BIOCHEMICAL CORRELATES OF ORGANIC ANION TRANSPORT IN DEVELOPING KIDNEY

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ABSTRACT

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By

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Renal organic anion transport capacity is less in the newborn than in the adult of most animal species. A primary stimulus to development of transport is substrate availability. Pretreatment of newborn animals with substrates for the transport system such as penicillin increases the rate of transport maturation. The purpose of this investigation was, therefore: 1) to formulate a maximally stimulating penicillin dose and dosage regimen; 2) to investigate the mechanisms of substrate stimulation of renal organic anion transport; 3) to evaluate substrate stimulation as a tool to facilitate isolation and characterization of components of the transport system.

Penicillin pretreatment of two-week-old rabbits significantly increased the ability of renal cortical slices to transport p-aminohippuric acid (PAH). The PAH slice to medium ratio (S/M) increased with dose to a maximum at 90,000 I.U. procaine penicillin G per animal. Increasing the dose to 180,000 I.U. with sodium penicillin G produced no greater increase. Stimulation was fully developed after 4 penicillin injections at 12-hour intervals. The maximal response

was observed 24 hours after the final injection, while after 72 hours the capacity of slices from treated animals to transport PAH was no different than control. To determine the effect of a maximal substrate challenge on the development of transport capacity, pregnant does were treated with 90,000 I.U. procaine penicillin G i.m. twice daily through the last half of gestation. Young animals received 2 penicillin injections prior to sacrifice. PAH S/M from pups 3 days, 1 and 2 weeks old were not significantly different from that normally observed at 4 weeks. Thus, intrinsic transport capacity for PAH is mature at 4 weeks of age. Only prior to this age may transport be enhanced by substrate. Maximal enhancement of PAH transport capacity occurs when 4 injections of 90,000 I.U. procaine penicillin G are administered at 12-hour intervals followed by sacrifice 24 hours after the final injection.

The effect of penicillin on PAH transport was investigated in rabbit renal cortical slices and separated proximal tubules. Slices were preincubated for 30 minutes, then PAH was added to produce concentrations in the medium of 1, 2 and 4 x 10^{-4} M and the slices incubated for 15 minutes. The effect of penicillin pretreatment on runout was observed by preloading slices in 6.3 x 10^{-4} M PAH for 90 minutes. Slices were transferred at 1-minute intervals through a series of 20 beakers plus an initial rinse beaker. Results were expressed as µg PAH per g tissue remaining in the slice and runout constants calculated. Penicillin pretreatment increased the rate of PAH uptake at each concentration in the medium. Passive diffusion of PAH into the cells and efflux from the cells was not significantly different from control. In separated proximal tubules PAH uptake

was analyzed using a double reciprocal plot. Tubule suspensions were preincubated for 15 minutes and incubated in medium containing 1, 4 or 8 x 10^{-4} M PAH. The maximal velocity of transport was increased following penicillin, but the apparent binding affinity of the system for substrate was unaffected. Furthermore, since substrate stimulation of PAH transport was blocked by cycloheximide, these kinetic data suggest that the effect of penicillin on the active transport system is quantitative (theoretical maximal velocity of transport) rather than qualitative (apparent affinity).

Proximal tubular ultrastructure was not changed by penicillin pretreatment. Either the effect was too subtle to be observed or it involved soluble rather than particulate proteins. The correlation between glutathione S-transferases, cytosolic drug metabolizing enzymes implicated as transport carriers, and organic anion transport was therefore investigated.

Glutathione (GSH) S-aryltransferase activity in 100,000 x g supernatant of renal homogenates, an estimate of GSH S-transferase concentration in the tissue, was less in newborn rats and rabbits than adults. Enzyme activity increased to adult values by 1 week of age in rats, prior to maturation of transport capacity. Enzyme activity in rabbit kidney was not different at 1 day and 2 weeks but was increased by 4 weeks coincident with transport maturation. In rats, 25 mg/kg 3-methylcholanthrene (3-MC) administered once a day for 3 days significantly increased enzyme activity but had no effect on transport capacity. Chronic ammonium chloride acidosis increased enzyme activity 8-fold but decreased transport capacity. Forty-eight hours following unilateral nephrectomy in rats, transport

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capacity was significantly increased with little effect on enzyme activity. L-Methionine-SR-sulfoximine (1.85 mmoles/kg) significantly reduced glutathione concentration in renal cortex but had no effect on transport capacity. Organic anion transport was greater in male mice than female, yet there was no difference in enzyme activity between sexes. 3-MC (10, 20, 30, 40 mg/kg) administered to 2-week-old rabbits twice daily for 3 days increased transport in a dose dependent manner. GSH S-transferase activity was also increased. Penicillin (90,000 I.U. twice daily for 2 days) similarly increased transport but had no stimulating effect on enzyme activity. The apparent lack of correlation between transport capacity and GSH S-transferase in several instances suggests that GSH S-transferase concentration is probably not the rate limiting step in renal organic anion transport.

Penicillin pretreatment of newborn rabbits had no effect on DNA/protein, RNA/protein, nor RNA/DNA ratios in kidney cortex. Incorporation of ^{14}C L-leucine into renal cortical slice protein was similarly unaffected by substrate stimulation. Using suspensions of separated proximal tubules, the incorporation of label into protein was slightly increased 24 hours after termination of penicillin treatment. *In vivo*, after 3 injections cf 90,000 I.U. penicillin, uptake of label into 100,000 x g pellet protein was enhanced compared with control but the difference was not statistically significant. Penicillin had no apparent effect when total renal cortical protein was assayed.

PAH S/M ratio was significantly enhanced 8 hours after a single injection of 90,000 I.U. penicillin. Stimulation was maximal after

24 hours and had returned to control 48 hours after treatment. ^{14}C L-leucine incorporation into 100,000 x g pellet protein was increased 16 hours after a single injection of penicillin, but the effect was variable. There was no consistent effect of penicillin on cytosolic proteins. The specific activity of intracellular leucine precursor pools was not altered by penicillin treatment. Sephadex filtration and gel electrophoresis of soluble and particulate proteins, respectively, following *in vivo* labeling, did not indicate localized uptake of radioactivity in protein fractions. Thus, either substrate stimulation of organic anion transport involves slight alterations in protein biochemistry that may not be detected by these methods, or penicillin acts through a mechanism not involving increased synthesis of a protein component of the transport system.

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By

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INTRODUCTION

Organic Anion Secretion in the Kidney

Active secretory systems in renal excretory function were first hypothesized by Heidenhain (1880) following the observation that injection of certain highly colored dyes resulted in localization of color within proximal tubular cells. Marshall and Vickers (1923) provided additional evidence for active secretion when they determined that the rate of excretion of phenolsulfonphthalein (PSP) was greater than could be accounted for by glomerular filtration alone. Subsequently, renal organic anion secretory processes were anatomically localized by stop flow analysis (Malvin et al., 1958) and micropuncture (Edwards and Marshall, 1924) to the proximal segment of the nephron. In early studies emphasis was placed on compounds such as p-aminohippurate (PAH) and PSP because of uncomplicated handling by tubular cells (i.e., unmetabolized) and because of a relative lack of the analytical technology required for quantification of other substances. As a result, as late as 1939, Shannon (1939) suggested that PSP secretion in the kidney might "be considered to be the result of incidents in the cell's genetic history" and lack a normal function because no endogenous analogue to PSP could be identified. Since then it has become increasingly apparent that animals and man produce or ingest large amounts of compounds which are not extensively metabolized and in many cases are pharmacologically

active. Metabolic alterations which do occur usually produce forms which are more easily eliminated from body fluids. Those compounds are then ideal substrates for the relatively rapid rates of elimination which may be attained through active secretion in the kidney.

The capacity of renal tubules to secrete organic anions and cations is nearly universal in occurrence among vertebrates. Similarly, the structural specificity for substrate appears to be common among species though the efficacy of transport may vary. In mammals, the primary function of the renal organic anion secretory system may be the elimination of various conjugates. The excretion of a large number of organic compounds of either exogenous or endogenous origin is facilitated by conjugation in the liver and kidney with glycine, glucuronate and sulfate (Sperber, 1959; Wesson, 1969). Conjugation, however, is not a prerequisite for transport. Barac-Nieto and Cohen (1968) studied renal extraction of non-esterified fatty acids and determined that probenecid, an effective competitive inhibitor of organic anion transport, decreased renal uptake. Subsequently, Barac-Nieto (1971) observed that palmitate competitively inhibited PAH uptake. These data suggest transport by a common mechanism. Selleck and Cohen (1965) hypothesized that the organic anion transport system was primarily involved in transfer of metabolic intermediates such as non-esterified fatty acids, citrate and α-ketoglutarate to sites of dissimilation in the kidney. The resulting pool of rapidly convertible intermediates may then provide a source of energy for other proximal tubular functions such as sodium and nutrient reabsorption.

Although a role for the organic anion secretory system in transport of endogenous compounds has been demonstrated, greater emphasis has been placed on excretion of exogenous compounds. In addition to the acidic dyes such as PSP and substituted hippurates, many drugs are eliminated by the renal secretory system. Penicillin was studied extensively after its discovery as an anti-infective agent. The renal clearance of penicillin is of the same magnitude as PAH and therefore plasma elimination is rapid (Rammelkamp and Bradley, 1943; Rantz and Kirby, 1944). Limited availability of drug following its introduction necessitated the use of other organic anions to competitively inhibit excretion and prolong plasma half life. This led to the development of probenicid, which remains a classical inhibitor of organic anion secretion (Beyer, 1947). Other drugs as well, such as thiazide diuretics and furosemide and salicylates, are actively secreted by the kidney at rates approaching that of PAH (Baer et al., 1959; Bowman, 1975; Hirsch et al., 1975; Schachter and Manis, 1958). Therefore, far from the view of Shannon (1939), organic anion secretion may be considered an important mechanism in drug elimination as well as regulation of body fluid concentrations of various endogenous compounds and their metabolites.

Characteristics of the Renal Organic Anion Transport System

Active transport is defined as carrier mediated translocation of substances across biological membranes against electrochemical concentration gradients (Wesson, 1969). The process is therefore dependent upon a constant supply of energy derived from cell

metabolism. In the kidney, PAH and related compounds are transferred from peritubular blood to tubular lumen by a mechanism which satisfies the requirements set forth in the definition of active transport. The net movement of PAH and related substances is "uphill", into the cell, against a concentration gradient (Foulkes and Miller, 1959; Foulkes, 1963). The rate of transport increases with substrate concentration until a maximum is reached at which the system becomes saturated (Weiner, 1973). Furthermore, secretion of one anion is competitively inhibited by the simultaneous administration of a second (Wesson, 1969). Though secretion is substrate selective, specific structural requirements have not been determined due to the diverse nature of transported substances. In this area, Taggart (1958) hypothesized that the determining factor in transport was the net negative charge on the carboxyl group of anionic compounds. Several years later, Despopolous (1965) concluded that all secreted ions were characterized by three oxygen or equivalent atoms spatially related to approximate the configuration of the PAH side chain. Both theories appear to be oversimplifications in that many transported substances do not meet these criteria. The data, however, are consistent with a process mediated by specific and discrete transport carriers.

Uncouplers of oxidative phosphorylation, hypoxia, cold and metabolic poisons decrease organic anion transport capacity *in vivo* and *in vitro*, suggesting strict dependence of the system on adequate availability of oxidative energy (Maxild and Moller, 1969; Mudge and Taggart, 1950a; Shideman and Rene, 1951; Taggart and Forster, 1950). The mechanisms of energy transfer, however, are unclear.

Maxild (1973) observed that inhibitors of mitochondrial ATP production such as 2,4-dinitrophenol and carbonylcyanide-M-chlorophenylhydrazone decreased PAH transport. Concentrations of inhibitor which produced 50% depression in transport capacity had no effect on ATP concentration. Similarly, anaerobic conditions decreased PAH transport and ATP in the cell, but upon return to aerobiosis, transport capacity was regained prior to regeneration of a full complement of ATP. Though transport appears to be intimately linked to respiratory electron transfer, endogenous ATP may not function as the primary energy donor. Therefore, it was suggested that decreased transport following treatment with metabolic inhibitors did not result from lower ATP concentration but was due to alterations in metabolic patterns (Weiner, 1973). Fluoroacetate, for example, inhibits tarnsport and results in the accumulation of Krebs cycle intermediates (Farah et al., 1953, 1955) which have been shown to be inhibitory to the PAH transport mechanism (Cross and Taggart, 1950). In any regard, the route and intermediates of energy transfer from the respiratory chain to the transport mechanism have not been identified.

