control tubules (1.65±0.15 μmole/g tubule). Palmitate incorporation into total tubule lipid was not statistically different in penicillin treated (TL = 4.06±0.25 μmole/g tubule) and control (TL = 3.58±0.14 μmole/g tubule) tubule suspensions. In four of the five pairs (drawn from the 13 pairs described in Figure 34) of tubule suspensions

Figure 33. Effect of penicillin pretreatment on the ability of proximal tubule suspensions to accumulate PAH (T/M) and oxidize or esterify palmitic acid. Beginning on day 11, 90,000 I.U. of procaine penicillin G was administered twice daily for 2 days. Animals were sacrificed 24 hours after the final injection. Tubules were incubated in a medium containing 1 mM palmitate-^{14}C (0.4 μCi/μmole) + 10 mM acetate. Results were expressed as the mean difference ± standard error for the difference between functions measured in control and treated tubule suspensions. The functions measured were: CO_2 = carbon dioxide; TL = total lipid; PL = phospholipid; CH = cholesterol + diglyceride + monoglyceride; NEFA = nonesterified fatty acid; TG = triglyceride.

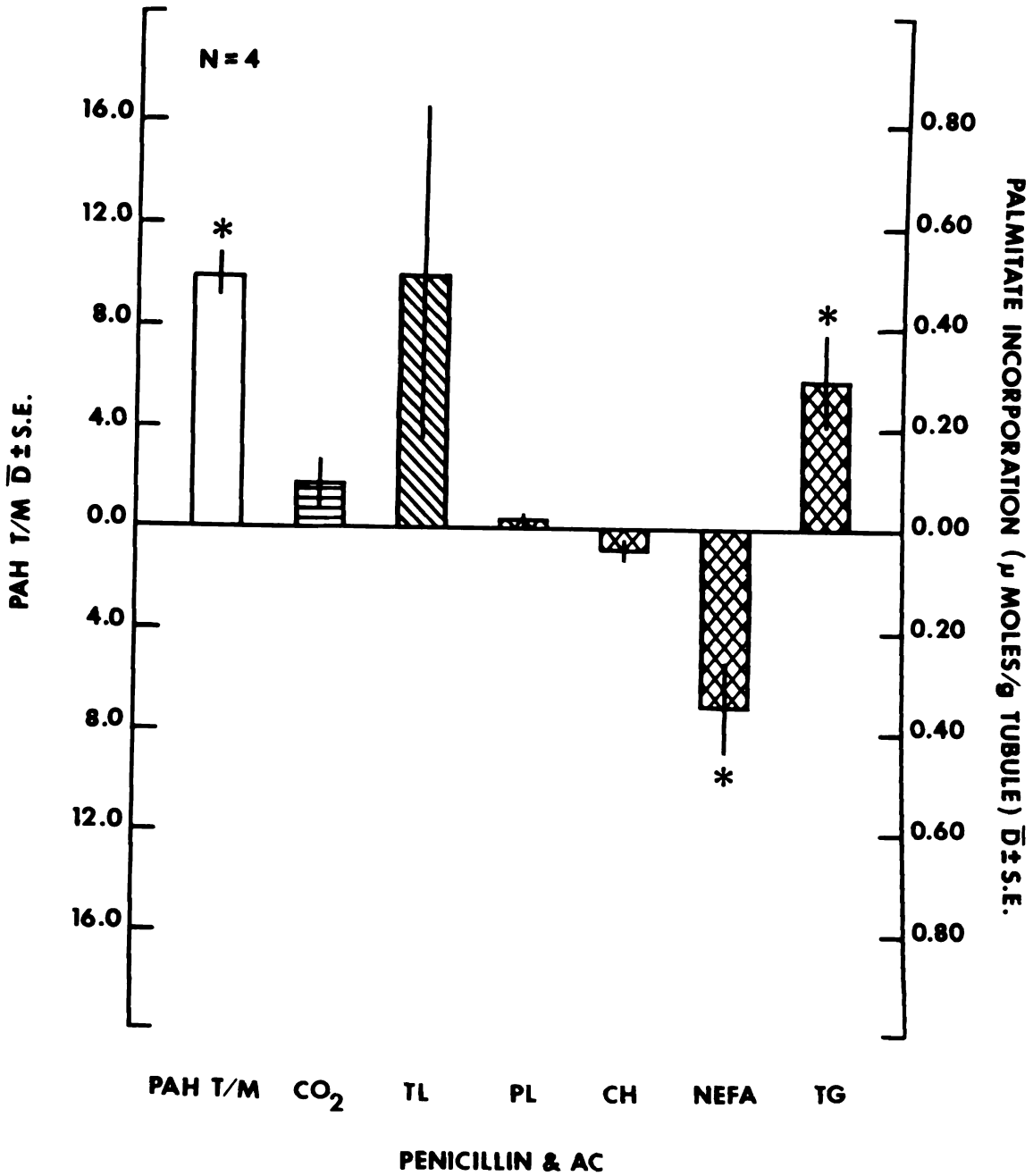
N = 4
16.0
12.0
8.0
4.0
0.0
4.0
8.0
12.0
16.0
PAH T/M D̄ ± S.E.
0.80
0.60
0.40
0.20
0.00
0.20
0.40
0.60
0.80
PALMITATE INCORPORATION (μ MOLES/g TUBULE) D̄ ± S.E.
PAH T/M
CO2
TL
PL
CH
NEFA
TG
PENICILLIN & AC

Figure 33

TABLE 10

Effect of Penicillin Pretreatment of 2-Week-Old Rabbits on PAH Accumulation and Palmitate Metabolism[a]

Treatment	PAH T/M	Palmitate Incorporation (mole/g tubule)					
		CO_2	TL	PL	CH	NEFA	TG
Control	5.00	0.29	4.24	0.74	0.15	0.75	1.63
Penicillin	14.86	0.38	4.73	0.74	0.11	0.40	1.93
$\bar{D}$	9.86[b]	0.09	0.50	0.01	-0.04	0.35[b]	0.30[b]
±	±	±	±	±	±	±	±
S.E.	1.06	0.05	0.33	0.02	0.02	0.09	0.09

[a]Four injections of 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Rabbits were sacrificed 24 hrs after the last injection. Paired tubule suspensions from control and penicillin treated animals were incubated in media containing 7.4×10^{-5}M PAH or 1 mM palmitate-1-^{14}C (sp.ac. 0.4 µCi/µmole) + 10 mM acetate. Values represent treatment means and mean difference ± standard error for 5 litters. See Table 9 for an exaplanation of the palmitate fractions.

[b]Significant difference, $p<0.05$.

Figure 34. Incorporation of palmitate-^{14}C into nonesterified fatty acid (NEFA) and triglyceride (TG) fractions from control and treated rabbit tubule suspensions. Beginning on day 11, 90,000 I.U. of procaine penicillin G was administered twice daily for 2 days. Animals were sacrificed 24 hours after the last injection. Tubules were incubated in a medium containing 1 mM palmitate-^{14}C (0.4 μCi/μmole) + 10 mM acetate. Individual data from 13 litters were plotted. Each pair of points represents the average value obtained for control and penicillin treated tubules within a litter.

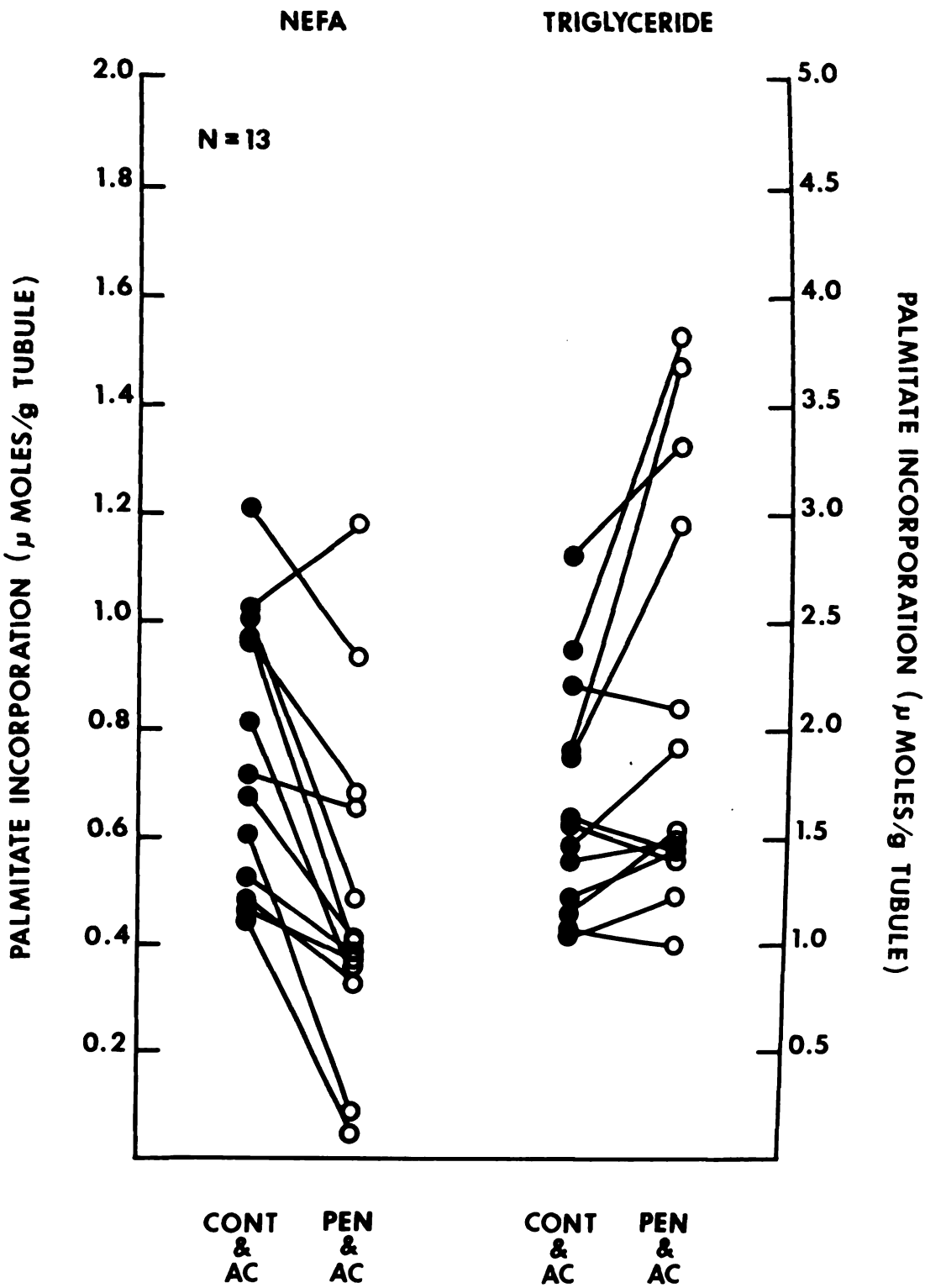
NEFA
TRIGLYCERIDE
N = 13
PALMITATE INCORPORATION (μ MOLES/g TUBULE)
PALMITATE INCORPORATION (μ MOLES/g TUBULE)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
CONT & AC
PEN & AC
CONT & AC
PEN & AC

Figure 34

examined, penicillin pretreatment decreased both, the tubule NEFA concentration and the amount of palmitate-^{14}C present as NEFA (Table 11). However, due to the large variability the observed differences were not statistically significant (Table 11). The specific activity of NEFA present in Folch extracts of control and penicillin treated tubules were not significantly different (Table 11). The penicillin pretreatment of neonatal rabbits had no effect on the plasma NEFA concentration (Table 12).