Localization of Transport and Identification of Possible Transport Carriers

Compounds which are secreted *in vivo* must cross the peritubular membrane, cell cytoplasm and luminal membrane before entering the tubular lumen. The first step in the secretory process appears to be active transport into the cell (Cross and Taggart, 1950; Forster and Copenhaver, 1956; Foulkes and Miller, 1959). Tune et al. (1969) used isolated microperfused rabbit proximal tubules and determined

that PAH concentration in proximal tubular cells was higher than in either the bathing medium or tubular perfusate. From these data a mechanism was proposed whereby PAH was actively transported across the peritubular membrane, accumulated intracellularly, and passively diffused down a concentration gradient from cell to lumen. The transport mechanism would then most likely be located within or in close proximity to the peritubular membrane.

Renal organic anion secretory rate is governed in part by the structure and conformation of substrate molecules (Wiener, 1973; Wesson, 1969). Proteins possess the structural complexity necessary for discrimination between closely related substances and are therefore implicated as transport carriers. A prerequisite for active transport is that a specific, reversible interaction occur between substrate and carrier. Measurement of binding affinity is then a useful tool in characterization of transport mechanisms. A carrier mediated mechanism has been proposed for the system which actively secretes N-methylnicotinamide (NMN) and other organic bases (Ross et al., 1975). Competitive binding studies utilizing organic base substrates and dibenamine, an irreversible inhibitor of base transport (Ross et al., 1968), suggest that a protein carrier exists in proximal tubular cells (Holohan et al., 1973; Magour et al., 1969; Ross et al., 1969). The renal transport system for organic bases possesses many of the same functional characteristics as that for organic anions (Peters, 1960; Rennick et al., 1954; Rennick and Moe, 1960). The process is energy dependent and substrate saturable. Transport of one organic cation is competitively inhibited by simultaneous administration of a second but not by organic anions (Farah

et al., 1959; Kandel and Peters, 1957; Rennick and Farah, 1956). Therefore, renal secretion of organic anions and cations comprise two similar but distinct systems. Investigations of substrate binding to components of the organic acid secretory system have been hindered by failure to develop an irreversible inhibitor of transport. Recently, however, a ligand binding assay for organic acids in the kidney was developed.

The first conclusive evidence of binding capacity for transported organic anions in renal subcellular fractions was obtained by Holohan et al. (1975) using Sephadex and Millipore filtration techniques. These investigators determined that of the fractions studied the 100,000 x g pellet of renal cortical homogenates possessed the highest binding capacity for PAH and NMN. There was no binding to proteins in the soluble fraction. The binding affinity constants in 100,000 x g pellet material were 25 and 20 mM for PAH and NMN, respectively, indicating relatively low affinity systems. The binding component was released from membranes by treatment with nonionic detergents and was heat labile and substrate specific. Though there was competition for binding among anionic and cationic compounds, there was no interaction between the groups, further demonstrating independence of the mechanisms. From the proposed model, that is, active transport across peritubular membranes, one would anticipate that the carrier protein would be associated with the membranous fraction of kidney homogenates as has been observed. However, a great deal of evidence has accumulated which suggests that cytosolic proteins may also be involved in organic anion transport.

In the liver, organic anions are actively cleared from sinusoidal blood and transported into bile by hepatic cells. Arias and co-workers (Levi et al., 1969; Reyes et al., 1969, 1971) isolated two cytosolic proteins, Y and Z, from liver homogenates which competitively bound organic anions. The protein present in highest concentration, protein Y, was designated ligandin. The concentration of ligandin in liver, as well as the rate of anion excretion, is increased after administration of various microsomal enzyme inducers (Klaasen, 1975; Reyes et al., 1969). A role for ligandin in biliary excretion of organic anions was proposed based on this evidence. A protein immunologically identical to ligandin was isolated from renal homogenates and a similar function in organic anion transport hypothesized (Kirsch et al., 1975a,b).

Renal and hepatic ligandin are immunologically identical to glutathione S-transferase B (Habig et al., 1974a), one of a class of drug metabolizing enzymes which conjugate various compounds to reduced glutathione (Habig et al., 1974b). In the kidney, glutathione S-transferases are located only in cells of the proximal tubules, the only area of the nephron which secretes organic anions (Fine et al., 1975; Kirsch et al., 1975a,b). Several lines of evidence support the hypothesis that these enzymes function as transport carriers in the kidney. Glutathione S-transferase activity was competitively inhibited by transported organic anions (Clifton et al., 1974, 1975a; Kaplowitz et al., 1975). After injection of labeled penicillin, a substrate of the transport system, 88% of the radioactivity was bound to the glutathione S-transferase containing fraction of renal cell homogenates (Kirsch

et al., 1975a,b). Probenecid, a competitive inhibitor of organic anion transport, reduced binding of penicillin to the renal enzyme (Kirsch et al., 1975a,b). Inducers of drug metabolizing enzymes in the kidney such as 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increased renal concentration of GSH S-transferases as well as urinary excretion, plasma disappearance and binding of organic anions (Clifton et al., 1975b; Kirsch et al., 1975b).

Therefore, two plasible explanations exist to describe the mechanism of PAH transport in the kidney. The first involves active transport at the peritubular membrane (Holohan et al., 1975; Tune et al., 1969); and the second, specific binding within the cell as the primary events in intracellular accumulation of organic anions (Kirsch et al., 1975a,b). The transport model proposed by Tune et al. (1969) does not include intracellular binding of PAH. Farah et al. (1963) and Foulkes and Miller (1959) suggested that two pools of PAH existed within the proximal tubular cell, one in rapid equilibration with extracellular fluid and the second, less readily exchangeable with the extracellular fluid and responsible for the high concentration of PAH within the cell. Whether these data are related to the observations of Arias and co-workers (Kirsch et al., 1975b) is unknown. The data, however, might suggest that renal organic anion transport is mediated by both membrane transport and intracellular binding.

Use of the Slice Technique

Kidney slices were first used to quantify transport when Forster (1948) determined that thin slices of frog kidney accumulated phenol

red within the lumen by a process which was dependent upon metabolic energy. Cross and Taggart (1950) utilized thin slices of rabbit renal cortex as a means to study organic anion transport in the mammalian kidney. This technique permitted investigation of transport phenomenon in the absence of complications due to renal blood flow, blood flow distribution and glomerular filtration which might alter availability of metabolites or substrate. In addition, the study of renal transport *in vitro* allows investigation of a wider range of experimental variables, some of which might be deleterious or impossible to produce in the intact animal. The ability to precisely control experimental conditions over long periods of time and to rapidly change these conditions is a further asset of the slice preparation. Major criticisms of the slice technique have involved objection to the artificial conditions imposed by *in vitro* studies.

The first step in renal organic anion transport involves active uptake of substrate into the tubular cell (Foulkes and Miller, 1959). Therefore, slices of renal cortex incubated in physiological, oxygenated medium accumulate transported organic anions resulting in high intracellular concentrations. Foulkes and Miller (1959) determined, in slices, that the movement of PAH across the luminal membrane of proximal tubular cells was very slow compared to flux across the peritubular membrane, probably due to collapse of the lumen in the absence of glomerular filtration. Net uptake studies then constitute an evaluation of active transport across the peritubular membrane. Uptake data are generally represented as a slice to medium (S/M) concentration ratio where S equals concentration in the slice and

M equals concentration in the medium. An S/M ratio greater than 1 is assumed to be indicative of active transport when non-specific cellular binding of substrate is negligible. Autoradiographic studies have localized PAH in tubular cells to the cytoplasm (Miatello et al., 1966). In addition, incubation under nitrogen, which effectively inhibits active transport, results in S/M ratios of unity (Cross and Taggart, 1950). Therefore, PAH enters the cell and is apparently not bound to an appreciable extent to cell membranes or intracellular organelles.

During incubation of tissue slices, substrate is accumulated until a steady state is reached, whereupon rates of active influx and passive efflux are equal. The final S/M ratio is a function of the relative rates of substrate flux across the membrane. An increase in the active uptake rate of substrate would be reflected as a higher S/M ratio and vice versa. Slice to medium ratio can then be used as an indication of transport capacity. A large amount of data are available which suggest that organic anion S/M ratio in kidney slices is closely representative of tubular transport *in vivo* (Berndt, 1976). Acetate and lactate enhance slice uptake of PAH (Cross and Taggart, 1950) and tubular transport in intact animals (Mudge and Taggart, 1950b), while succinate and fumarate block transport in both preparations. Metabolic inhibitors such as 2,4-dinitrophenol and fluoroacetate, as well as competitive inhibitors such as probenecid and penicillin decrease transport capacity measured both *in vitro* and *in vivo* (Beyer et al., 1944; Cross and Taggart, 1950; Farah et al., 1953; Mudge and Taggart, 1950a; Weiner, 1973). Furthermore, organic anionic compounds which are accumulated in slices are secreted in the

intact animal while no slice accumulation is observed in species which are unable to secrete the agent (Mudge et al., 1971). Therefore, steady state uptake of organic anions in slices appears to serve as an adequate representation of intact organ function. However, slices may not be the best estimate of renal function *in vivo*.

Slices of renal cortex consist of several cell layers. Therefore, cells on the interior of the slice are dependent upon diffusion for contact with medium. If oxygen penetration becomes rate limiting, function through the slice may be altered and measurements reflect an average value for a heterogeneous population of cells. Burg and Orloff (1962) described a technique for enzymic digestion of renal cortex resulting in a free suspension of separated proximal tubules. In this preparation each cell is in constant contact with bathing medium. Separated proximal tubules consume oxygen, maintain electrolyte concentration gradients, and accumulate PAH (Burg and Orloff, 1962; Burg and Orloff, 1969; Ecker and Hook, 1974a; Guder et al., 1971; Huang and Lin, 1965). Barriers to diffusion and much of the non-transporting tissue present in slices (distal tubules, connective tissue, glomeruli) are eliminated when using separated tubules. In addition, PAH uptake is more rapid and greater tissue to medium concentration ratios are attained (Huang and Lin, 1965). Therefore, though uptake of organic anions in renal cortical slices is indicative of *in vivo* secretion, perhaps a better estimate may be obtained using separated proximal tubules. As estimates of tubular function other than transport (i.e., metabolism, protein synthesis), the separated proximal tubule preparation may be a superior model.

Development and Substrate Stimulation of Renal Organic Anion Transport

At birth the kidneys of most animal species are structurally and functionally immature. The ability of the newborn kidney to excrete a large load of sodium and water is less than would be predicted on size alone (Dean and McCance, 1949; Edelmann and Spitzer, 1969). In addition, mechanisms for tubular secretion of drugs such as penicillin and sulfas are quantitatively immature. Several investigators observed that clearance and extraction of PAH in the newborn was less than in the adult (Alexander and Nixon, 1962; Calcagno and Rubin, 1963; Hook et al., 1970; Horster and Lewy, 1970; Levine and Levine, 1958). Transport capacity measured as the accumulation of PAH in renal cortical slices or separated proximal tubules was also less in the newborn (Ecker and Hook, 1974a; Hirsch and Hook, 1970a; Hirsch and Pakuts, 1974; Kim et al., 1972; Rennick et al., 1961). The relatively low functional capacity in young animals has been attributed to structural immaturity and to the presence of nephrogenic and incompletely differentiated tissue in the outer cortex of the kidney. In humans the full complement of glomeruli and tubules is present at birth and the major changes observed are increases in size and differentiation (Edelmann, 1969), whereas in rats at birth nephrogenesis is a prominent characteristic (Baxter and Yoffey, 1948; Bogomolova, 1966). Cortical tissue is not fully developed in rats until 28 days of age. Up to one year is required for the cortex to reach its adult thickness and for the brush border to reach adult heights (Bogomolova, 1966). Rennick et al. (1961) have shown greater *in vitro* ability of inner, more mature, juxtamedullary tissue to transport PAH than the histologically immature

outer cortex. Though renal tubular cells in young animals appear by light microscopy to be small (Hirsch et al., 1971), electron microscopic studies in newborn and fetal animals have shown complete ultrastructural differentiation with only small quantitative differences from mature kidney (Bernstein, 1971). Structural development does not appear to be the sole or principal determinant of transport capacity.