3) Effect of Iodipamide Pretreatment of 2-Week-Old Rabbits

Incorporation of palmitate-1-^{14}C into tubule lipid fractions was not altered by prior treatment of rabbits with iodipamide (Figure 35). Palmitate-^{14}C incorporation into NEFA fractions of control (0.92±0.09 μmole/g tubule) and iodipamide treated (0.89±0.18 μmole/g tubule) tubule suspension was not significantly different ($\bar{D}$±S.E. = -0.03±0.19 μmole/g tubule) (Figure 35). Iodipamide pretreatment did not significantly alter the amount of label recovered as TG from control (1.57±0.13 μmole/g tubule) or treated (1.87±0.36 μmole/g tubule) tubules ($\bar{D}$±S.E. = 0.30±0.27 μmole/g tubule). PAH accumulation by iodipamide pretreated tubules (T/M = 3.60±0.39) was not significantly different than control (T/M = 3.97±0.50) (Figure 35).

4) Effect of Penicillin Pretreatment of Adult Rabbits

Palmitate-^{14}C incorporation into lipid fractions of adult tubules was not altered by prior penicillin pretreatment (Figure 36). Recovery of label present as NEFA was not different in control (1.02±0.39 μmole/g tubule) or pretreated (1.41±0.42 μmole/g tubules) tubule suspensions ($\bar{D}$±S.E. = 0.39±0.38 μmole/g tubule) (Figure 36).

TABLE 11

Effect of Penicillin Pretreatment of 2-Week-Old Rabbits on the Concentration of Nonesterified Fatty Acids in Separated Proximal Tubules and Incorporation of Palmitate-^{14}C into Tubule Nonesterified Fatty Acids[a]

Treatment	[NEFA] (μmole/g tubule)	[NEFA-^{14}C] (μCi/g tubule)	Sp. Ac. (μCi/ mole)
Control	3.98	0.32	0.08
Penicillin	3.73	0.29	0.06
$\bar{D}$	-0.24	-0.09	-0.02
±	±	±	±
S.E.	0.18	0.05	0.01

[a]Four injections of 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Animals were sacrificed 24 hrs after the last injection. Paired, control and treated tubule suspensions were incubated in a medium containing 1 mM palmitate-1-^{14}C (sp.ac. 0.4 μCi/μmole) + 10 mM acetate. Values represent the means and mean difference ± standard error obtained from 5 litters.

Table 12

Effect of Penicillin Pretreatment of 2-Week-Old Rabbits on Plasma Concentration of Nonesterified Fatty Acids[a]

Treatment	(n)[b]	Plasma [NEFA] (nmole/ml)
Control	16	443 ± 47
Penicillin	19	449 ± 49

[a]Four injections of 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Animals were stunned 24 hrs after the last injection, immediately exsanguinated and a mixed arterial and venous blood sample collected in a heparinized tube. Assays were performed on frozen plasma samples.

[b](n) = number of animals.

Figure 35. Effect of iodipamide pretreatment on PAH accumulation (T/M) and palmitate oxidation and esterification by suspensions of renal proximal tubules. Iodipamide was administered twice daily for 3 days beginning on day 10 of age. Animals were sacrificed 24 hours after the last injection. Experiments were similar to those of Figure 33. Results were expressed as the mean difference ± standard error for the difference between functions measured in control and treated tubule suspensions.

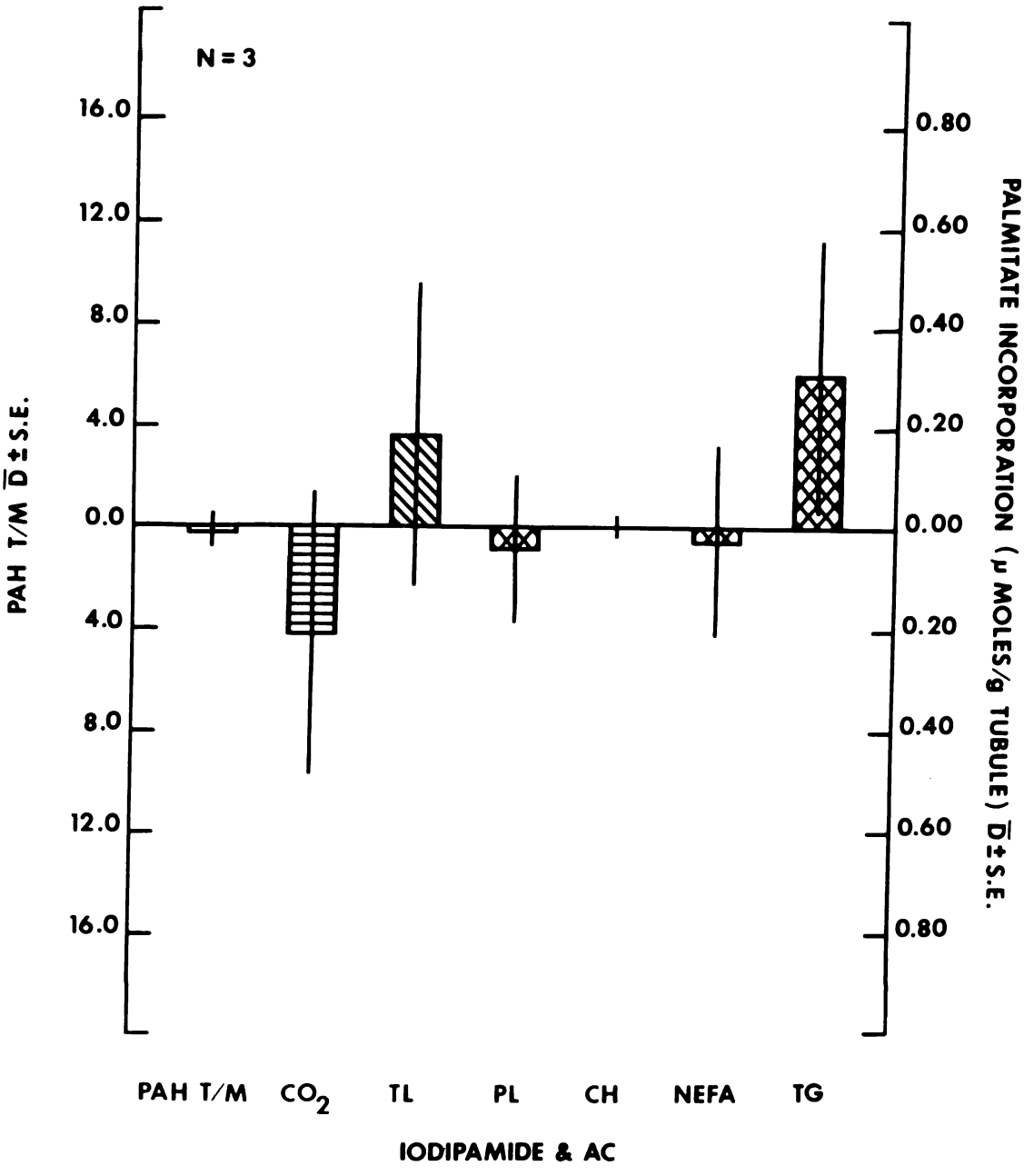
N = 3
16.0
12.0
8.0
4.0
0.0
4.0
8.0
12.0
16.0
PAH T/M D̄ ± S.E.
0.80
0.60
0.40
0.20
0.00
0.20
0.40
0.60
0.80
PALMITATE INCORPORATION (μ MOLES/g TUBULE) D̄ ± S.E.
PAH T/M
CO2
TL
PL
CH
NEFA
TG
IODIPAMIDE & AC

Figure 35

Figure 36. Effect of penicillin administration to adult rabbits on PAH accumulation (T/M) and palmitate oxidation and esterification by suspensions of renal proximal tubules. Pairs of adult, female, New Zealand white rabbits were used. Procaine penicillin G, 450,000 I.U./kg body wt. was administered to one rabbit twice daily for 2 days. Controls received isovolumnic amounts of 0.9% NaCl. Animals were sacrificed 24 hr after the last injection. Experiments were similar to those in Figure 33. Results were expressed as the mean difference ± standard error for the difference between functions measured in control and treated tubule suspensions.

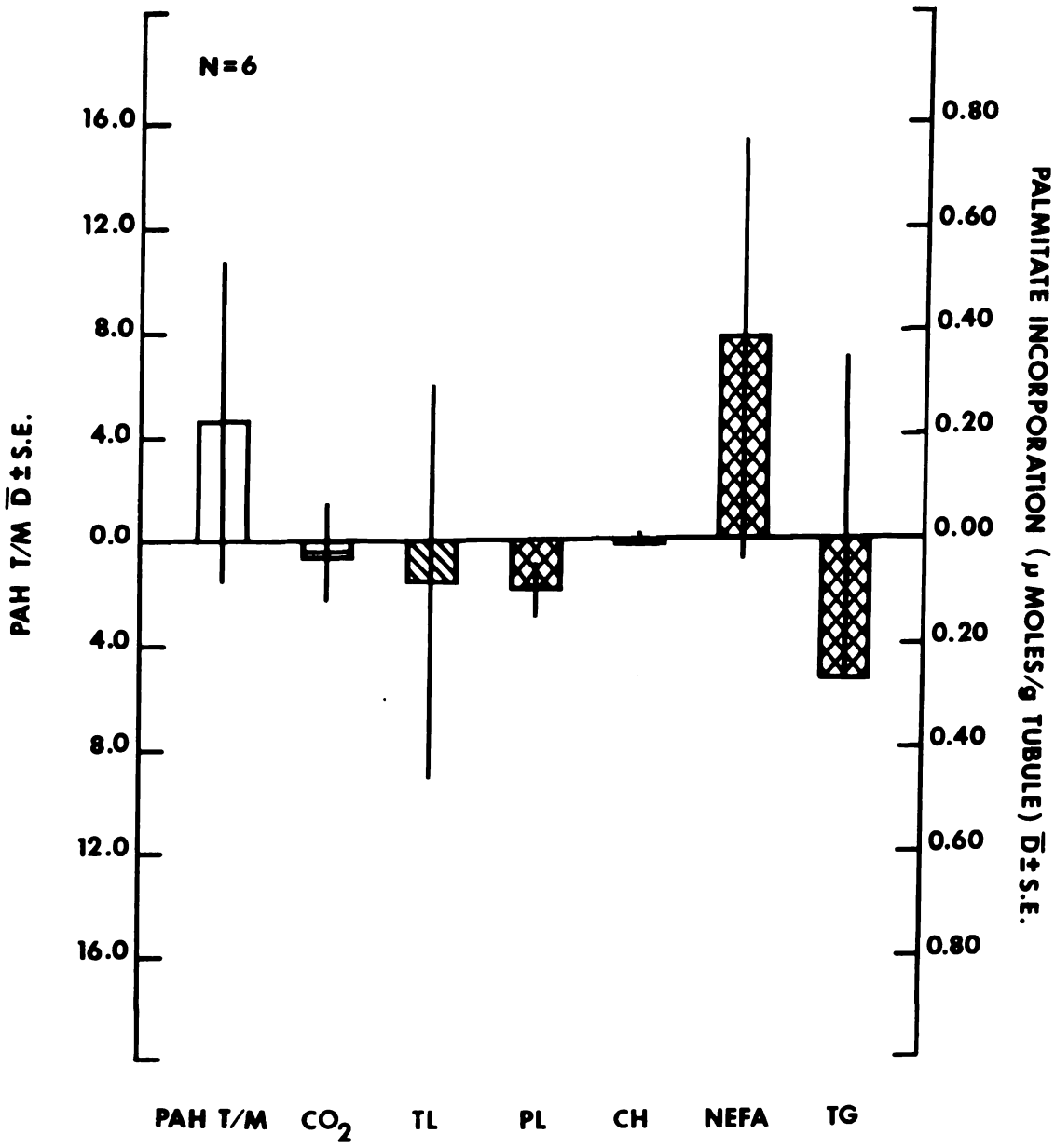
N=6
16.0
12.0
8.0
4.0
0.0
4.0
8.0
12.0
16.0
PAH T/M D̄ ± S.E.
0.80
0.60
0.40
0.20
0.00
0.20
0.40
0.60
0.80
PALMITATE INCORPORATION (μ MOLES/g TUBULE) D̄ ± S.E.
PAH T/M
CO2
TL
PL
CH
NEFA
TG
PENICILLIN & AC
ADULT

Figure 36

Similarly, palmitate-^{14}C incorporated into TG fractions from penicillin treated (2.05±0.54 μmole/g tubule) and control (2.32±0.61 μmole/g tubule) tubule suspension was not different ($\bar{D}$±S.E. = -0.26± 0.61 μmole/g tubule). Penicillin pretreatment of adult rabbits also did not alter tubule PAH accumulation (cont. T/M = 14.16±2.70; treat. T/M = 18.72±6.25) (Figure 36).