Transport capacity exhibits a characteristic pattern of development *in vitro* (Hirsch and Hook, 1970a). In rabbits PAH S/M ratio developed slowly with age until at 2 to 3 weeks a rapid increase was observed. Maximal PAH S/M ratio occurred at 4 weeks of age and this was followed by a decline to adult values. Similar patterns of development *in vitro* were observed using rats, dogs and pigs (Kim et al., 1972; Rennick et al., 1961). The rapid increase in transport capacity occurring during early development is apparently a result of an increased availability of substrate (Ecker and Hook, 1974b; Hook, 1974). During the neonatal period renal vascular resistance falls and renal blood flow increases, presumably presenting a larger substrate load to the tubules (Edelmann, 1969). Several examples are available in the literature of substrate induced adaptations in enzyme activity and transport function (Heppel, 1969; Knox et al., 1956; Tepperman and Tepperman, 1963). To investigate the effect of increased substrate load on renal organic anion transport development, Hirsch and Hook (1969, 1970a,b,c) challenged the system with large doses of penicillin, an organic anion rapidly transported in the kidney, and observed a significantly increased PAH S/M ratio. The effect of penicillin on transport was not unique

in that other transported substances, such as PAH and probenecid, also increased the rate of maturation (Hirsch and Hook, 1970a; Hook and Bostwick, 1973). If animals were sacrificed several days after the termination of substrate treatment, no stimulation of transport was apparent, providing further evidence for the substrate dependence of transport rate (Hirsch and Hook, 1969, 1970c). If precautions were taken to prevent young rabbits from contacting solid food, the rate of transport maturation was decreased (Ecker and Hook, 1974b). During normal development, then, environmental substances such as derived from food probably contribute to functional maturation. Several investigators have since determined that PAH transport *in vivo*, measured as elimination from plasma, extraction or clearance in several animal species, was also increased by penicillin pretreatment (Bond et al., 1976; Kaplan et al., 1975; Lewy and Grosser, 1974). Thus, by several methods the data demonstrate that organic anion transport maturation is dependent upon substrate availability and may be increased by substrate pretreatment.

The stimulating effect of penicillin pretreatment on organic anion transport in young animals is in several ways analogous to induction of drug metabolizing enzymes in the liver. Agents such as phenobarbital and 3-methylcholanthrene enhance microsomal drug metabolizing capacity in liver through increased synthesis of enzyme protein (Chiesara et al., 1967; Conney, 1967; Fouts and Rogers, 1965; Funcke and Timmerman, 1973; Hayes and Campbell, 1974; Pantuck et al., 1968). The effect of substrate stimulation on renal protein biochemistry was investigated by Hirsch and Hook (1970b). Penicillin pretreatment of newborn rats was associated with an increased kidney

weight to body weight ratio. Concomitant administration of cycloheximide, a potent inhibitor of protein synthesis (Ennis and Lubin, 1964), blocked the stimulating effect of penicillin. The protein content of the 100,000 x g pellet of renal cortical homogenates was increased following penicillin, suggesting that the effect was confined to microsomal proteins and that the transport mechanism was also localized to membranes. Interpretation of these data was difficult, however, because the protein content of all subcellular fractions was slightly increased, though the effect was only significant in the 100,000 x g pellet. In addition, cycloheximide decreased transport capacity in controls. Therefore, to obtain a more specific estimate of protein synthesis, rates of incorporation of labeled amino acids into protein were determined. The uptake of ^{14}C L-leucine and ^{14}C L-glutamine into the trichloroacetic acid insoluble fraction of renal cortical homogenates was increased following penicillin (Hirsch and Hook, 1970b). There was no stimulation of amino acid uptake in medullary tissue consistent with the inability of medullary slices to transport PAH (Cross and Taggart, 1950). Ammonium chloride acidosis increased kidney weight as well as labeled amino acid uptake (Bignall et al., 1968) but had no stimulating effect on PAH transport (Hirsch and Hook, 1970b). Therefore, the effect of penicillin is probably not the result of non-specific increases in protein content. From these data Hirsch and Hook (1970b) concluded that the stimulating effect of penicillin on PAH transport was the result of increased synthesis of specific transport proteins.

Substrate Stimulation as a Tool in the Investigation of Renal Organic Anion Transport Mechanisms

Isolation and characterization of components of the renal organic anion transport system have been hindered by an apparent low binding affinity of substrate (Holohan et al., 1975) and failure to develop an irreversible inhibitor of transport. Substrate stimulation may provide an alternate method. Enhancement of organic anion transport capacity by penicillin is specific; that is, organic base transport is unaffected (Hirsch and Hook, 1970a,c), and is apparently mediated through an increased synthesis of protein components of the system. Therefore, it should be possible to stimulate transport and label the induced protein by supplying radioactive precursors. The incorporation of label may then provide a marker to facilitate further investigation. In earlier studies rats were used as the test animal (Hirsch and Hook, 1970b). However, rabbits are more susceptible to substrate stimulation and may similarly exhibit more pronounced changes in renal cortical biochemistry following substrate stimulation. Therefore, in the present investigation, rabbits were used exclusively in studies of substrate stimulation.

The specific objectives of this study were first to reevaluate substrate stimulation in order that maximal enhancement of transport capacity might be produced. Once an optimal treatment regimen was determined, the next objective was to investigate the mechanism of penicillin enhancement of transport, including the effect of penicillin on membrane flux of PAH and related alterations in proximal tubule ultrastructure.

Several investigators suggested that cytosolic drug metabolizing enzymes in the kidney functioned as primary acceptor proteins for

transport (Clifton et al., 1975a; Kirsch et al., 1975b). Conclusive evidence, however, was not obtained. Therefore, another objective of this study was to determine the relationship between glutathione S-transferase concentration in kidney cortex and organic anion transport capacity. Finally, the effect of substrate stimulation on amino acid incorporation and protein synthesis *in vivo* and *in vitro* was to be investigated. Gel electrophoresis and Sephadex filtration were to be used to partially purify labeled proteins from kidney homogenates. From these data the feasibility of using substrate stimulation as a tool to isolate components of the transport system was to be evaluated.

METHODS

General

1. Animals

New Zealand White rabbits, Sprague-Dawley rats and Swiss Webster mice were purchased from local breeders. Animals were maintained in departmental animal quarters. Rabbits and rats were bred, or litters were purchased with lactating females. Young animals remained with their mothers during treatment and until the time of experimentation. Within each litter, half the pups were treated with drugs and the remainder received drug vehicle or saline as control. Unless otherwise noted, all animals were male; littermates were of both sexes.

2. Description of the *in vitro* slice technique

Animals were killed by a blow to the head and the kidneys removed, weighed and placed in ice cold isotonic saline. Thin slices of renal cortex were prepared free hand and briefly kept in a further aliquot of ice cold saline until incubation. Slices of renal cortex (100-200 mg wet weight) were incubated in 2.7 ml of the phosphate buffer medium[1] described by Cross and Taggart (1950) which contained

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[1] 97 mM NaCl, 40 mM KCl, 0.7 mM $CaCl_2$, 7 mM NaH_2PO_4-Na_2HPO_4 (pH 7.4).

7.4×10^{-5} M p-aminohippuric acid (PAH) and/or 6.0×10^{-6} M ^{14}C N-methyl-nicotinamide (NMN). In some cases 10 mM sodium acetate was included in the medium. The incubation period was 90 minutes at 25°C under 100% oxygen. After incubation slices were removed from the medium, blotted and weighed. Tissue and a 2 ml aliquot of medium were homogenized in a 3 ml volume of 10% trichloroacetic acid (TCA). Water was added to a final volume of 10 ml and samples centrifuged. Aliquots of tissue and medium supernatant were assayed for PAH by the method of Smith et al. (1945). ^{14}C NMN was quantified by adding 1 ml tissue or medium supernatant to scintillation vials containing 10 ml modified Bray's solution (6 g 2,5-diphenyloxazole and 100 g naphthalene per liter of dioxane). Radioactivity was determined in a Beckman LS-100 liquid scintillation spectrometer using external standardization. Results were expressed as a slice to medium (S/M) concentration ratio where S equals milligrams per gram tissue (wet weight) and M equals milligrams per milliliter medium.

L-leucine S/M ratios were determined by incubating slices in Krebs Henseleit bicarbonate buffer[2] containing 55 μM ^{14}C L-leucine (sp. act. 1.45 μCi/μm) for 60 minutes at 37°C under 100% oxygen. Slices were homogenized and assayed as described for NMN and S/M ratios calculated as the ratio of DPM per milligram tissue divided by DPM per milliliter medium.

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[2] 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 10 mM glucose, 1 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 (pH 7.4).

3. Description of the *in vitro* separated proximal tubule technique

Separated proximal tubules were prepared by a technique similar to that of Burg and Orloff (1962) with some of the modifications employed by Huang and Lin (1965). Animals were killed by a blow to the head and the kidneys exposed through a midline incision. The abdominal aorta was clamped rostral and cannulated caudal to the renal arteries. The kidneys were perfused with ice cold physiological saline until clear of blood. The infusion was continued using 0.3% collagenase (Nutritional Biochemical Corp., Cleveland, OH) dissolved in Ringer's solution.[3] The renal vein was clamped and the perfusion continued until the kidney became turgid. The infusion was stopped and the renal vein clamped. After approximately 3 minutes the kidney was removed and cortical tissue dissected free. Tissue was finely minced and incubated for 45 minutes at 25°C while being slowly stirred and oxygenated by bubbling 100% oxygen through the suspension. After digestion the suspension was centrifuged for 60 seconds at 500 rpm and the supernatant discarded. The pellet was rinsed three times by resuspension in 5% calf serum-Ringer's solution. During the third wash the tissue suspension was filtered through two layers of surgical gauze prior to centrifugation. The pellet of tubules was resuspended in sufficient Ringer's-acetate medium containing PAH to produce a 2 to 4% (w/v) suspension. The suspension was incubated at 25°C while being continually gassed and mixed by bubbling with 100% oxygen. Following

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[3] 120 mM NaCl, 16.2 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 5.5 mM dextrose, 10 mM Na acetate, 10.0 mM KH_2PO_4-Na_2HPO_4 (pH 7.4).

incubation the samples were centrifuged at 0°C for ten minutes at 10,000 x g in the special centrifuge tubes described by Burg and Orloff (1962). To estimate trapped medium in the pellet 0.1 ml 5% inulin per 4 ml incubation medium was added immediately prior to centrifugation. Tissue water content was determined by drying the tissue pellet to constant weight. The pellet was homogenized in 5 ml 3% TCA and the protein precipitate removed by centrifugation. The supernatant and a sample of medium treated in a similar manner were assayed for PAH (Smith et al., 1945) and inulin (Schreiner, 1950).

Pharmacodynamic Analysis of Substrate Stimulation of Renal Organic Anion Transport by Penicillin

1. Effect of dosage and treatment schedule

The relationship between penicillin dose and PAH S/M in rabbits was determined using both procaine penicillin G and sodium penicillin G. Treatment was begun on the 10th day of life and continued at 12-hour intervals for a total of 6 injections. In all cases, animals were sacrificed 24 hours after the final injection. Procaine penicillin G (15,000, 30,000 and 90,000 I.U.) was given subcutaneously. Sodium penicillin G (30,000, 100,000 and 180,000 I.U.) was injected intraperitoneally.

PAH S/M ratios were determined after 2, 4, 6 or 8 injections of 90,000 I.U. of procaine penicillin G, subcutaneously, to newborn rabbits. Injections were given at 12-hour intervals beginning on the 10th day of life. Animals were sacrificed 24 hours after final injection and run in pairs, control and treated together.

PAH S/M was determined at several intervals after 90,000 I.U. of procaine penicillin G, given subcutaneously, twice daily for 2 days. Treatment was begun on the 11th day of life. Pairs of control and treated animals together were sacrificed and PAH S/M determined 12, 24, 36 and 72 hours after the final injection.

The effect of a single injection of penicillin on PAH S/M was determined by administering 90,000 I.U. procaine penicillin G subcutaneously on the 12th day of life. Animals were sacrificed and transport capacity determined 2, 4, 8, 12, 16, 24 and 48 hours following treatment.

Residual penicillin in various tissues 24 hours after a subcutaneous injection was determined by combining 1 μc of ^{14}C-benzyl penicillin (penicillin G) with the final injection of procaine penicillin. Samples of bladder urine, blood, fat, kidney cortex, liver and muscle were obtained, solubilized in 1 ml of Soluene at 37°C and counted in 10 ml of toluene counting solution containing 5 g of 2,5-diphenyloxazole (PPO) and 200 mg of dimethyl 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP) per liter. Results were expressed as disintegrations per minute per gram of tissue.

2. Substrate stimulation during development

To elucidate the effect of a maximal penicillin challenge on PAH S/M, pregnant does were injected intramuscularly with 90,000 I.U. of procaine penicillin G twice daily from day 14 of gestation to delivery. Within a litter, pups were sampled at 3 days, 1, 2 and 4 weeks. Two days before experimentation, a pair of pups was randomly selected. One member of the pair received two injections of

90,000 I.U. of procaine penicillin G at 12-hour intervals, and the other served as a saline control. Animals were sacrificed 24 hours after the second injection.

Investigation of Mechanisms of Penicillin Stimulation of PAH Transport

1. Uptake and efflux of PAH by slices

Rabbits 10 days old received 30,000 I.U. procaine penicillin G subcutaneously twice daily for 3 days. Control littermates received saline. Animals were killed 24 hours after the final injection. To estimate the maximal rate of uptake into slices, tissue was pre-incubated for 30 minutes. PAH was then added to achieve medium concentrations of 1.0, 2.0, and 4.0 x 10^{-4} M and the slices incubated for another 15 min. Duplicate tissue samples were incubated simultaneously in a two-chambered Dubnoff metabolic shaker, one under a gaseous phase of 100% oxygen and the other under 100% nitrogen. The oxygen-requiring component of PAH transport was determined by calculating the difference between PAH uptake under oxygen and nitrogen. The rate of transport was expressed as micrograms PAH taken up per gram of tissue per minute of incubation.

Runout of PAH was determined using the method of Farah et al. (1963) with some of the modifications devised by Berndt (1965). Slices were preloaded by incubating 300-600 mg of tissue in 6 ml of medium containing 6.3 x 10^{-4} M PAH for 90 minutes. Tissue was removed from the incubation medium, rinsed, and placed in a net fashioned of nylon mesh. The tissue was transferred at 1-minute intervals with continuous shaking through a series of 20 beakers each containing 4.0 ml of PAH-free medium. At the conclusion of

the runout experiment, the tissue was removed from the net, blotted, weighed, and treated as before. Tissue and runout beakers were assayed for PAH and the results expressed as micrograms PAH remaining in the slices per 100 mg tissue.

2. Kinetic analysis of PAH uptake by separated proximal tubules

At 11 days of age treatment was begun. Procaine penicillin G was administered to rabbits in a dose of 90,000 I.U. twice daily for 2 days. Animals were killed 24 hours after the final injection. PAH uptake in separated proximal tubules was determined by preincubating an aliquot of suspension under 100% oxygen at 25°C for 15 minutes. PAH was then added to produce concentrations in the medium of 1, 4, and 8 x 10^{-4} M and the incubation continued for another 15 minutes. The tubule suspension was then centrifuged at 2°C for 10 minutes at 10,000 g in the special centrifuge tubes described by Burg and Orloff (1962) and the tissue pellet assayed for PAH. To estimate trapped medium in the pellet, inulin was added to the suspension immediately prior to centrifugation. Results were expressed as micrograms PAH per gram of tissue (dry weight).

3. Inhibitory effect of cycloheximide

The effect of cycloheximide on penicillin enhancement of PAH S/M was determined in rabbits by administering 0.18 mg cycloheximide (approx. 0.4 mg/kg) dissolved in saline, intraperitoneally, followed by 90,000 I.U. procaine penicillin G, subcutaneously, or saline for control. Two injections (12-hour intervals) of each drug were administered beginning on the 12th day of life. Animals were tested 24 hours after the final injection.

4. Proximal tubular ultrastructure

Adult female and 2-week littermate rabbits were used. Adults were not treated. Beginning on the 11th day of life, 90,000 I.U. procaine penicillin G was administered twice daily for 2 days. Animals were sacrificed 24 hours after the final injection. Aliquots of tubule suspension were prepared and shipped to the microscopy laboratory in a double blind fashion.[4]

The code was not broken until all analyses were complete. Tissue was prepared for electron microscopy by fixing the suspension of tubules in ice cold 1% osmium tetroxide in Millionig's buffer at pH 7.3 before dehydration in alcohol. After fixation, tubules were rinsed in buffer and embedded in epoxy resin by standard techniques. Sections for electron microscopy were stained with uranyl acetate and lead citrate by a modified Reynolds technique. Several sections of each tissue sample were taken and multiple photographs were developed from each section.

5. Na, K-ATPase

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 after the final injection. Na, K-ATPase activity was determined in a crude homogenate of separated tubules. Freshly prepared tubules were homogenized in a

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[4]Electron micrographs were prepared and analyzed by Dr. Jay Bernstein, Director, Department of Anatomic Pathology, William Beaumont Hospital, Royal Oak, MI 48072. Electron microscopic investigations were supported in part by the William Beaumont Hospital Research Institute and the Kidney Foundation of Michigan.

solution containing 0.25 M sucrose, 5 mM EDTA, and 30 mM histidine (pH 6.8). Enzyme activity was measured in medium containing 5 mM ATP, 30 mM Tris, and 5 mM $MgCl_2$ at pH 7.4. Half the beakers contained 115 mM NaCl and 10 mM KCl (total ATPase) and half contained neither NaCl nor KCl (Mg-ATPase) in a total volume of 3 ml. The incubation mixtures were preincubated for 5 minutes and the reaction was started by adding ATP. The reaction was allowed to proceed for 15 minutes and then stopped by adding 1.0 ml 10% TCA. After centrifugation, the supernatant was analyzed for phosphate (P_i) (Chen et al., 1956) and an aliquot of homogenate was analyzed for protein (Lowry et al., 1951). Na, K-dependent ATPase was represented as the difference between total and Mg-dependent ATPase. Results were expressed as micromoles of P_i released per milligram of protein in 10 minutes.

Evaluation of the Role of Glutathione (GSH) S-Transferases As Determinants of Renal Organic Anion Transport

1. Description of GSH S-aryltransferase assay technique

Animals were killed by a blow to the head and the kidneys removed. One kidney was minced in ice cold 0.25 M sucrose -0.01 M KH_2PO_4 (pH 7.4) buffer, rinsed and homogenized in 2 volumes of the same buffer. The second kidney was used for PAH S/M ratio determination as previously described. In very young animals kidneys from several pups were pooled.

GSH S-transferase concentration in renal tissue was estimated as GSH S-aryltransferase activity by the method of Booth et al. (1961) or Habig et al. (1974b). Whole kidney homogenates were

centrifuged at 2°C for 90 minutes at 100,000 x g in a Beckman L3-50 ultracentrifuge. The 100,000 x g supernatant was assayed for enzyme activity. Enzyme incubation medium contained 0.1 M pyrophosphate buffer (pH 8.0), 5 mM reduced glutathione and 1 mM 1,2-dichloro-4-nitrobenzene (dichloronitrobenzene) as substrate. Alternatively, 1-chloro-2,4-dinitrobenzene (dinitrochlorobenzene) was used as substrate in a medium of 0.1 M potassium phosphate (pH 6.5), 1 mM reduced glutathione and 1.0 mM dinitrochlorobenzene. Three milliliter aliquots of medium were preincubated for 3 minutes. One hundred microliters of enzyme preparation was added and initial optical density readings recorded. Enzyme activity was measured at 37°C and 25°C and optical density measured at 344 and 340 nm for dichloronitrobenzene and dinitrochlorobenzene substrate, respectively. Dilutions of 100,000 x g supernatant using sucrose-phosphate buffer were required when dinitrochlorobenzene was used as substrate to maintain OD changes within measurable limits. After 15 minutes incubation final optical density was determined. Sucrose-phosphate buffer was used as blank. Product formation was estimated from the change in optical density during incubation minus non-enzymatic formation determined in the blank. Protein concentration in the 100,000 x g supernatant or a dilution of the supernatant was estimated by the method of Lowry et al. (1951). Data were expressed as net change in optical density per milligram protein per 15 minute incubation.

2. Studies on possible correlations between PAH transport capacity and GSH S-transferases

a. Effect of age. The ability of renal cortical slices to accumulate PAH and GSH S-transferase concentration in renal cortical homogenates was quantified using rat and rabbit pups randomly selected from litters at the designated ages. PAH S/M ratio and enzyme activity were determined in kidneys from the same animal except in 1-day rats where kidneys from 4 pups were pooled. Rabbits 1 day, 2 weeks and 4 weeks old and rats 1 day, 1 week and adult were used.

b. Effect of 3-methylcholanthrene. Weanling rats weighing approximately 50 g and 2-week-old rabbits were used. 3-Methylcholanthrene (3-MC) was dissolved in corn oil and injected intraperitoneally. Controls received a comparable volume of corn oil. Rats were administered 25 mg/kg once daily for 3 days. Rabbits were treated with 10, 20, 30 or 40 mg/kg twice daily for 3 days beginning on the 11th day of life. All animals were killed 24 hours after the final injection.

c. Effect of 2,3,7,8 tetrachlorodibenzo-p-dioxin. Adult rats weighing approximately 150 g were injected intraperitoneally with 10 or 25 µg/kg 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) dissolved in acetone-corn oil (1:9). Alternatively, 1 or 5 µg TCDD was administered intragrastrically using a gavage needle. Volume was never greater than 1 ml when administered orally. Controls received vehicle alone. Animals were allowed free access to food and water. S/M ratios were determined 3 or 7 days after treatment using renal

cortical slices taken in a plane both parallel and perpendicular to the major renal axis. Extracellular water in the slices was estimated by inulin space. Inulin in tissue and medium supernatant was assayed by the method of Schreiner (1950).

d. Effect of chronic metabolic acidosis. Adult rats weighing approximately 150 g were made acidotic by supplying 0.28 M ammonium chloride as sole drinking fluid for 7 days. Controls were allowed tap water. Animals had free access to food and were killed on the 7th day of treatment.

e. Effect of substrate stimulation by penicillin. Rabbits were injected subcutaneously with 90,000 I.U. procaine penicillin twice a day for 2 days. Animals were sacrificed 24 hours after the final injection.

f. Sex difference. PAH S/M ratio in adult male and female mice was determined by incubating slices in medium containing 7.4 x 10^{-5} M ^{14}C-PAH (sp. act. 1.4 μCi/μmole) and measuring radioactivity in tissue and medium supernatants using liquid scintillation spectrometry.

g. Effect of uninephrectomy. Adult rats weighing approximately 150 g were lightly anesthetized with ether and the left kidney removed through a flank incision. Caution was exerted not to disturb the adrenal. The abdominal wall was sutured using 00 silk and the skin incision closed with wound clips. Control animals were sham operated and the kidney left undisturbed. The animals were killed 48 hours after surgery. Either PAH S/M ratio or GSH

S-aryltransferase activity were determined in the remaining kidney. Only the unoperated kidney was used in sham operated animals.

3. PAH transport capacity following GSH depletion

Adult rats weighing approximately 150 g were used. L-methionine-SR-sulfoximine was dissolved in distilled water and administered intraperitoneally in a dose of 1.85 mmoles/kg. Controls received saline. After 2 hours animals were killed by a blow to the head and the kidneys removed. Renal cortical slices from both kidneys were pooled and divided into 3 portions. One was assayed for glutathione and the others incubated for 60 minutes at 25°C under 100% oxygen in phosphate buffer medium as described earlier. Medium in one incubation vessel contained 7.4×10^{-5} M PAH for determination of PAH S/M ratio. The other contained no PAH and was used for determination of tissue glutathione concentration after incubation. Glutathione was estimated by the method of Hewitt et al. (1974). A 5% homogenate of renal cortex in 6% TCA was spun for 10 minutes at 3000 rpm in an International centrifuge. Aliquots of supernatant were diluted to 2.0 ml with 6% TCA and 8.0 ml 0.3 M Na_2HPO_4, 1.0 ml 0.04% 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNTB) in 10% sodium citrate added. GSH was quantified as the increase in absorbance at 412 nm.