5) Effect of Penicillin Pretreatment of 2-Week-Old Rabbits Palmitate Metabolism in an Acetate-free Medium

Incubation of tubule suspensions from control and penicillin pretreated rabbits in an acetate free incubation medium produced a pattern of palmitate-^{14}C distribution (Figure 37,38) similar to that observed in previous studies (Figure 33,34). In each experiment label recovered as NEFA was less in tubules from treated rabbits than in paired controls (Figure 37,38). Similarly, palmitate-^{14}C recovered as TG was greater in treated tubule suspensions than impaired control tubule suspensions (Figure 37,38). However, due to variability, these differences were not statistically significant. Addition of acetate (10 mM) to the incubation medium appeared to increase palmitate-^{14}C incorporation into total lipid of control tubule suspensions by increasing label recoverable as NEFA and TG (Figure 39). The observed differences were not statistically significant (Figure 39). Acetate had no effect on incorporation of label into lipid fractions from penicillin treated tubules (Figure 40).

Figure 37. Effect of penicillin pretreatment on the ability of proximal tubule suspensions to esterify palmitate in an acetate free medium. Beginning on day 11, 90,000 I.U. of procaine penicillin G was administered twice daily for 2 days. Animals were sacrificed 24 hr after the last injection. Tubules were incubated in medium containing 1 mM palmitate-1-^{14}C (sp.ac. 0.4 μCi/μmole). Results were expressed as mean difference ± standard error of the difference between functions measured in control and treated tubule suspensions.

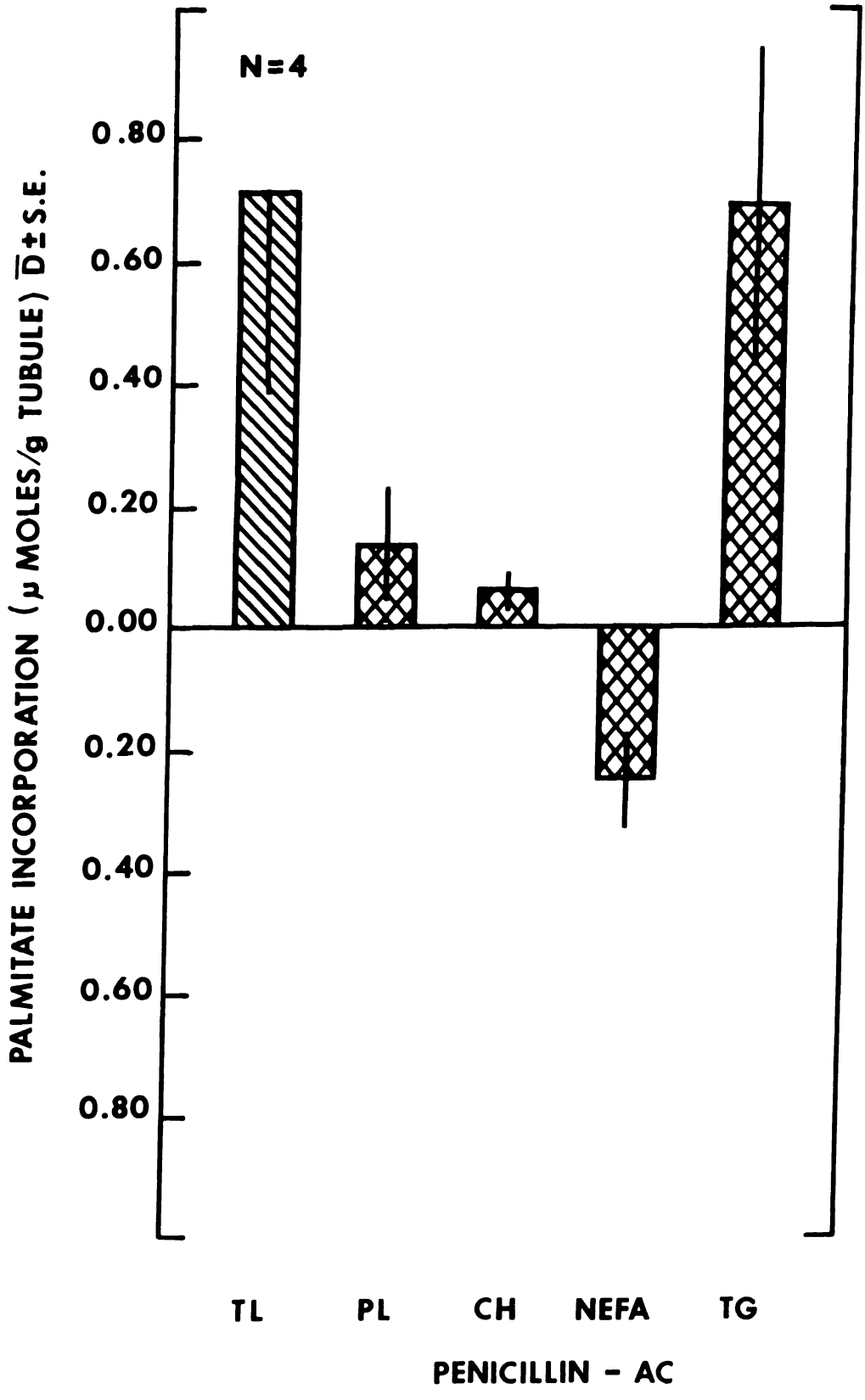
N=4
0.80
0.60
0.40
0.20
0.00
0.20
0.40
0.60
0.80
PALMITATE INCORPORATION (μ MOLES/g TUBULE) D̄ ± S.E.
TL
PL
CH
NEFA
TG
PENICILLIN - AC

Figure 37

Figure 38. Incorporation of palmitate-1-^{14}C into nonesterified fatty acid (NEFA) and triglyceride (TG) fractions from control and treated rabbit tubule suspensions. Procaine penicillin G was administered twice daily for 2 days, beginning on day 11 of age. Animals were sacrificed 24 hr after the last injection. Tubules were incubated in an acetate-free medium containing 1 mM palmitate-1-^{14}C (sp.ac. 0.4 μCi/μmole). Individual data points from 4 litters were plotted. Each pair of points represents the average value obtained for control and penicillin treated tubules within a litter.

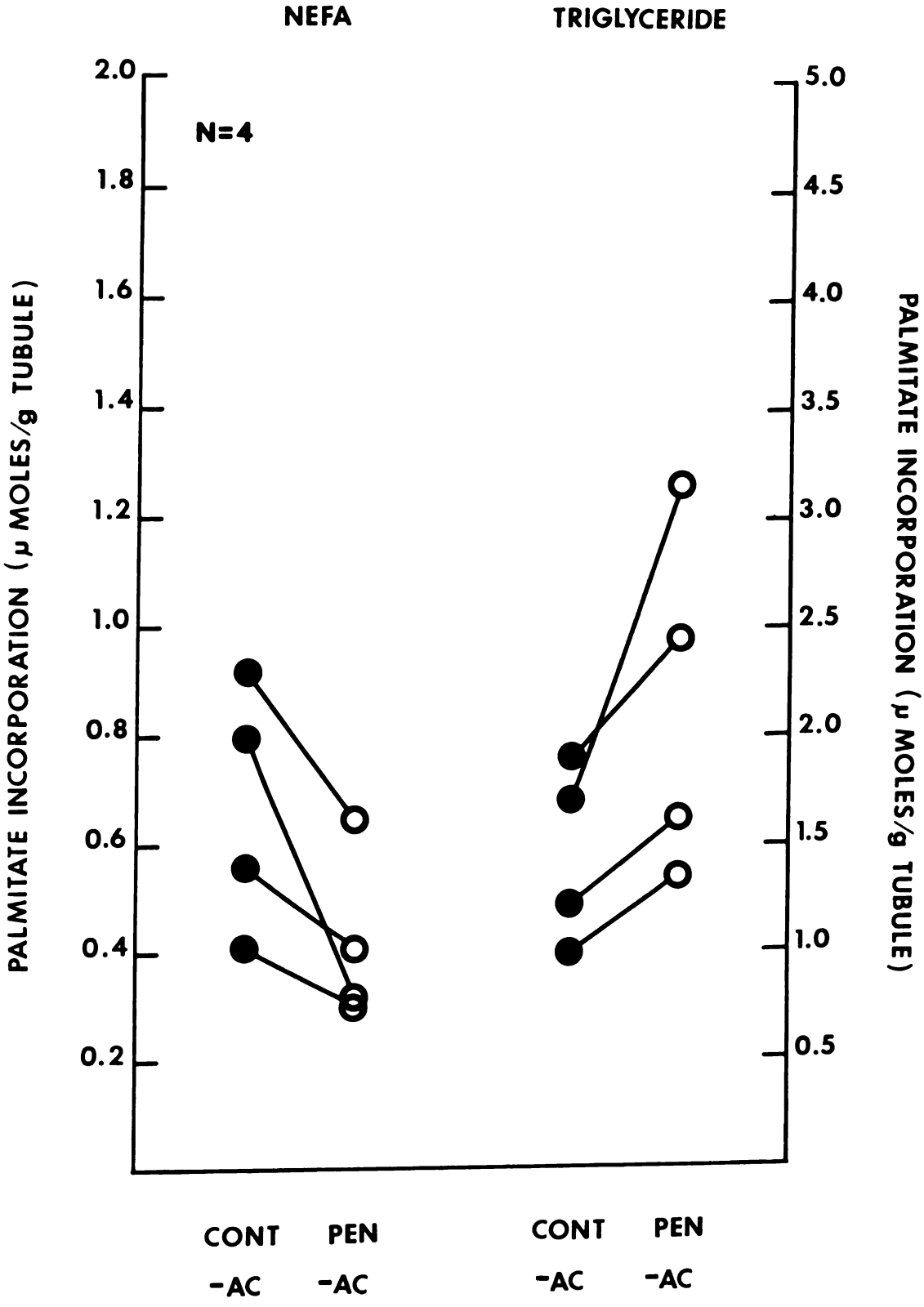
NEFA
TRIGLYCERIDE
N=4
PALMITATE INCORPORATION (μ MOLES/g TUBULE)
PALMITATE INCORPORATION (μ MOLES/g TUBULE)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
CONT -AC
PEN -AC
CONT -AC
PEN -AC

Figure 38

Figure 39. Effect of acetate on palmitate esterification by tubule suspensions prepared from young (2 week) rabbits. Rabbits were the saline-treated controls used in experiments described in Figure 37 and 38. One-half of the tubules prepared were incubated in medium containing 1 mM palmitate whereas the remaining tubules were incubated with 1 mM palmitate + 10 mM acetate. Results were expressed as the mean difference ± standard error of the difference between functions measured in tubules incubated with and without acetate.