4. Effect of age and penicillin pretreatment on penicillin binding to GSH S-transferases

Binding of penicillin to soluble proteins was estimated in adults and 2-week-old rabbits. The effect of substrate stimulation on binding was determined in 2-week-old pups treated with 90,000 I.U.

procaine penicillin G subcutaneously twice daily for 2 days. Animals were sacrificed 24 hours after the final injection. Controls received saline.

Kidneys were minced in ice cold 0.25 M sucrose-0.01 M KH_2PO_4 (pH 7.4) and rinsed several times. Tissue was homogenized in a further aliquot of buffer. Homogenate was centrifuged at 100,000 x g for 90 minutes at 2°C. An aliquot of 100,000 x g supernatant containing 50 mg soluble protein was taken, to which was added 0.88 µmole penicillin (0.57 µCi/µmole) and 1 mM reduced glutathione. Cold sucrose-phosphate buffer was added to make the final volume 5 ml. The mixture was allowed to stand in ice for 4 hours and layered on a 3 x 100 cm Sephadex G-100 column equilibrated wtih 0.01 M KH_2PO_4 (pH 7.4). Flow rate was adjusted to 15 ml/hr by varying the height of the buffer reservoir and 3 ml samples of eluate collected. Samples were assayed for protein by the method of Lowry (1951). Radioactivity was determined in 1 ml fractions of eluant samples which were added to 10 ml modified Bray's and counted in a Beckman LS-100 liquid scintillation spectrometer. Data were represented as mg/ml protein and CPM/ml ^{14}C-penicillin in each sample. GSH S-transferase concentration in eluant fractions was estimated as GSH S-aryltransferase activity using dinitrochloro-benzene as enzyme substrate. Data were expressed as ΔOD/mg protein/ 15 minutes.

Incorporation of Amino Acids and Protein Synthesis Following Substrate Stimulation by Penicillin

1. Description of techniques for partial purification and quantification of DNA, RNA and protein

Lipid, RNA and DNA were extracted from TCA precipitates of tissue by a modified Schneider procedure (Fleck and Munro, 1962; Schneider, 1957). Renal cortical tissue was homogenized in 10% TCA and the insoluble pellet resuspended in 10 ml 95% ethanol. The suspension was allowed to stand at room temperature for 30 minutes and centrifuged at 2°C in a Beckman J-21 centrifuge at 10,000 rpm. After centrifugation the ethanol extraction was repeated. The pellet was resuspended in 10 ml ethanol:ether (3:1) and heated in a 60°C water bath for 30 minutes. After heating the suspension was allowed to stand in ice for 20 minutes and centrifuged. The pellet was solubilized in 2 ml 1 N KOH and incubated at 37°C for approximately 14 hours. After incubation 0.4 ml 6 N HCl and 2 ml 5% TCA were added. The suspension stood in ice for 30 minutes. After centrifugation the supernatant was assayed for riboses by the orcinol procedure (Keleti and Lederer, 1974). The acid insoluble precipitate was resuspended in 10 ml 5% TCA and heated in a 90°C water bath for 20 minutes. The suspension stood in ice for 30 minutes after heating and the supernatant was assayed for 2-deoxy-sugars by the diphenylamine reaction (Burton, 1956; Keleti and Lederer, 1974). The final pellet was solubilized in 1 N KOH and aliquots taken for protein determination by the method of Lowry (1951). When amino acid incorporation into protein was determined, 200 µl solubilized material was neutralized with 1 N HCl and counted

in 10 ml PCS liquid scintillation cocktail (Amersham Searle). Data were represented as DPM/mg protein or DPM/µg DNA.

The effect of age and penicillin pretreatment on DNA/protein, RNA protein and RNA/DNA ratios in rabbits was also determined. Adults were used without treatment. Young rabbits were treated with 90,000 I.U. procaine penicillin G twice daily for 2 days beginning on the 11th day of life and killed 24 hours after the final injection.

2. Incorporation of ^{14}C L-leucine *in vitro*

Littermate rabbits were treated with 90,000 I.U. procaine penicillin G subcutaneously twice daily for 2 days beginning on the 11th day of life. Animals were killed and renal cortical slices prepared 24 hours after the final injection. Incorporation of amino acids into separated proximal tubular protein from control and penicillin treated rabbits was determined 3 and 24 hours after treatment.

Tissue slices were incubated in phosphate buffer medium containing 2.7×10^{-4} M L-leucine (sp. act. 0.14 µCi/µmole) for 60 minutes at 25°C under 100% oxygen. Separated proximal tubules were incubated in medium containing 1.26×10^{-4} M L-leucine (sp. act. 0.27 µCi/µmole) for 60 minutes at 25°C while being constantly gassed and mixed by bubbling with 100% oxygen. After incubation tissue was homogenized in 10% TCA and lipid, RNA and DNA extracted. The final protein pellet was solubilized and assayed for protein and radioactivity.

3. Incorporation of ^{14}C L-leucine *in vivo*

Rabbits were treated with either 1 or 3 injections of 90,000 I.U. procaine penicillin G at 12-hour intervals. Treatment was begun such that the experiment was run on the 14th day of life. All animals were sacrificed 16 hours after the final injection.

Amino acid uptake was quantified by anesthetizing animals with 2 g/kg urethane, i.p. The left femoral vein was exposed and 8 μCi/kg ^{14}C L-leucine (sp. act. 302 μCi/μmole) in normal saline injected. Fifteen minutes after injection of label the animals were killed, the kidneys quickly removed, and cortex dissected free. Cortical tissue was minced in ice cold 0.25 M sucrose, washed with several changes of 0.25 M sucrose and homogenized. The homogenate was centrifuged at 10,000 x g for 20 minutes at 2°C and the pellet discarded. The supernatant was centrifuged at 100,000 x g for 60 minutes at 2°C in a Beckman L3-50 ultracentrifuge. Following centrifugation a sample of supernatant was precipitated with an equal volume of cold 10% TCA, allowed to stand in ice for 30 minutes and the pellet washed three times by resuspension with TCA. The 100,000 x g pellet was resuspended in 0.25 M sucrose and centrifuged at 100,000 x g for 60 minutes at 2°C. Following centrifugation the supernatant was discarded and the pellet solubilized in 2 ml 1 N KOH. The precipitate from the soluble fraction was treated similarly. Solubilized material was incubated at 37°C for approximately 20 hours and reprecipitated by addition of 0.4 ml 6 N HCl and 2 ml 5% TCA. The resulting pellets were washed 3 times with 3 ml cold 10% TCA and solubilized in 1 ml 1 N KOH. Aliquots were taken for assay of protein by the method of Lowry (1951) and radioactivity by liquid

scintillation spectrometry. Results were represented as DPM/mg protein.

In an alternate experiment animals were injected i.v. with ^{14}C L-leucine as described and sacrificed after 60 minutes. Cortex was dissected free and homogenized in 10% TCA. The acid insoluble pellet was extracted for lipid, RNA and DNA as described earlier. Results were represented as DPM/mg protein.

4. Leucine pool size[5]

The specific activity of amino acid pools was determined in animals treated with 1, 2 or 3 injections of penicillin. Sixteen hours after the final injection 8 μCi/kg ^{14}C L-leucine (sp. act. 302 μCi/μmole) were injected i.v. and the animals sacrificed after 15 minutes. Kidneys were perfused with 20 ml ice cold physiological saline and the cortex dissected free. One gram cortical tissue was homogenized in 10 ml 5% sulfosalicylic acid and kept in ice for 30 minutes. The suspension was centrifuged at 10,000 x g for 20 minutes at 2°C and the supernatant was dried. The residue was dissolved in 2 ml lithium citrate buffer, pH 2.0. Aliquots were taken for ^{14}C determination using liquid scintillation spectrometry. Leucine was determined using a Technicon Autoanalyzer. Results were represented as DPM/nmole leucine.

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[5]Leucine determinations were conducted by Dr. W. Bergen, Department of Animal Husbandry, Michigan State University, East Lansing, MI 48824.

Separation of Protein Fractions of Renal Cortical Homogenates Following Substrate Stimulation by Penicillin and *in vivo* Labeling with ^{14}C L-leucine

1. Labeling of renal cortical protein and subcellular fractionation

Rabbits were treated with 90,000 I.U. procaine penicillin G subcutaneously on the 13th day of life and sacrificed 16 hours after the injection. Animals were anesthetized with 2 g/kg urethane and the left femoral vein exposed. Three injections of 8 µCi/kg ^{14}C L-leucine (sp. act. 302 µCi/µmole) were administered intravenously at 20-minute intervals. Twenty minutes after the third pulse animals were killed, kidneys quickly removed and cortex dissected free. Cortical tissue was minced and washed in 0.25 M sucrose. Minced tissue was homogenized in 0.25 M sucrose and the homogenate centrifuged at 10,000 x g for 20 minutes at 2°C. The supernatant was centrifuged at 100,000 x g for 90 minutes at 2°C. Supernatant and pellet proteins were analyzed by Sephadex filtration and gel electrophoresis, respectively.

2. Sephadex filtration

A 5 ml aliquot of 100,000 x g supernatant was layered on a 3 x 100 cm Sephadex G-100 column equilibrated with 0.01 M KH_2PO_4 (pH 7.4). Elution rate was 15 ml/hr and 3 ml samples were collected. Protein and radioactivity concentrations in eluant fractions were determined by standard procedures and data plotted as CPM and mg/ml protein versus sample number.

3. Gel electrophoresis

The 100,000 x g pellet was resuspended in sufficient 10 mM Tris 1 mM EDTA (pH 8.0) to result in a final concentration of from 3 to 8 mg/ml protein. The suspension was dialyzed against 10 mM Tris 1 mM EDTA pH 7.5 overnight to remove K^+. The final electrophoresis sample was 1% sodium dodecyl sulfate (SDS), 7% sucrose, 10 mM Tris, 1 mM EDTA and contained from 2-5 mg/ml protein. Immediately prior to electrophoresis sufficient concentrated mercaptoethanol was added to result in 2% final concentration and the sample heated in boiling water for 15 minutes. After cooling, bromphenol blue was added as tracking dye.

Polyacrylamide gels (5-6% acrylamide) containing 1% SDS at pH 7.4 were prepared by the method of Fairbanks et al. (1972) in glass tubes 6 mm inner diameter by 75 mm in length. Gels were electrophoresed for 30 minutes in buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, and 1% SDS (pH 7.4) prior to addition of sample. A 20-30 µl sample was layered on the gel and electrophoresed at 5 mA/gel. Usually 4 hours were required for the tracking dye to migrate the length of the gel. Gels were removed from the tubes, fixed in 10% TCA overnight, and stained in a solution of 0.4% Coomassie Brilliant Blue and 5% TCA. Gels were destained using a Bio-Rad diffusion destainer in 10% TCA, 33% methanol solution. Alternatively, gels were sliced using a Bio-Rad gel slicer immediately following TCA fixation. Gel slices were heated at 50°C in 1 ml Soluene 100 for 3 hours and allowed to stand at room temperature overnight. Radioactivity was determined by

liquid scintillation spectrometry after addition of 10 ml PCS liquid scintillation cocktail (Amersham-Searle).

Statistical Analyses

Data were analyzed statistically by either Student's *t*-test, paired or group comparison, or randomized complete block analysis of variance. Treatment means were tested using the Student Newman Keul's test (Steels and Torrie, 1961). The 0.05 level of probability was used as the criterion of significance.

RESULTS

Pharmacodynamic Analysis of Substrate Stimulation

1. Effect of dosage and treatment schedule

PAH S/M ratio in 2-week rabbit kidney cortex was enhanced by the administration of both procaine penicillin G and sodium penicillin G. A dose-dependent relationship existed between penicillin dose and PAH S/M ratio in both cases. Maximal accumulation of PAH by kidney slices occurred after treatment with 90,000 I.U. of procaine penicillin in the presence of 10 mM sodium acetate. The presence of acetate in the incubation medium increased the S/M ratio in slices from treated animals but did not alter the dose-response relationship (Figure 1). Increasing the dose of penicillin to 180,000 I.U. with the soluble form increased the PAH S/M ratio above control (Figure 2), but the magnitude of the stimulation was no greater than that observed after 90,000 I.U. of procaine penicillin G (Figure 1).

The effect of penicillin on PAH S/M ratio appeared maximal after 2 injections of 90,000 I.U. of procaine penicillin G (Figure 3). Continuing treatment for 4 days (8 injections) produced no greater effect. Enhancement of PAH S/M ratio after penicillin was greatest 24 hours after the final injection, although the effect was significant at both 12 and 36 hours (Figure 4). After 72 hours,

Figure 1. Effect of procaine penicillin dose on accumulation of PAH by rabbit renal cortical slices. Procaine penicillin G was administered twice daily for 3 days. Rabbits were 10 days old at the beginning of treatment and were killed 24 hours after the last injection. Each bar represents the mean (± S.E.) obtained from 3 litters.

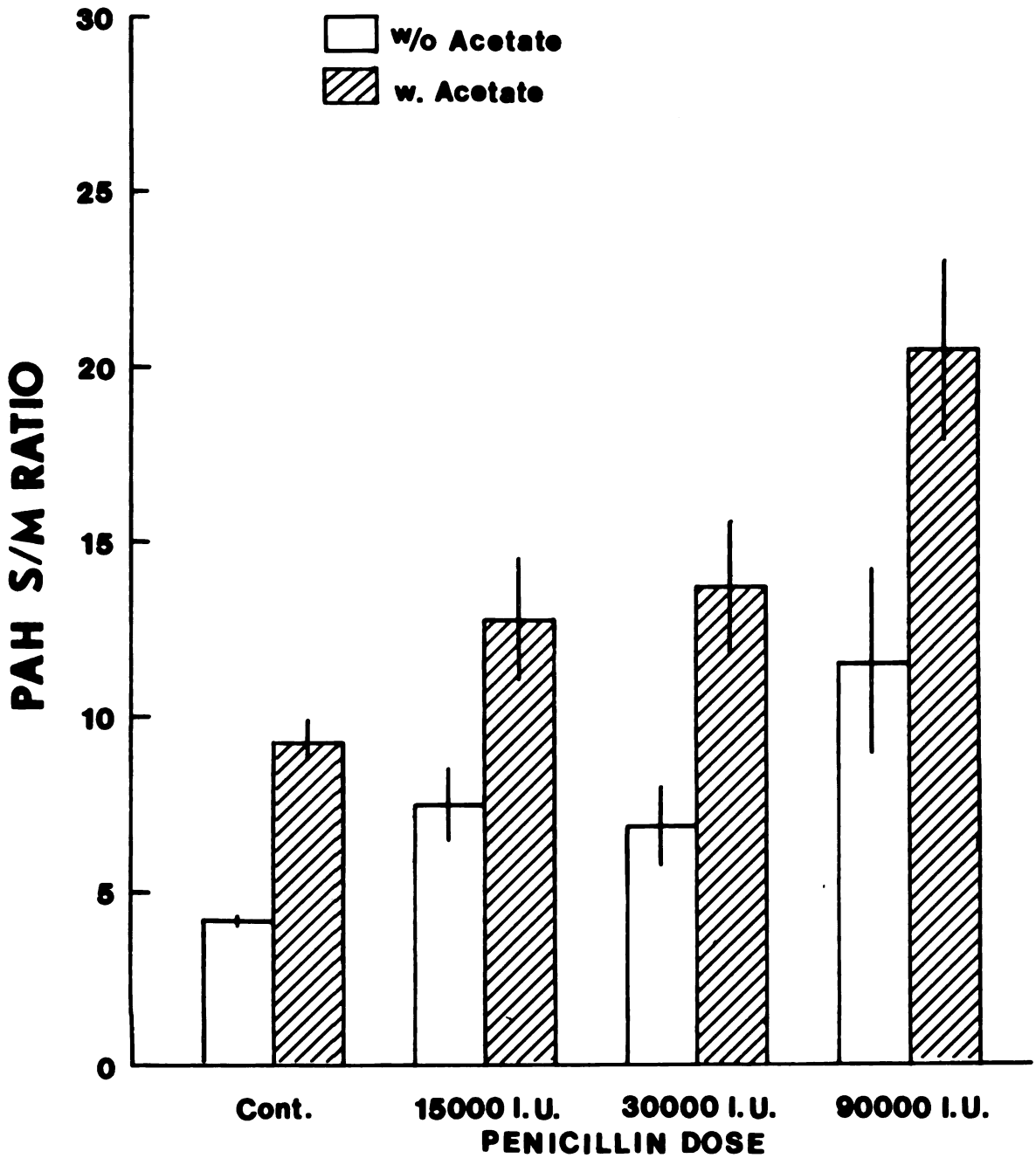
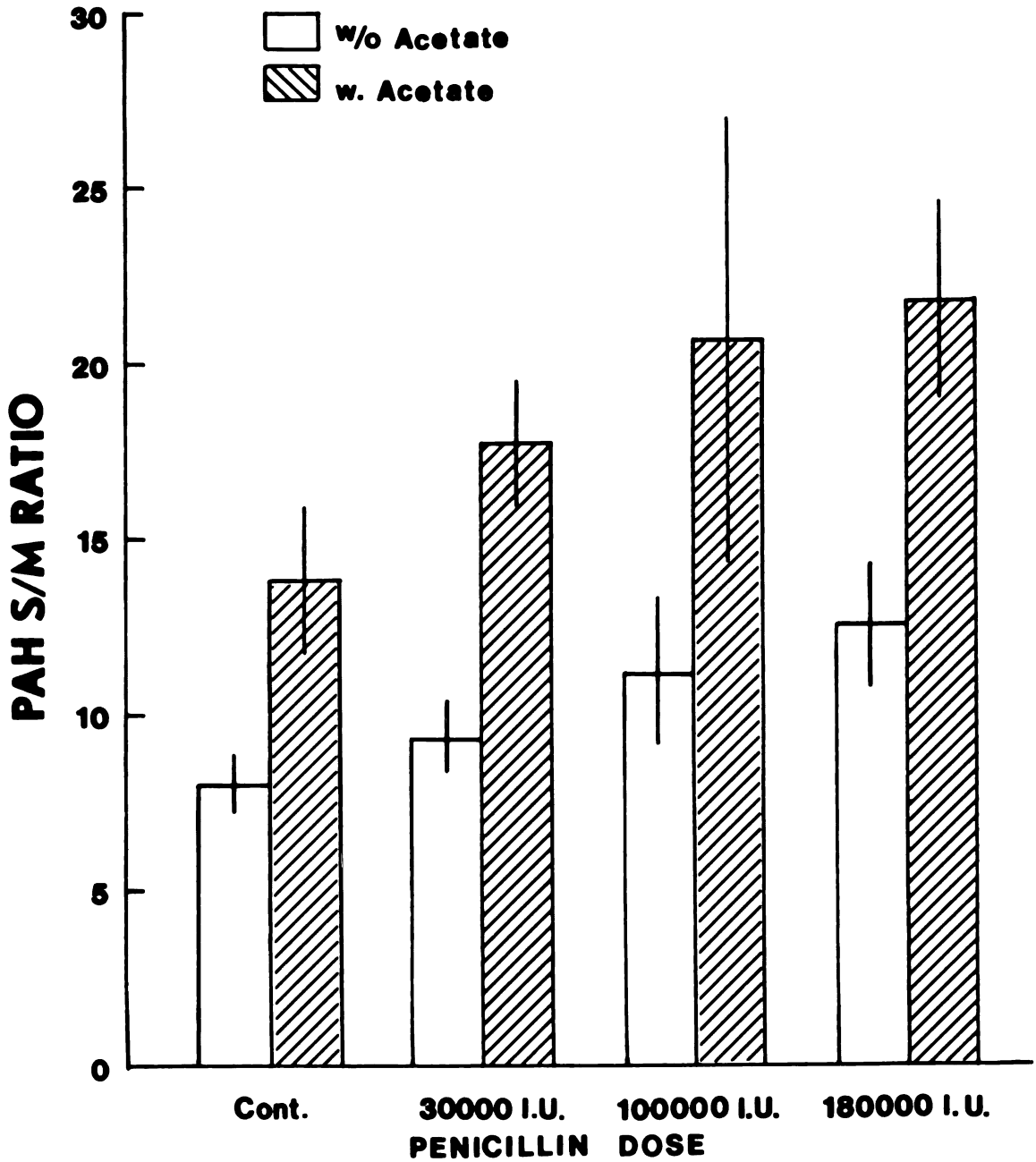

w/o Acetate
w. Acetate
PAH S/M RATIO
30
25
20
15
10
5
0
Cont.
15000 I.U.
30000 I.U.
90000 I.U.
PENICILLIN DOSE

Figure 1

Figure 2. Effect of sodium penicillin dose on accumulation of PAH by rabbit renal cortical slices. Sodium penicillin G was administered intraperitoneally following the same regimen as in Figure 1. Each bar represents the mean (± S.E.) obtained from 3 litters.


30
25
20
15
10
5
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PAH S/M RATIO
w/o Acetate
w. Acetate
Cont.
30000 I.U.
100000 I.U.
180000 I.U.
PENICILLIN DOSE

Figure 2

Figure 3. Effect of treatment duration on enhancement of PAH S/M ratio. Beginning on the 10th day of life, rabbits were administered 2, 4, 6 or 8 injections of 90,000 I.U. procaine penicillin G at 12-hour intervals. Animals were sacrificed 24 hours after the final injection. Each bar represents mean (± S.E.) obtained from 3 litters.

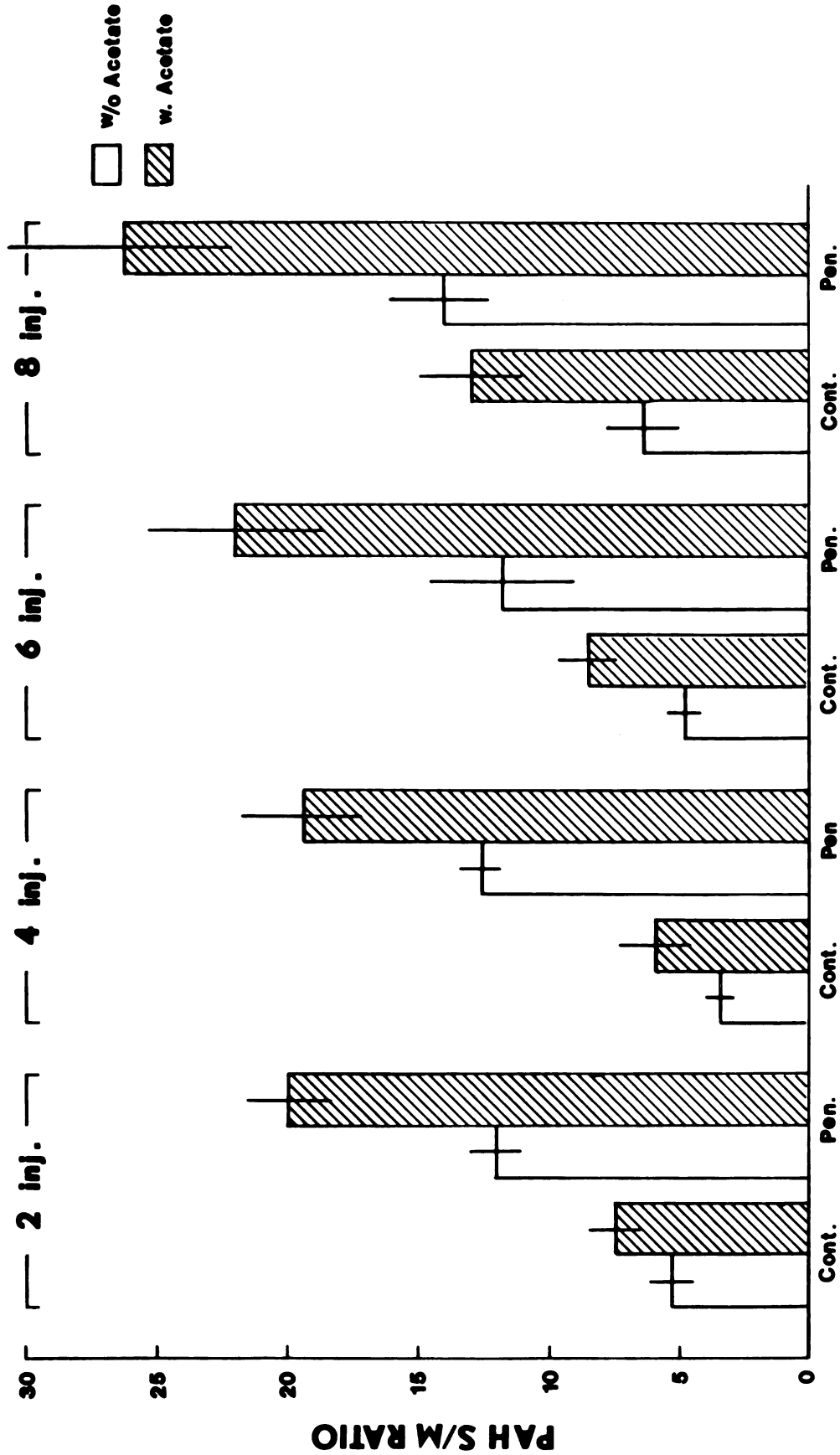

w/o Acetate
w. Acetate
2 inj.
4 inj.
6 inj.
8 inj.
Cont.
Pen.
Cont.
Pen.
Cont.
Pen.
Cont.
Pen.
PAH S/M RATIO
0
5
10
15
20
25
30

Figure 3

Figure 4. Accumulation of PAH by rabbit renal cortical slices 12, 24, 36 and 72 hours after termination of treatment. Beginning on the 11th day of life, rabbits were treated with 90,000 I.U. of procaine penicillin G subcutaneously twice daily for 2 days. Each bar represents mean (± S.E.) obtained from 3 litters.