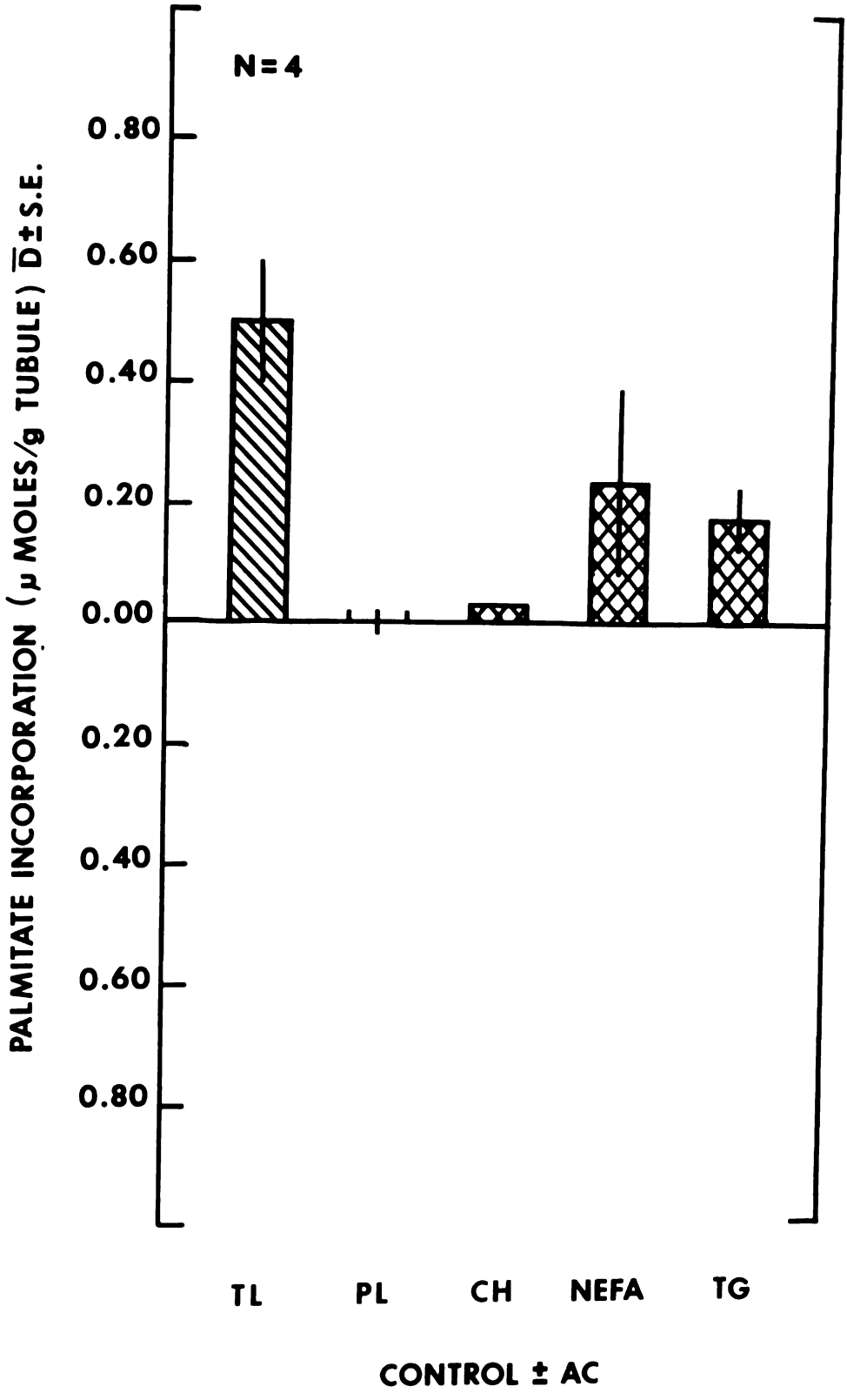
N=4
0.80
0.60
0.40
0.20
0.00
0.20
0.40
0.60
0.80
PALMITATE INCORPORATION (μ MOLES/g TUBULE) D̄ ± S.E.
TL
PL
CH
NEFA
TG
CONTROL ± AC

Figure 39

Figure 40. Effect of acetate on palmitate esterification by tubule suspensions prepared from neonatal (2 week) rabbits. Rabbits were the penicillin treated animals described in Figure 37 and 38. Experiments were handled in the same fashion as Figure 39. Results were expressed as mean difference ± standard error of the difference between functions measured in tubules incubated with and without acetate.

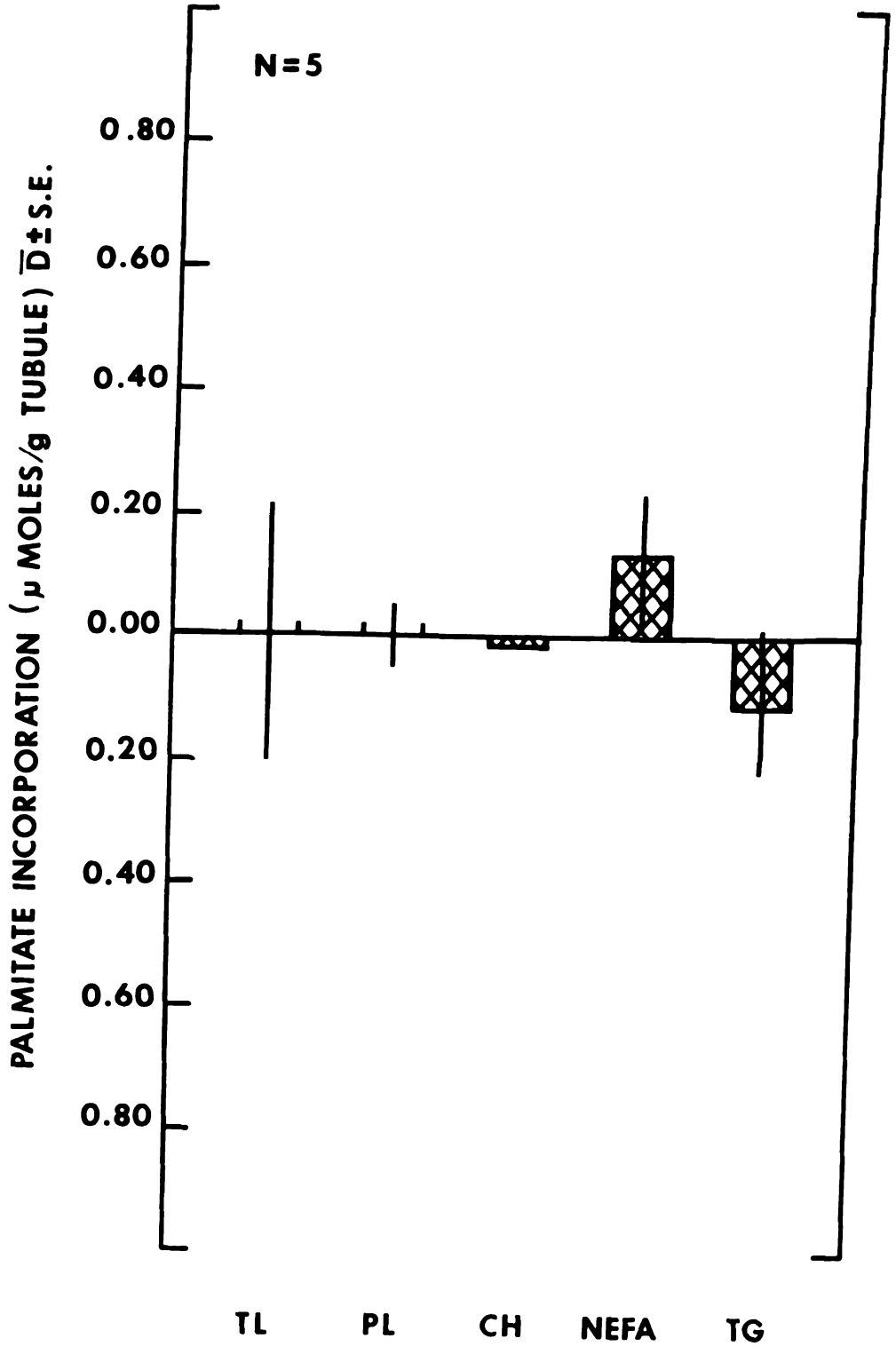
N=5
0.80
0.60
0.40
0.20
0.00
0.20
0.40
0.60
0.80
PALMITATE INCORPORATION (μ MOLES/g TUBULE) D̄ ± S.E.
TL
PL
CH
NEFA
TG
PENICILLIN ± AC

Figure 40

DISCUSSION

Analysis of renal proximal tubular transport capabilities in developing animals has been successfully used to demonstrate the existence of functionally separate transport systems for sugars and amino acids (Segal et al., 1971) and for organic anions and cations (Rennick et al., 1961; Hook, 1974). Determination of the patterns of development of amino acid reabsorption demonstrated multiple transport systems within a single class of transported solutes. For example, Segal and Smith (1969a; 1969b) used patterns of development to delineate differences between transport systems for cystine and lysine reabsorption in neonatal rat kidney cortex. In addition, Baerlocher et al. (1971a; 1971b) demonstrated that the separate high affinity transport systems for proline and glycine reabsorption were absent in newborn rats and developed independently, with proline transport appearing at one week of age and glycine transport capacity observed not until 3 weeks of age. These investigations suggested that analysis of the developemtnal patterns of renal organic anion secretion might aid in the discrimination of multiple organic anion transport systems.

Hirsch and Hook (1969; 1970) and Johnson et al. (1974) demonstrated that treatment of neonatal rabbits, rats and dogs with substrates of the organic acid transport system (e.g., PAH, penicillin) selectively enhanced maturation of slice PAH accumulation without altering accumulation of organic cations. Selective substrate

stimulation of the organic anion transport system following administration of exogenous anionic substrate loads has been subsequently confirmed in several in vitro and in vivo preparations (Ecker and Hook, 1974; Pegg and Hook, 1975; Bond et al., 1976; Lewy and Grosser, 1974). These investigations suggested that multiple anion transport systems might be defined by differential response to administration of exogenous substrates of each suspected transport system. Theoretically, pretreatment with a substrate from one transport system should enhance accumulation of that anion group only.