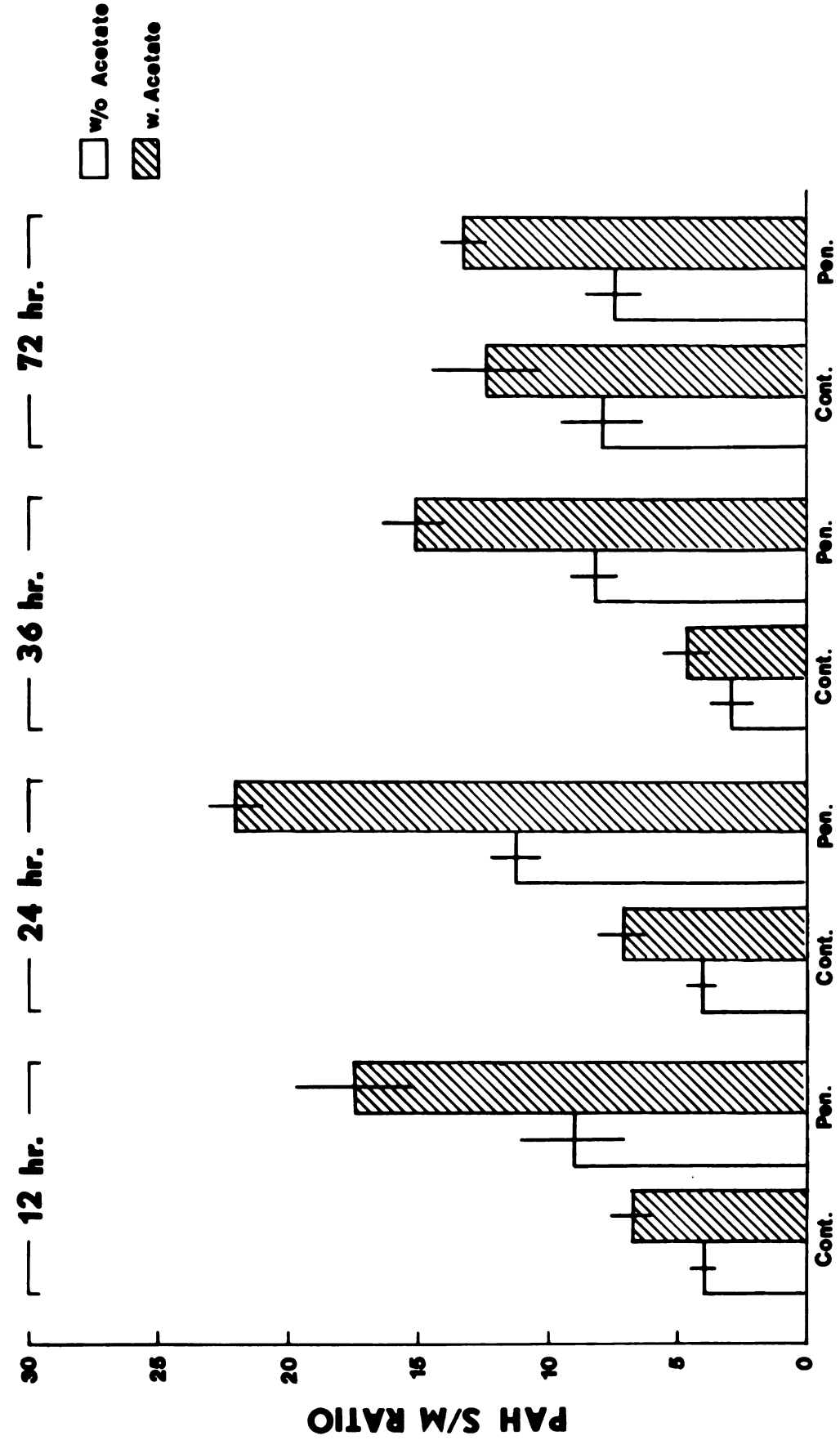

w/o Acetate
w. Acetate
12 hr.
24 hr.
36 hr.
72 hr.
Cont.
Pen.
Cont.
Pen.
Cont.
Pen.
Cont.
Pen.
PAH S/M RATIO
0
5
10
15
20
25
30

Figure 4

there was no significant difference between control and penicillin-treated tissue with or without acetate.

PAH S/M ratio was significantly increased 8 and 24 hours after a single 90,000 I.U. injection of procaine penicillin (Figure 5). Though PAH S/M in penicillin treated slices was greater than control after 12 and 16 hours, the stimulation was not significant due to high variability and low number of replications. Maximal stimulation following a single injection occurred 24 hours after treatment. There was no significant difference between control and treated after 48 hours.

No radioactivity over background was observed in renal cortical slices 24 hours after the injection of 1 μCi of ^{14}C-benzyl penicillin. All of the tissues except bladder urine were similarly devoid of any activity. Samples from bladder urine contained measurable, although physiologically insignificant, traces of radioactivity 24 hours after injection.

2. Substrate stimulation during development

The effect of acute administration of penicillin on PAH S/M ratio was determined in animals whose mothers had received chronic penicillin treatment during gestation. S/M ratio in the pups born of these mothers was significantly greater ($P<.05$) than the S/M ratio normally observed at birth (Hirsch and Hook, 1970c). When littermates were treated acutely with procaine penicillin G the S/M ratio was significantly elevated at 3 days, 1 and 2 weeks of age (Figure 6). There was no significant difference in the S/M ratio of the treated animals. That is, the PAH S/M ratio was as high at

Figure 5. Accumulation of PAH by rabbit renal cortical slices 2, 4, 8, 12, 16, 24 and 48 hours following penicillin treatment. On the 12th day of life, rabbits received a single injection of 90,000 I.U. procaine penicillin G or saline for control. The upper panel represents PAH S/M ratio versus time for control and penicillin treated animals. The difference in mean value of S/M ratios from control and treated animals is plotted versus time in the lower panel. Each S/M value represents the mean ($\pm$ S.E.) of at least 3 determinations.

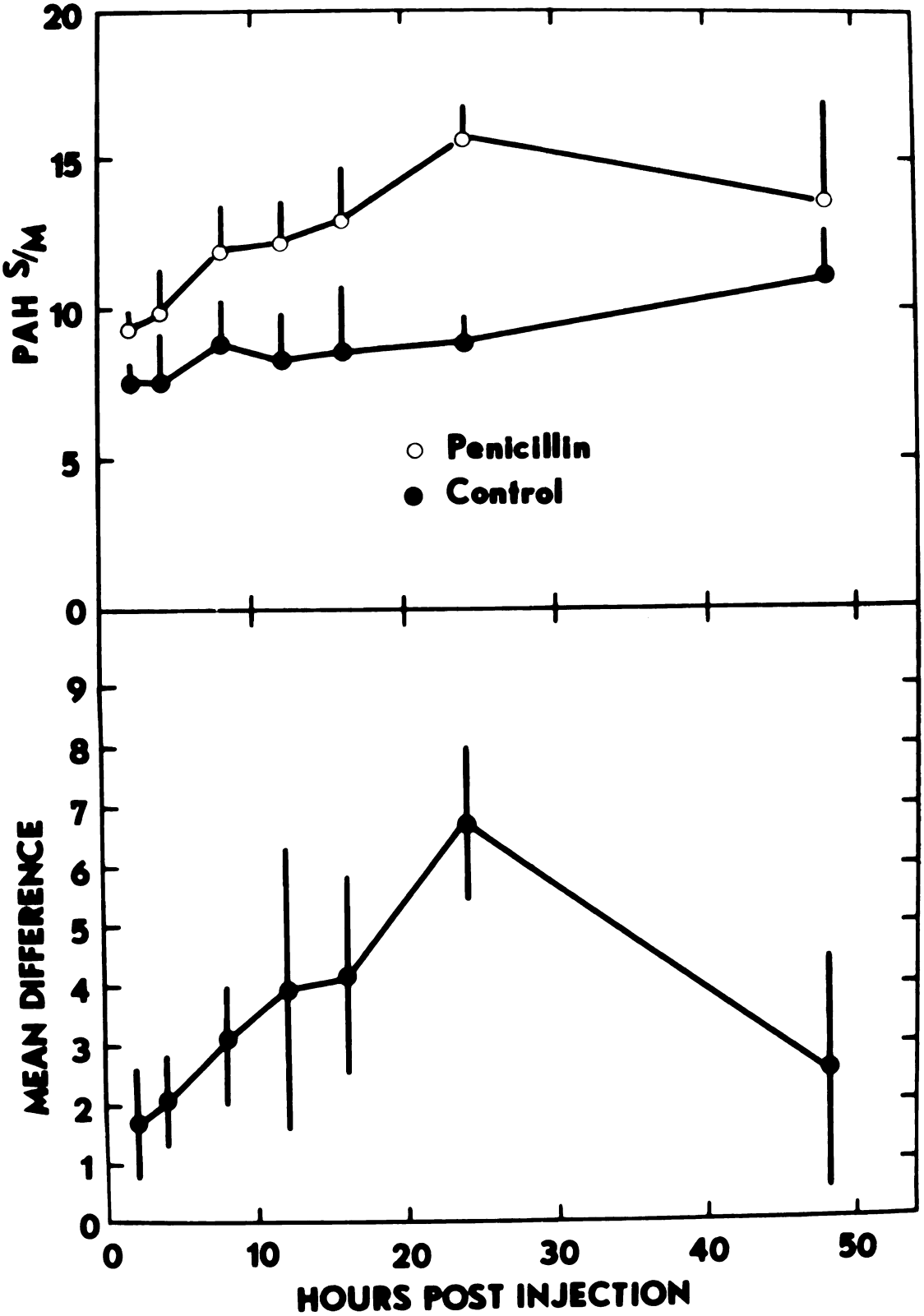

20
15
10
5
0
PAH S/M
Penicillin
Control
9
8
7
6
5
4
3
2
1
0
MEAN DIFFERENCE
0
10
20
30
40
50
HOURS POST INJECTION

Figure 5

Figure 6. Effect of penicillin on development of PAH S/M by rabbit renal cortical slices. Pregnant does were injected intramuscularly with 90,000 I.U. of procaine penicillin G twice daily from day 14 of gestation to delivery. Within a litter, pups were sampled at 3 days, 1, 2 and 4 weeks. Two days before experimentation, a pair of pups was randomly selected. One member of the pair received 2 injections of 90,000 I.U. procaine penicillin G at 12-hour intervals and the other served as control. Animals were sacrificed 24 hours after the second injection. O, accumulation of PAH in cortical tissue from pups receiving prenatal and postnatal penicillin; ●, animals receiving prenatal penicillin and postnatal saline. Each point represents the mean (± S.E.) obtained for 3 litters.