The literature contains a vast amount of experimental evidence supporting the dissimilarity of secretory transport systems for PAH and UA in man and lower animals. The delay in appearance of active accumulation of UA by renal cortical slices compared to PAH accumulation (Figure 1) coupled with the marked differences in developmental patterns for these anions supports the possibility of separate secretory systems for these anions in rabbit proximal tubules. Additional support for this concept can be derived from the failure of PEN (group I) and probenecid to alter slice accumulation of UA (group II). Similarly, the lack of effect of UA pretreatment on PAH accumulation (Figure 9) is in accordance with this hypothesis. However, other investigators have reached different conclusions. Based on inhibitor studies in rabbit tissue Møller (1967a,b) and Sheikh and Møller (1971) concluded that a common transport system for UA and PAH was involved in tubular secretion of these anions and that the affinity of UA for the system was less than the affinity of PAH. Nevertheless, sufficient differences in the characteristics of uptake of PAH and UA exist to suggest that the transport processes are not absolutely identical (Mudge et al., 1973). Optimal in vitro incubation conditions for UA

and PAH accumulation differed with respect to potassium concentration (Berndt and Beechwood, 1965), medium pH (Berndt and Beechwood, 1965; Copenhaver and Davis, 1965) and metabolic substrates (Platts and Mudge, 1961). Berndt (1965) also demonstrated that efflux of UA from preloaded rabbit cortex slices occurred through a process substantially different from the process mediating PAH efflux.

Different affinities for a single common transport system could not be responsible for differences in optimal incubation medium conditions for maximal UA or PAH accumulation or for the differences in developmental accumulation patterns or response to substrate stimulation. Addition of acetate to the incubation medium (Kim and Hook, 1972), normal maturation of renal cortical slice anion accumulation (Ecker and Hook, 1974) and penicillin stimulation of immature tubule anion accumulation (Pegg et al., 1976a) are all processes which alter the maximum velocity of transport rather than affinity of the carrier system for anionic substrates. Thus, accumulation of UA and PAH should be affected in a similar fashion by these treatments if only a single transport system exists. The differences in response of slice UA and PAH accumulation might therefore be better explained by postulating the existence of two separate, but overlapping, transport systems for PAH and UA in rabbit renal proximal tubules as has been done for kidneys of other species.

An alternative explanation for the above observations (Figures 1 and 4) is that cortical slice UA accumulation resulted from the reabsorptive transport of UA (Berndt and Beechwood, 1965). However, Shiekh and Møller (1971) demonstrated that at high medium UA concentrations (greater than 0.1 mM UA) UA was accumulated intracellularly

as a consequence of the secretory process. Therefore, under the experimental conditions used in these investigations (0.5 mM) development of UA accumulation should reflect maturation of the UA secretory system.

Bárány (1972; 1973a; 1973b; 1974a,b; 1975) described a similar group of separate, but overlapping, transport systems to explain the effects of various anions on the slice accumulation of iodipamide. One system (H-system), sensitive to iodohippurate inhibition, was assumed to be the classical hippurate transport system (Bárány, 1972). The second, hippurate insensitive system (L-system), was inhibited by anions normally excreted by the liver (Bárány, 1973a,b). Thus, bile acids (e.g., chenodeoxycholate), cholographic agents and flavaspidic acid produced marked inhibition of iodipamide accumulation by the L-system (Bárány, 1973b; 1974b; 1975). Benzylpenicillin and PAH had little effect on iodipamide accumulation by the L-system. In addition, pyrazinoic acid, m-hydroxybenzoic acid and salicylic acid also had little effect on iodipamide accumulation via the L-system, suggesting that the L-system was not involved in UA accumulation in rabbit kidney cortex (Bárány, 1973b). The independent pattern of maturation for CDC (group III) accumulation as compared to PAH or UA (Figures 1, 2) supports this hypothesis. Selective enhancement of PAH accumulation without alteration of CDC accumulation after PEN pretreatment (Figure 7) was consistent with separate H and L transport systems. This position is strengthened by the lack of stimulation of PAH by iodipamide (Figure 11).

Several aspects of CDC accumulation by renal cortical slices evident during the course of these investigations deserve comment. Bárány (1975) observed considerable nonactive slice accumulation of CDC at 0° or following incubation under 100% nitrogen. Similar results were obtained in the present studies (Figure 7). However, the nonactive portion of CDC accumulation did not appear to participate in the age-related changes of L-system transport capacity since no differences in CDC S/M ratios were observed at 0°. As expected, penicillin administration to neonatal rabbits had no effect on the nonactive component of CDC accumulation (Figure 7). Finally, the marked large CDC S/M ratios obtained were apparently not caused by sequestration of CDC, preventing egress from the tissues, since runout of CDC from preloaded adult rabbit slices was not different than runout of PAH (unpublished results). Similarly, Berndt et al. (1968) demonstrated that reduced efflux was not responsible for the large tissue-to-medium iodipamide gradients generated in rabbit cortical slices.

In general, accumulation of the remaining anions utilized in this investigation paralleled the responses of PAH, UA and CDC and were consistent with the basic hypothesis. That is, Group I anion accumulation (PSP, Figure 4; PEN, Figure 5) was enhanced by prior administration of Group I substrates but was not altered by group II (UA, Figure 9, SULFI, Figure 8) substrate pretreatment. Similarly PEN administration did not enhance ASA (group II) accumulation (Figure 5).

Three exceptions to the general response pattern were noted during these investigations. Penicillin pretreatment significantly increased slice accumulation of sulfisoxazole (group II, Figure 6). Enhanced slice accumulation of SULFI was probably related to a small increase in the fraction of SULFI accumulated by the PEN activated group I transport system. Bárány (1973b,1974a) observed considerable overlap between the H- and L- transport systems (as much as 20% of iodipamide accumulation occurred via the hippurate system).

Stimulation of PAH transport capacity subsequent to ASA (Figure 10) and high dose (200 mg/kg) PABA (Figure 12) administration were the second and third apparent inconsistencies. A major route of PABA biotransformation in rabbits resulted in formation of PAH (Wan et al., 1972). Hirsch and Hook (1969) demonstrated that administration of PAH to neonatal rabbits stimulated slice PAH accumulation. Thus, the substrate stimulation observed at high PABA doses most likely resulted from a secondary response to an increased PAH load.

The frank toxicity exhibited by ASA suggested a possible mechanism for enhanced PAH accumulation following ASA treatment. ASA has been found to be nephrotoxic in several species (Abel, 1971) and this toxic effect was greater in newborn rats (Yeary et al., 1966). Several nephrotoxic agents including gentamicin (Cohen et al., 1975), neomycin (Lapkin et al., 1975), and potassium dichromate (Hirsch, 1973) increased in vitro PAH accumulation either specifically or secondarily following renal compensation to incurred damage. Possibly ASA induced alterations in PAH accumulation occurred as part of a toxic response rather than by substrate stimulation. Alternatively, biotransformation of ASA to anionic metabolites transported by the

group I system could result in substrate stimulation in a fashion similar to PABA. Following administration, ASA was rapidly hydrolyzed to salicylate (Mandel, 1971) which has been shown to be actively accumulated by rat renal cortical slices (Putney and Borzelleca, 1973). Interestingly, the glycine and glucuronic acid metabolites of salicylate (Hollister and Levy, 1965) were also observed to be actively transported by a mechanism qualitatively similar to that for PAH (Despopoulos, 1960,1965). The rapid hydrolysis of ASA by plasma and tissue esterases also suggested that slice ASA accumulation and the effects of ASA pretreatment reflect, at least in part, active salicylate transport by renal cortex.

Enhancement of transport system maturation as a response to exogenous substrate loads was apparently a function of the group I system alone. Administration of urate (Figure 9) or iodipamide (Figure 11) did not alter the rate of maturation of their respective transport systems. This lack of response could be attributed to inadequate (or excessive) drug administration, resulting in an insufficient stimulus for induction of extra transport capacity (or in competitive suppression of slice anion accumulation). Alternatively, duration of drug administration may have been inappropriate for optimal enhancement of maturation.

However, the group II and group III transport systems may be inherently resistant to potential inducing stimuli such as exogenous substrate loads. This appears to be the case for renal organic cation secretion. Challenge of neonatal rats and rabbits (unpublished results) and adult rats (Pegg et al., 1976b) with large doses of cationic substrates had no effect on intrinsic slice capability to

accumulate actively secreted organic cations. Similar observations have been obtained during investigations on induction of hepatic xenobiotic biotransformation pathways (Conney, 1967). Thus, while repeated administration of drugs such as diphenylhydantoin, phenylbutazone, tolbutamide and aminopyrine were capable of inducing enzymes that catalyzed their own biotransformation, repeated administration of aspirin, trimethadione and sulfanilamide had no subsequent effect on their metabolic fate within the body (Conney, 1967). These results suggest that while further evaluation of the group II and III transport systems is required with regard to induction potential and member substrates, the highly selective response of group I anion accumulation to substrate stimulation provides an exquisitely sensitive tool for determination of physiologic substrates, functions and biochemical correlates of transport.

The graphical representation (Figure 17) of the rate of oxygen-dependent slice PAH uptake (Figure 16) was qualitatively consistent with the existence of two active modes of PAH transport. However, the validity of using Michaelis-Menten kinetics to describe PAH accumulation in this slice system must be considered (Weiner, 1973; Christensen, 1960). The kinetic analysis of PAH transport was performed on data obtained in experiments designed to achieve a maximum rate of active uptake while minimizing the passive diffusion component of PAH uptake. Ross and Weiner (1972) demonstrated that freshly prepared renal cortical slices exhibit decreased adenylate energy charges, probably due to hypoxia during preparation. Aerobic incubation for 30 minutes prior to estimating uptake rate allowed the slices to regain normal adenylate energy charges and presumably normal levels of tissue

constiuents necessary for maximal rates of transport. The kinetic analysis of slice PAH accumulation is complicated by the presence of nonsecretory tissue in slices and the PAH content of interstitial space. Therefore, PAH accumulation under nitrogen was determined to partially correct for diffusional processes.

Since the observed K_m and V_{max} of the low capacity PAH uptake mode were within the range of values found by other investigators for PAH secretion (Gerencser et al., 1973; Park et al., 1971), this portion of the curve may represent the group I transport system described above. Bárány (1972,1973b) found little competitive interaction between PAH and the L-transport system (Hippurate-insensitive system) even at medium PAH concentrations greater than those used in this study (Figure 16). Thus, PAH transport by the L-system would be negligible at best. Therefore the high capacity mode of PAH uptake (Figure 17) likely represents PAH transport by the overlapping group II substrate system.

Initial interest in the biochemical mechanisms mediating renal extraction of nonesterified fatty acids (NEFA) was derived from investigations (Lee et al., 1962; Nieth and Schollmeyer, 1966; Barac-Nieto and Cohen, 1968; Weidemann and Krebs, 1969) which suggested that NEFA were the major substrate whose oxidation supported most renal work functions. Although subsequent studies (Barac-Nieto and Cohen, 1971; Park et al., 1973; Bertermann et al., 1975) have demonstrated that oxidation of palmitate to supply energy was not the major fate of palmitate in the renal cortex, net renal NEFA uptake was directly proportional to renal tubular sodium reabsorption (Barac-Nieto and Cohen, 1968). Therefore the nonoxidative fates of NEFA have been

suggested to play a significant role in support of renal sodium reabsorption (Cohen, 1975).

The organic acid secretory system has been suggested to participate either in the entry of NEFA into proximal tubule cells or in the intracellular binding of fatty acids prior to activation and dissimilation (Cohen and Kamm, 1976). Support for this hypothesis has been derived primarily from analyses of NEFA uptake and metabolism by kidney preparations in the presence of inhibitors of organic acid transport (e.g., probenecid, PSP, chlorothiazide, furosemide) (Barac-Nieto and Cohen, 1968; Dies et al., 1970; Pakarinen, 1970; Heinemann et al., 1975). However, interpretation of these experiments has been complicated by actions of these compounds unrelated to their specific inhibitory action on renal PAH transport. In contrast, administration of penicillin to immature rabbits specifically enhanced the PAH transport capacity of the kidney (Hirsch and Hook, 1970a,b; Ecker and Hook, 1974b; Pegg and Hook, 1975b; 1977; Pegg et al., 1976). Therefore, an alteration in proximal tubule palmitate accumulation or metabolism following induction of PAH transport capacity via substrate stimulation would be expected if palmitate interacts with (i.e., is a substrate of) the PAH transport system.

The experiments were designed to maximize the possibility of detecting a penicillin induced alteration in palmitate utilization. Brendel and Meezan (1975) demonstrated that proximal tubules isolated nonenzymatically were morphologically intact and had intact basement membranes in contrast to tubules isolated with the use of collagenase treatment. This preparation of tubules equalled or surpassed those isolated with the use of collagenase in metabolic functions such as

oxidative metabolism and gluconeongenesis and in transport functions such as PAH and 2-deoxyglucose uptake (Brendel and Meezan, 1975). Electron micrographs of randomly selected proximal tubule suspensions (Figures 29-32) confirmed the observations of Brendel and Meezan (1975). That is, tubules prepared by this procedure had intact brush border and basement membranes. Therefore, suspensions of proximal tubules prepared by the method of Brendel and Meezan were used in this series of experiments. Weidemann and Krebs (1969) demonstrated that oleate utilization by rat kidney cortex slices incubated in a phosphate buffered medium occurred at a high rate only at a fatty acid-to-albumin molar ratio of 7. Even at such a high oleate-to-albumin molar ratio, endogenous lipids still provided half the fuel for oxidation (Weidemann and Krebs, 1969). Similarly, Barac-Nieto (1976) found that both the rates of esterification and oxidation of palmitate by kidney slices were limited when a low fatty acid-to-albumin molar ratio (palmitate]/[BSA] = 1.25) was used. Both the esterification and oxidation rate were increased when the molar ratio in the medium was increased (Barac-Neito, 1976). Therefore, in order to insure a high rate of palmitate-1-^{14}C utilization a palmitate-to-albumin ratio of approximately 6 was used in these experiments. Since this ratio was much higher than those existing in vivo, extrapolation of these results to the in vivo situation should be made cautiously. These experiments were designed to explore only potential capabilities of the metabolic systems so that an interaction between renal palmitate utilization and PAH transport might be further defined (Barac-Nieto, 1976).

Proximal tubular palmitate esterification to triglyceride was significantly enhanced when tubule PAH transport capacity was increased by penicillin (Figures 33, 34, Table 10). This observation was consistent with the hypothesis that the metabolic fate of palmitate within the kidney was linked to transport by (or at least, interaction with) the PAH secretory system. The inability of iodipamide pretreatment to alter either tubule PAH accumulation or palmitate metabolism (Figure 35) strengthened this position. Similarly, since penicillin pretreatment does not alter organic anion secretion in mature rabbits (Hirsch and Hook, 1969; Pegg and Hook, 1975b) no effect on tubular capability to accumulate PAH and metabolize palmitate was expected, or observed (Figure 36). When data from inhibitor studies were examined (Heinemann et al., 1975) an analagous response was observed. That is, esterification of palmitate-1-^{14}C to triglycerides was inhibited under conditions that would be expected to completely inhibit PAH accumulation (Heinemann et al., 1975). Thus, inhibitors of PAH accumulation increased palmitate-^{14}C recovered as NEFA and decreased radiolabel present as triglyceride relative to the amounts present in uninhibited slices (Heinemann et al., 1975). Recently Wagner and Heinemann (1977) demonstrated that increasing medium potassium (and thereby tissue potassium) concentration promoted the accumulation and subsequent esterification and oxidation of palmitic acid. When medium potassium concentrations was increased from 6 mM K^+ to 50 mM K^+, esterification of palmitate-^{14}C to triglyceride was significantly increased (Wagner and Heinemann, 1977). Interestingly, the accumulation of PAH by renal cortical slices is modulated by medium potassium concentration (Cross and Taggart, 1950; Taggart et

al., 1953). Cross and Taggart (1950) demonstrated that the optimal medium potassium concentration for maximal PAH accumulation was 40 mM K^+, or similar to the 50 mM K^+ at which Wagner and Heinemann (1977) observed the maximal rate of palmitate esterification to triglyceride. These results are completely consistent with the hypothesis that palmitate interacts with the PAH transport system. The increased rate of palmitate esterification to triglyceride (Table 9) observed in tubules from 4 weeks as compared to 2 week old rabbits corresponded with the marked increase in PAH transport capacity between 2 and 4 weeks of age (Table 9).

The mechanism by which substrate stimulation with penicillin enhanced palmitate esterification remains obscure. Several investigators have demonstrated that addition of metabolic substrate such as glucose, lactate and glutamine to incubation medium could alter both the rate and eventual disposition of palmitate by kidney cortex slices (Weidemann and Krebs, 1969; Chorvathova et al., 1975; Barac-Nieto, 1976). Since acetate was routinely added to the palmitate medium in these experiments, penicillin treatment might have altered acetate metabolism by renal tubules. Secondary products of acetate metabolism could have then altered palmitate metabolism nonspecifically. However, the same pattern of alterations in palmitate esterification was produced when tubules from penicillin treated and control rabbits were incubated in an acetate-free medium (Figures 37, 38). Therefore, penicillin did not appear to alter palmitate esterification in this fashion. Since substrate stimulation with penicillin had no effect on body weight (Hirsch and Hook, 1969) and did not change plasma NEFA concentration (Table 12) alterations in diet or an extrarenal effect(s)

of penicillin (e.g., increased lipolysis of adipose tissue triglyceride) were not indicated. Penicillin stimulation of PAH transport capacity in neonatal rabbits has been shown to be dependent on an intact protein synthetic mechanism (Hirsch and Hook, 1970c; Pegg and Hook, 1975b). Hirsch and Hook (1970c) also demonstrated that penicillin pretreatment significantly increased the protein content of the 100,000 x g pellet from renal cortical homogenates of nursing rats. In addition, penicillin treatment significantly increased the incorporation of ^{14}C-amino acid into the acid-insoluble fraction of similar homogenates (Hirsch and Hook, 1970c). These observations coupled with the effect of penicillin on palmitate esterification to triglyceride suggest that penicillin might increase the synthesis of one or more enzymes involved in triglyceride formation (e.g., acyl-CoA: glycerophosphate acyl-transferase, acyl-CoA: 1-acylglycerophosphate acyltransferase; phosphatidate phosphohydrolase) (Hubscher, 1970; Kupke, 1972; Bremer et al., 1976). Alternatively, substrate stimulation with penicillin might increase the activity of existing enzyme(s) by stimulating synthesis of a peptide activator (Gelehrter, 1976).

Palmitic acid has been shown to competitively inhibit PAH uptake by rat renal cortical slices (Barac-Nieto, 1971). Penicillin pretreatment of immature rabbits increased PAH transport capacity of proximal tubules, enhanced esterification of palmitate-^{14}C to triglyceride and appeared to reduce the intracellular concentration of nonesterified fatty acids in proximal tubules (Table 11). These observations could indicate that the stimulation of PAH transport capacity by substrate stimulation resulted from an increased transfer of an inhibitor (NEFA) from an intracellular binding site shared with

PAH to a storage depot (TG) with which PAH did not interact. Barac-Nieto (1971) postulated a similar mechanism, that is, removal of NEFA from a common intracellular binding site, to explain the stimulation of slice PAH accumulation in the presence of exogenous carnitine. These results might suggest that agents or treatment regimens which result in a reduction of the intracellular concentration of NEFA, regardless of the mechanism, would enhance PAH transport capacity. The lack of effect of acetate on palmitate esterification and recovery of label associated with NEFA (Figures 39, 40) was consistent with the proposal (Hirsch and Hook, 1970b; Pegg and Hook, 1975b) that acetate and substrate stimulation enhanced PAH transport capacity by separate mechanisms.

Addition of sodium acetate to the incubation medium increased final medium pH (Figure 18). Several investigators (Copenhaver and Davis, 1965; Bowman et al., 1973) demonstrated that maximal PAH accumulation by rabbit renal cortical slices occurred at pH 8. However, Mudge and Taggart (1950) concluded that enhanced PAH transport could not be explained solely by the alkalinizing effect of acetate. A similar conclusion was obtained when the effect of various TCA cycle intermediates on PAH accumulation was examined (Figures 22,24,25). Low concentrations of citrate, α-ketoglutarate and succinate significantly increased PAH accumulation without appreciably altering the final medium pH (Figures 22,24,25). This marked dissociation between medium pH and PAH accumulation suggests that these intermediates do not stimulate PAH accumulation merely by alkalinizing the incubation medium. The parallel changes in medium pH and PAH accumulation with acetate (Figures 14,18,19) suggested that stimulation of PAH accumulation

resulted from the metabolism of acetate rather than from a direct effect of acetate on the transport system. Maxild (1973) and Gerencser et al. (1977) demonstrated that acetate stimulation of PAH accumulation did not occur by increasing renal cortical tissue ATP but was nevertheless, dependent on oxidative energy metabolism. Cohen and Randall (1964) postulated that renal metabolic changes could affect PAH transport if specific substances were to accumulate or disappear from within the kidney. Therefore, it would appear reasonable to suspect that acetate stimulated PAH accumulation by formation of a positive modulator of anion transport.

Dixit and Stern (1974) demonstrated that formation of citrate was a likely intermediate step in renal metabolism of acetate both in vivo and in vitro. Preparation and incubation of rabbit renal cortical slices significantly reduced tissue citrate concentration (Figure 20). Acetate (10 mM) significantly elevated both citrate concentration and the ability of rabbit slices to accumulate PAH (Figure 20). Although Cross and Taggart (1950) demonstrated that 0.01 M citrate slightly depressed PAH accumulation, a more physiological concentration of citrate (Cohen and Barac-Nieto, 1973) significantly increased PAH S/M ratios (Figure 21). A dose-response curve (Figure 22) described a biphasic relationship between PAH accumulation and medium citrate concentration. Low concentrations of citrate in the medium stimulated while higher concentrations depressed PAH accumulation.

The changes in slice citrate concentration and PAH accumulation produced by acetate coupled with the stimulating effect of physiological concentrations of citrate on PAH accumulation suggested that

transport of organic anions is regulated by the renal cortical concentration of citrate. This hypothesis was supported by the observation that the PAH transport capacity of a given species was correlated with the endogenous renal cortical citrate contraction (Table 2). Similar correlations between acetate stimulation of slice PAH accumulation and citrate concentration (Table 2) coupled with enhanced PAH accumulation in the presence of added citrate (Table 3) strengthened the hypothesis.

Alterations in organic anion transport (Weiss and Preuss, 1970) and renal citrate metabolism (Simpson, 1967; Simpson and Sherrard, 1969; Simpson and Angielski, 1973; Pahsley and Cohen, 1973) in various acid-base states also support the hypothesis that citrate could act as an intracellular modulator of PAH transport.

Maintenance of systemic acid-base homeostasis is one of the major regulatory functions of the kidney. Several excretory and nonexcretory (i.e., metabolic) approaches are utilized to fulfill this function. For example, the enhanced excretion of ammonium (NH_4^+) ions following increased production of ammonia (NH_3) by deamination and deamination of glutamine in renal cortical mitochondria is an example of a major excretory mechanism operating in systemic acidosis. The product of renal glutamine metabolism is α-KG, a low pKa dicarboxylic acid. If each mole of α-KG produced from glutamine were released into the renal venous blood it would neutralize 2 moles of bicarbonate (HCO_3^-), thus reducing the efficiency of renal HCO_3^- regeneration (Cohen and Kamm, 1976). However, renal cortical metabolism undergoes several alterations in order to eliminate this problem. Renal cortical

tissue exhibits a markedly increased rate of gluconeogenesis and CO_2 production from α-KG and other organic acids (e.g., citrate, lactate) in response to the reduction in intracellular fluid pH (ICF pH) and/or bicarbonate concentration (ICF $[HCO_3^-]$) produced by metabolic acidosis, respiratory acidosis or K^+-depletion (Cohen and Kamm, 1976). The enhanced rate of conversion of these organic acids to neutral products (glucose, CO_2) during acidosis increases the efficiency of renal NH_3 production and HCO_3^- regeneration and thus acts as a major nonexcretory (metabolic) mechanism in the kidney for pH regulation (Cohen and Kamm, 1976). However, these metabolic adaptations also offer an explanation for the reduced PAH transport capacity observed in chronically acidotic or K^+-depleted rats. Both metabolic acidosis (Hems et al., 1971; Adler et al., 1971; Relman, 1972; Cohen and Kamm, 1976) and K^+-depletion (Adler et al., 1974; Cohen and Kamm, 1976) significantly depressed ICF pH and/or ICF$[HCO_3^-]$ and significantly reduced renal citrate concentration. The concentration of all other TCA cycle intermediates was also reduced. The experiments described above (Table 5) confirmed the relationship between reduced cortical citrate concentration and reduced PAH transport capacity in chronic metabolic acidosis and provide additional evidence that citrate could act as an intracellular modulator of PAH accumulation. The inability of acetate to stimulate PAH accumulation in slices from acidotic rats was particularly intriguing (Table 5). Metabolic acidosis reduced renal cortical oxaloacetate concentration below the limit of detection (Relman, 1972). Since oxaloacetate is necessary for citrate synthesis, addition of exogenous acetate would not be expected to elevate citrate concentration in slices from acidotic rats and therefore would not be

expected to elevate slice PAH accumulation. Conversely, acetate did elevate both citrate concentration and PAH accumulation in slices from normal rats (Table 2). Thus, it would appear that either sufficient endogenous oxaloacetate was present in kidney cortex to permit citrate synthesis from exogenous acetate or that endogenous precursors supplied the requisite oxaloacetate. Interestingly, PAH accumulation by slices from acidotic rats was significantly stimulated by addition of citrate or lactate (Hewitt and Hook, unpublished results). Since lactate increased both slice citrate concentration and PAH accumulation to a greater extent than acetate (Table 4), the slice oxaloacetate concentration may limit acetate-induced stimulation of PAH accumulation. However, Cross and Taggart (1950) suggested that acetate rather than oxaloacetate was one of the rate limiting components of the PAH transport system since oxaloacetate had only 25% of the stimulatory ability of an equimolar concentration of acetate.

In contrast to acidosis, the kidney conserves acidic substrates during metabolic alkalosis (Cohen and Kamm, 1976). Gluconeongensis from organic acids was depressed whereas conversion of glucose and glycerol to lactic acid was enhanced. The renal utilization of citrate appeared to be particularly sensitive to alterations in ICF[HCO_3^-] whether produced *in vivo* or *in vitro* (Simpson, 1967). For example, citrate oxidation and utilization by renal cortical slices and/or mitochondria was decreased when medium [HCO_3^-] was elevated (Simpson, 1967; Pashley and Cohen, 1973). The 2-3 fold increase in renal cortical citrate concentration observed during metabolic alkalosis was consistent with decreased citrate metabolism (Simpson, 1963; Adler *et al.*, 1971; Relman, 1972). The rise in tissue citrate (and

other organic acids) concentration during alkalosis would tend to correct the metabolic alkalosis by neutralizing a portion of the HCO_3^- generated in the kidney and thus would serve as a nonexcretory mechanism for regulating systemic acid-base balance (Cohen and Kamm, 1976). The increased hippurate accumulation by kidney cortex slices from alkalotic rats (Weiss and Preuss, 1970) is consistent with the suggestion that enhanced tissue citrate concentration, arising from either renal metabolic adaptations or exogenous substrate, modulates PAH transport capacity.

Additional evidence for citrate involvement in acetate stimulation is provided in Figure 23. Pre-loading of slices with citrate increased PAH accumulation above NaCl pre-incubated controls while reducing stimulation of accumulation produced by acetate. These data suggested that acetate stimulation of PAH accumulation has a maximal value determined by cortical citrate concentration or possibly by a limited capacity of the slices to contain higher concentrations of PAH.

Although the data presented above support the concept that citrate concentration functions as a positive modulator of PAH accumulation, they did not permit the elimination of other possible metabolites (e.g., α-ketoglutarate) which follow citrate in the TCA cycle from consideration as possible regulators of PAH transport.

Various investigators (Cross and Taggart, 1950; Farah et al., 1955; Koishi, 1959) demonstrated that several renal metabolic substrates (e.g., α-ketoglutarate, octanoate) enhanced PAH accumulation by cortical slices when present at low medium concentrations. Dose-response curves for α-ketoglutarate and succinate (Figures 24, 25) confirmed the biphasic relationship between PAH accumulation and medium concentration of various TCA cycle intermediates.

Progressive alkalinization of the incubation medium occurred as medium substrate (citrate, α-ketoglutarate or succinate) concentration was increased to 5.0 mM (Figures 22,24,25). However, substrate concentrations greater than 5.0 mM (Figure 22) did not produce additional increases in final medium pH. The relationship between substrate concentration and final medium pH cannot be adequately explained. Accumulation of these substances in vitro is, at least in part, via a peritubular transport system(s) similar to that for PAH (Selleck and Cohen, 1965: Balagura-Baruch et al., 1973). Maximal alkalinization of the medium could conceivably be reflecting maximal transport or metabolism of these compounds. Although medium pH reached optimal levels for PAH accumulation at high medium substrate concentrations (Figures 22,24,25), PAH S/M ratios were markedly depressed. Competition for a limited number of transport sites could explain the observed inhibition of PAH accumulation.

Acetate (10.0 mM) stimulated PAH accumulation and increased slice citrate concentration suggesting that restoration of slice citrate to endogenous levels (or moderately increasing citrate stores above endogenous concentrations) was the key step in PAH accumulation. Citrate might act as a positive modulator of PAH accumulation. However, citrate could stimulate accumulation of PAH by acting as precursor for a subsequent, positive modulator of anion transport. This possibility was examined by determining slice PAH accumulation and citrate concentration when a TCA cycle intermediately downstream from citrate was present as substrate. α-Ketoglutarate was chosen as a representative intermediate. In addition, the effect of glucose on these parameters

was determined. α-Ketoglutarate (0.5 mM) significantly stimulated PAH accumulation but did not alter slice citrate concentration (Figure 26). Glucose, a substrate not extensively utilized by kidney cortex (Cohen and Barac-Nieto, 1973), had no effect on either PAH accumulation or slice citrate concentration. These results demonstrated that intermediates following citrate in the TCA cycle could serve as modulators of PAH accumulation through a mechanism independent of replacement of depleted cellular citrate stores.

Slices were incubated with acetate as substrate (Figure 27) to determine if citrate and α-ketoglutarate could act as modulators through two, similar, but distinct mechanisms or if citrate stimulated PAH accumulation solely by increasing slice α-ketoglutarate concentration. Incubation of slices in 10.0 mM acetate increased slice PAH accumulation, citrate concentration and α-ketoglutarate concentration (unpublished results). However, Kim and Hook (1972) demonstrated that maximal stimulation (100%) of PAH accumulation by rabbit renal cortical slices was achieved at a medium concentration of 2.0 mM acetate. Thus, total tissue capacity for PAH, rather than modulator concentration could have been the limiting factor in PAH accumulation when 10.0 mM acetate was present as substrate. Therefore, slices were incubated with 1.0 mM acetate in order that tissue metabolite concentrations could be determined under conditions where the intrinsic slice capacity for PAH was not the limiting factor in PAH accumulation. Tissue citrate concentration was depleted in control slices (Figure 27) whereas α-ketoglutarate concentration was not different than the endogenous concentration. Acetate (1.0 mM) produced submaximal

stimulation of PAH accumulation and increased both tissue citrate and α-ketoglutarate. Although with acetate, the α-ketoglutarate concentration was greater than the endogenous concentration, acetate did not completely restore slice citrate concentration. The increase in PAH accumulation (51%) appeared to correlate better with the submaximal increase in slice citrate concentration (42%) than the increase in slice α-ketoglutarate content concentration (220%) (Figure 27). These investigations suggested that a single mechanism for modulation of PAH accumulation probably does not exist. Citrate, α-ketoglutarate, and most likely other TCA cycle intermediates, apparently modulate PAH accumulation through at least two similar, but separate, mechanisms.

SPECULATION

Separate mechanisms for stimulation of PAH accumulation by TCA cycle intermediates could be envisioned if these intermediates served as counter-anions for PAH accumulation by their respective mitochondrial translocating systems.

Three different carriers which catalyze the permeation of TCA cycle intermediates through the inner mitochondrial membrane have been characterized: (a) for dicarboxylates (malate, succinate); (b) for α-ketoglutarate; (c) for tricarboxylates (citrate, isocitrate) (Klingenberg, 1970). These anion translocators bring about an energy-dependent, one-to-one exchange of an intramitochondrial for an extramitochondrial anion (Klingberg, 1970; Meijer and Van Dam, 1974). Therefore, a major modulating factor for the transport of one anion is the concentration of the appropriate counter-anion (Meijer and Van Dam, 1974). Theoretically then, citrate or α-ketoglutarate could serve as counter-anions for PAH, first accumulating within the mitochondrial matrix space and then exchanging with cytosolic PAH. Compartmentalization of PAH within the mitochondrial matrix space would represent the energy-dependent intracellular concentrating mechanism in the model of PAH transport proposed by Foulkes and Miller (1959). Enhanced accumulation of PAH in the presence of acetate or low concentrations of citrate, α-KG and succinate (Figures 22, 24, 25) would result from an increased matrix space concentration of counter-anion(s) available for

exchange with PAH. Conversely, inhibition of PAH accumulation at high medium concentrations of TCA cycle intermediates (Figures 22,24,25) might represent competition with PAH for the available carrier sites on the inner mitochondrial membrane.

Some support for such a hypothesis can be derived from experimental data present in the literature. Simpson and Angielski (1973) demonstrated the existence of a citrate translocating system in mitochondria isolated from rabbit renal cortex. The tricarboxylate carrier system, at least in hepatic mitochondria, was extremely unspecific (Kleineke et al., 1973). In addition to citrate, threo-D_3-isocitrate, L-malate, phosphoenolpyruvate (PEP), several structurally related anions including phenyl-PEP, D-2-phosphoglycerate (D-2-PGA), D-3-PGA and L-2-PGA were able to exchange with intramitochondrial tricarboxylates via the tricarboxylate carrier system (Kleineke et al., 1973). An interaction of secreted organic anions with renal anion translocators can also be inferred from the data of Pakarinen and Runeberg (1969). PSP and probenecid inhibited the oxidation of citrate, α-ketoglutarate and succinate by mitochondria prepared from guinea pig kidney cortex. Inhibition of α-KG and succinate by PSP was concentration dependent, with 50% inhibition occurring at a medium concentration of 1.5 mM PSP. Citrate oxidation was relatively resistant to PSP, with a 40% inhibition achieved with 3.0 mM PSP in the medium. Interestingly, at lower medium PSP concentrations (0.75 mM and 1.5 mM PSP) citrate oxidation was stimulated (Pakarinen and Runeberg, 1969). The effects of probenecid on oxidation of these intermediates differed slightly. The concentration of probenecid (0.3 mM) required to inhibit α-KG oxidation 50% was much less than that of

PSP (1.5 mM). However, at this probenecid concentration, citrate and succinate oxidation were only slightly depressed (10-20%). Probenecid, at a concentration (0.75 mM) which inhibited α-KG oxidation by 90% did not produce a further reduction of either citrate or succinate oxidation (Pakarinen and Runeberg, 1969). These observations are consistent with (but do not prove) the concept that secreted organic anions inhibit the translocation of TCA cycle intermediates into the mitochondria and thereby reduce the oxidation. The concentration related effects discussed might be interpreted as demonstrating a much higher affinity of the α-ketoglutarate translocator (as opposed to the citrate and dicarboxylate translocators) for probenecid and PSP. However, the inhibition of citrate and succinate oxidation suggested that all of the translocators were subject to inhibition. The observation that rabbit kidney cortex mitochondria accumulated PAH (Tables 7,8), against a concentration gradient, was also consistent with an interaction of secreted organic anions with mitochondrial anion translocating systems.

In this model, mitochondrial PAH accumulation has been postulated to depend on the concentration of citrate (and other counter-anions) within the matrix space. As mentioned above, Weiss and Preuss (1970) demonstrated that PAH transport capacity was increased in alkalosis. This observation appeared to be consistent with the proposed model of PAH accumulation since renal cortical citrate concentration was also found to be elevated in alkalosis (Simpson, 1963; Adler et al., 1971; Relman, 1972). However, the matrix space citrate concentration of rabbit kidney cortex mitochondria has been shown to be significantly decreased under alkalotic conditions (Simpson and Angielski, 1973).

Simpson and Angielski (1973) demonstrated that the steady-state matrix space citrate concentration was regulated by alterations in medium [HCO_3^-]. Increased medium [HCO_3^-] decreased matrix space citrate concentration whereas lowering medium [HCO_3^-] below normal increased the concentration of citrate in the matrix space. Since regulation occurred in the presence or absence of inhibition of TCA cycle activity, Simpson and Angielski (1973) concluded that bicarbonate altered citrate transport by the tricarboxylate translocator of the inner mitochondrial membrane. These investigators suggested that the increased [HCO_3^-] of alkalosis enhanced the rate of citrate transport out of the matrix space. No evidence for inhibition of citrate entry into the matrix space by [HCO_3^-] was presented. Thus, PAH accumulation by the citrate translocator might be modulated by the rate of citrate efflux from the mitochondria rather than by the steady-state matrix space citrate concentration.

The movement of citrate from the matrix space to the medium (cytoplasm) has also been shown to be modulated by long chain fatty acyl-CoA. The hepatic mitochondrial tricarboxylate carrier was reversibly inhibited by long-chain fatty acyl-CoA when added *in vitro* or in states where these intermediates were elevated *in vivo* (Halperin *et al.*, 1972; Cheema-Dhaldi and Halperin, 1976; Wojtczak, 1976). Inhibition was concentration-related, competitive in nature and dependent on the chain length of the fatty acid moiety (Wojtczak, 1976). If fatty acyl-CoA inhibit citrate transport from mitochondria in kidney as they do in liver, then mitochondrial PAH accumulation would presumably, be inhibited by fatty acyl-CoA. The degree of inhibition would be determined by the concentration of fatty acyl-CoA. As

mentioned above, palmitate competitively inhibits slice PAH accumulation (Barac-Nieto, 1971). Since palmitate is rapidly activated and metabolized by kidney tissue (Heinemann et al., 1970; Cohen and Kamm, 1976), addition of palmitate to incubation media would be expected to increase tissue fatty acyl-CoA concentration and thereby inhibit PAH accumulation. Similarly, factors which depress the concentration of this intermediate by enhancing catabolism (carnitine) or esterification (penicillin substrate stimulation) would be expected to secondarily increase the rate of citrate exchange and thereby enhance mitochondrial PAH accumulation.

An interaction of PAH with mitochondrial anion translocators need not be postulated to explain substrate stimulation by penicillin. The first step in NEFA utilization by cells is probably transfer of NEFA from albumin, the intravascular carrier, to binding sites on the cell surface (Spector, 1971; Heinemann et al., 1975). Following binding, the water-insoluble NEFA must cross an aqueous medium (cytoplasm), in which they would be expected to exhibit only limited solubility, to reach their intracellular sites of dissimilation. Intracellular movement of NEFA is apparently facillated by binding of NEFA to specific cytoplasmic proteins which reduce their hydrophobicity (Ockner et al., 1972; Ockner and Manning, 1974,1976; Mishkin et al., 1972,1975). Two such proteins, Y protein (GSH-S-transferase B; Ligandin) and Z-protein (FABP = fatty acid binding protein) have been isolated from the cytoplasm of several tissues, including kidney cortex (Ockner et al., 1972; Ockner and Manning, 1974, 1976; Mishkin et al., 1972,1975). Analysis of binding properties has demonstrated an approximately equal affinity of these proteins for NEFA (association

constant $\approx 10^6 M^{-1}$ for oleate) (Kamisaka et al., 1975a,b; Listowsky, 1976). As mentioned in the introduction, Y-protein has been suggested to be a major component of the renal organic anion transport system. As such, Y-Protein (and Z-protein) binds numerous small organic anions, including PAH (Kirsch et al., 1975; Arias et al., 1976). However, the affinity of Y-protein for these organic acids was found to be approximately 1000-fold less than the affinity for NEFA (association constant $\approx 10^3 M^{-1}$) (Kamisaka et al., 1975a; Listowsky et al., 1976). At the PAH concentrations normally observed in vitro and in vivo, NEFA would act essentially as an irreversible inhibitor of PAH binding. If binding of PAH to Y- (or Z-) protein was a requisite step in PAH accumulation, the transport capacity of the system would be determined by the amount of NEFA bound to these proteins whereas the rate of PAH accumulation would be determined by the rate at which the NEFA was removed. Thus, stimulation of reaction sequences utilizing NEFA either for oxidation (carnitine) or esterification (penicillin substrate stimulation) would result in an increased PAH transport capacity and an increased rate of PAH accumulation. This model of PAH transport would also be consistent with Foulkes and Miller's proposed model of PAH accumulation. In this example, PAH binding to Y- (or Z-) protein would represent the intracellular concentrating mechanism. The reaction sequences responsible for the ATP dependent activation of NEFA prior to metabolism would serve as the energy-requiring component and as the rate-limiting step in PAH accumulation since they would catalyze the removal of an otherwise irreversible inhibitor of transport.

The two models proposed here are obviously based on a number of assumptions for which little or no experimental data are available. These hypotheses have been discussed primarily because they represent two, among several, possible mechanisms for PAH accumulation which fit the majority of the experimental data, can be tested experimentally and therefore be used to further characterize the biochemical sequences responsible for secretion of organic anions.

SUMMARY

The purpose of this investigation was three-fold: 1) to demonstrate the existence of multiple transport systems for the secretion of organic acids within the kidney; 2) to provide additional evidence that palmitate is an endogenous substrate of the PAH transport system; 3) to provide evidence that an intracellular modulator of the PAH transport system exists and to elucidate the role of this modulator in acetate-induced stimulation of PAH accumulation by renal cortical slices. The newborn rabbit kidney was used as an experimental model because maturation of anion transport can be selectively accelerated by administration of exogenous functional loads.

Three distinct developmental patterns of organic anion accumulation by rabbit renal cortical slices were observed. Anions were tentatively placed into three groups based on similarities in patterns of development of slice accumulation. Group I contained PAH, PSP and PEN; group II contained UA, ASA and SULFI; and CDC was placed alone in group III. The effect of administering various agents to newborn rabbits was evaluated according to this tentative classification.

PEN pretreatment markedly enhanced maturation of group I anion accumulation without altering slice accumulation of UA, ASA or CDC. PEN stimulation of SULFI accumulation was minimal and probably related to accumulation by the overlapping group I system.

Pretreatment of neonatal rabbits with most group II or group III anions did not alter group I anion accumulation. ASA stimulation of PAH accumulation was probably secondary to the marked toxicity observed following ASA pretreatment. Group II and group III anion administration had no effect on maturation of their respective transport systems. Graphical representation of the rate of slice PAH uptake was consistent with the existence of two active modes of PAH transport. The low capacity PAH uptake mode represented the group I transport system whereas the high capacity uptake mode probably represented PAH transport by the overlapping group II substrate system. These results supported the concept of multiple renal secretory systems for organic anions.

Analysis of renal NEFA extraction and metabolism in the presence of inhibitors of organic acid and transport suggested that the PAH secretory system participated either in the entry of NEFA into proximal tubule cells or in the intracellular binding of the fatty acids prior to activation and dissimilation. Palmitate metabolism by proximal tubule suspensions prepared from penicillin treated newborn rabbits supported this suggestion. Penicillin pretreatment increased PAH accumulation by suspensions of proximal tubules and enhanced palmitate esterification to triglycerides. Palmitate-^{14}C recovered as NEFA in penicillin treated tubules was decreased. Age-related changes in palmitate esterification to TG correlated with similar changes in tubule PAH accumulation. Neither iodipamide administration to immature, or penicillin treatment of mature, rabbits altered tubule PAH accumulation or palmitate esterification. These results

suggested that PAH and palmitate shared, at least in part, a common transport system. In addition, penicillin apparently enhanced PAH accumulation by enhancing maturation of an enzymatic pathway(s), resulting in increased transfer of endogenous inhibitors (NEFA) from an intracellular binding site shared with PAH.

Accumulation of PAH by renal cortical slices from several species was increased when acetate was included in the incubation medium. The parallel changes in medium pH with acetate suggested that stimulation of PAH accumulation resulted from metabolism of acetate to a positive modulator of transport rather than a direct effect of acetate on the transport system. The PAH transport capacity of slices from various species was correlated with the endogenous renal cortical citrate concentration. Preparation and incubation of slices depleted tissue citrate concentration. Acetate increased citrate concentration and PAH accumulation by kidney cortex slices. Physiological concentrations of citrate increased PAH accumulation and final medium pH. Increasing concentrations of citrate produced a biphasic effect on PAH accumulation. These data suggested that citrate may act as an intracellular modulator of organic anion transport. This hypothesis was tested with other stimulators of PAH accumulation. Physiologic concentration of α-ketoglutarate or succinate increased slice PAH accumulation. Higher concentrations of either substrate inhibited PAH accumulation. Final medium pH increased with increased medium concentration of both substrates. α-Ketoglutarate increased PAH accumulation but had no effect on slice citrate concentration. Slices incubated without substrate were depleted of citrate but not α-ketoglutarate.

Acetate significantly increased slice concentration of both α-keto-glutarate and citrate. Thus, the group I anion transport system could be modulated by several metabolic intermediates acting through similar, but separate, mechanisms. Rabbit kidney cortex mitochondria accumulated PAH against a concentration gradient. This observation suggested that TCA cycle intermediates might serve as counter-anions for PAH accumulation by their respective mitochondrial translocating systems.

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