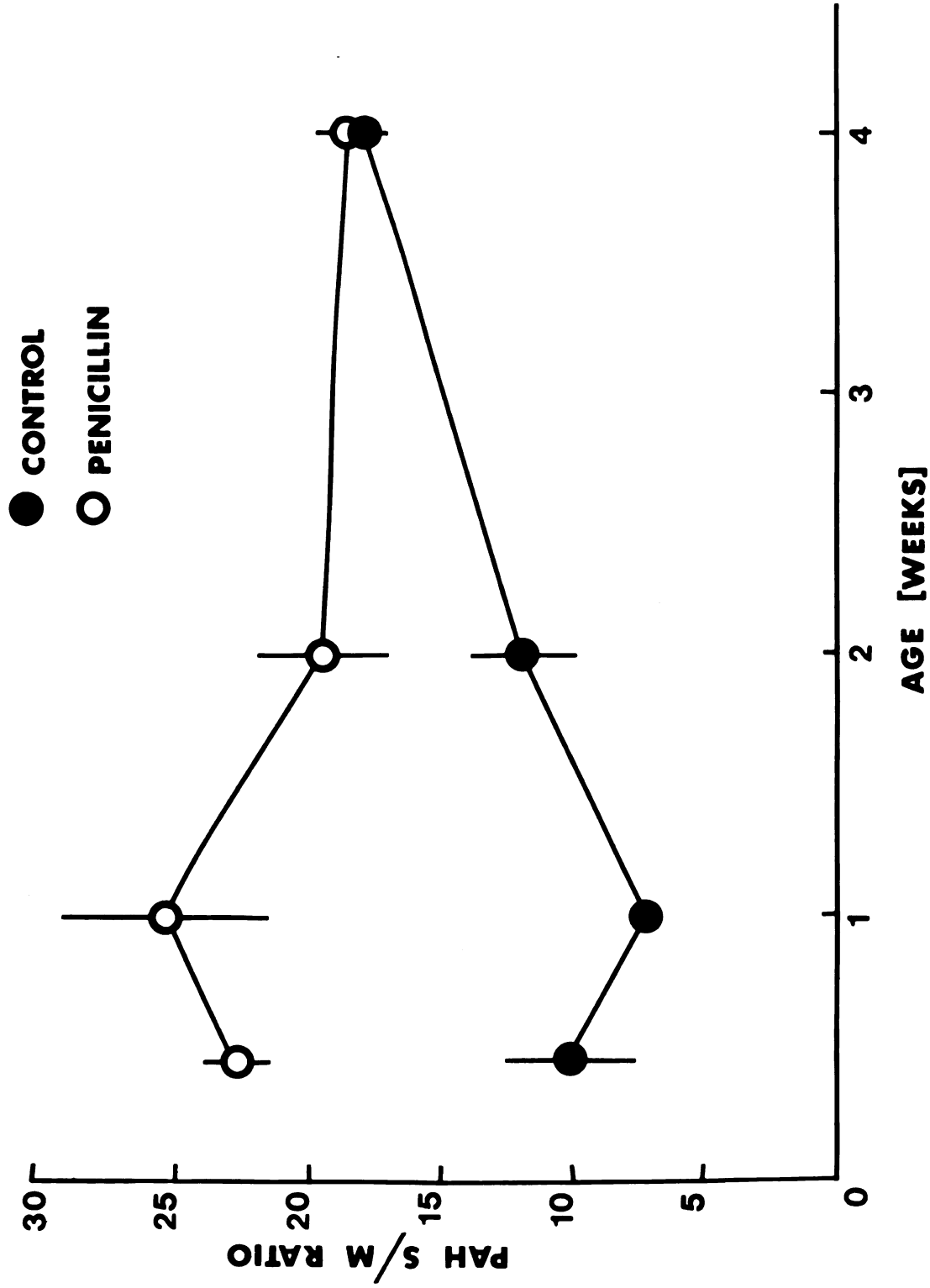

CONTROL
PENICILLIN
PAH S/M RATIO
30
25
20
15
10
5
0
1
2
3
4
AGE [WEEKS]

Figure 6

3 days of age as at 4 weeks. Animals 4 weeks of age did not respond to acute treatment with penicillin.

Investigation of Mechanisms in Penicillin Stimulation of PAH Transport

1. Uptake and efflux of PAH by slices

The rate of PAH uptake increased with increasing substrate concentration. Renal cortical slices from penicillin-treated 2-week rabbits took up 2.50 $\pm$ 0.14, 3.65 $\pm$ 0.10, and 5.51 $\pm$ 0.09 µg PAH g^{-1} min^{-1} at medium concentrations of 1.0, 2.0, and 4.0 x 10^{-4} M, respectively (Figure 7). The rate of PAH uptake in these slices was significantly greater at each of the medium concentrations than in slices from control animals (1.90 $\pm$ 0.10, 3.02 $\pm$ 0.17, and 4.87 $\pm$ 0.17 µg g^{-1} min^{-1}). The rate of PAH uptake under nitrogen was not affected by penicillin pretreatment (Figure 7). The difference in uptake between oxygen and nitrogen incubation, the best estimate of the oxygen-requiring, active component of uptake, was significantly enhanced by penicillin at each concentration (Figure 7).

The runout of PAH from preloaded slices exhibited an initial fast component followed by a slower component. The data were linear when plotted on a semilogarithmic scale (Figure 8). First-order rate constants were calculated for the linear portion of the curve. The runout constant for control tissue (0.022 $\pm$ 0.002 min^{-1}) was not significantly different from that for penicillin-treated tissue (0.023 $\pm$ 0.003 min^{-1}). Farah et al. (1963) suggested that the rapid component of PAH runout was due to a loosely bound intracellular pool. Contributions to enhanced PAH accumulation following penicillin might be made by either decreased runout or enhanced intracellular

Figure 7. p-Aminohippurate (PAH) uptake in 2-week control and penicillin-pretreated rabbit renal cortical slices. Beginning on the 10th day of life, animals received 30,000 I.U. procaine penicillin G twice daily for 3 days. Animals were killed 24 hours after the final injection. In each experiment, slices from control and treated animals within a litter were pooled and distributed into 12 beakers, 4 at each concentration of PAH and 2 under each gaseous phase. Duplicate values were averaged. Each bar represents means ($\pm$ S.E.) of pups from 8 litters. Vertical bars represent S.E. of O_2-N_2 uptake.

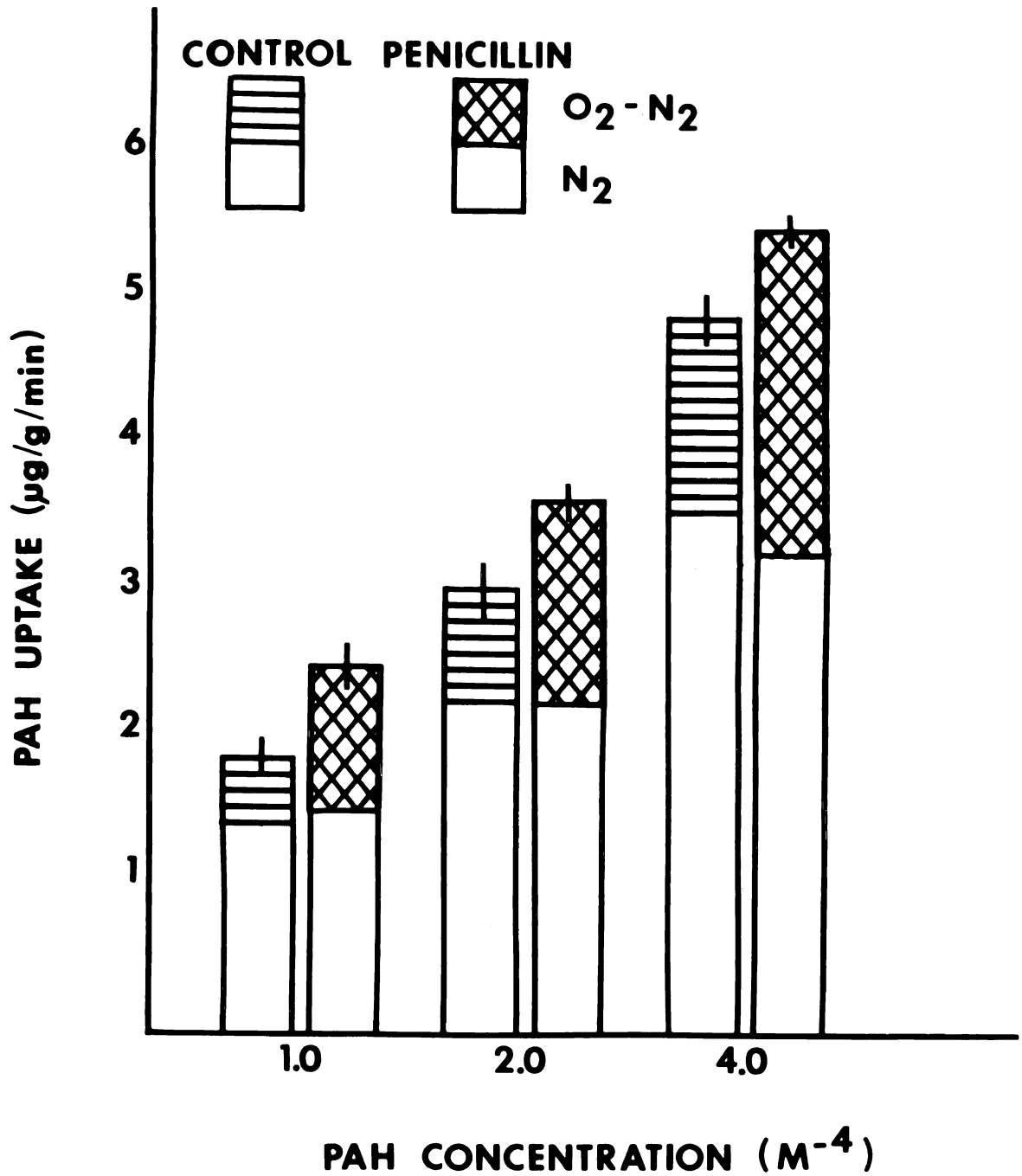

CONTROL
PENICILLIN
$O_2 - N_2$
N_2
PAH UPTAKE (µg/g/min)
1
2
3
4
5
6
1.0
2.0
4.0
PAH CONCENTRATION (M^{-4})

Figure 7

binding. Both phenomena would result in a slower rate of PAH runout. These possibilities may be excluded since an alteration in runout was not observed (Figure 8).

2. Kinetic analysis of PAH uptake in separated proximal tubules

Penicillin treatment of newborn rabbits significantly increased the rate of PAH uptake at each PAH concentration in the medium when compared to saline control. A double reciprocal plot of the data (Figure 9) suggested that there was an increase in the apparent maximal rate of uptake from 3.03 in the control to 6.25 µg/g/min after penicillin treatment. There appeared to be, however, no change in the apparent affinity of the carrier for substrate (2.10×10^{-4} M).

3. Inhibitory effect of cycloheximide

Administration of 0.18 mg of cycloheximide concurrently with 90,000 I.U. of procaine penicillin effectively blocked the stimulating effect of penicillin on PAH accumulation. Cycloheximide had no effect on control PAH S/M (Figure 10).

4. Proximal tubular ultrastructure

Treatment of 2-week rabbit kidneys with collagenase produced a homogeneous suspension of tubules, with no significant contamination with connective tissue or undifferentiated cells. It was evident from electron microscopy that proximal tubular cells from 2-week animals were differentiated to a degree comparable to adult tissues (Figures 11 and 12). Brush border, apical vacuoles, and intracellular organelles were well formed in the young animals. The brush border was less dense, however, than in the adult, and the basilar plasma membrane was less infolded. The basement membranes,

Figure 8. Runout of p-aminohippurate (PAH) from renal cortical slices from control and treated littermates. Beginning on the 10th day of life, animals received 30,000 I.U. procaine penicillin G twice daily for 3 days. Animals were killed 24 hours after the final injection. Slices were preloaded with PAH for 90 minutes, rinsed, and transferred through a series of beakers containing no PAH at 1-minute intervals. Concentration of PAH in the slices as a function of runout time was determined and a first-order rate constant (K) calculated. Points and regression lines represent means from 4 litters. Constants represent means $\pm$ S.E. and are not significantly different from each other ($P<0.05$).

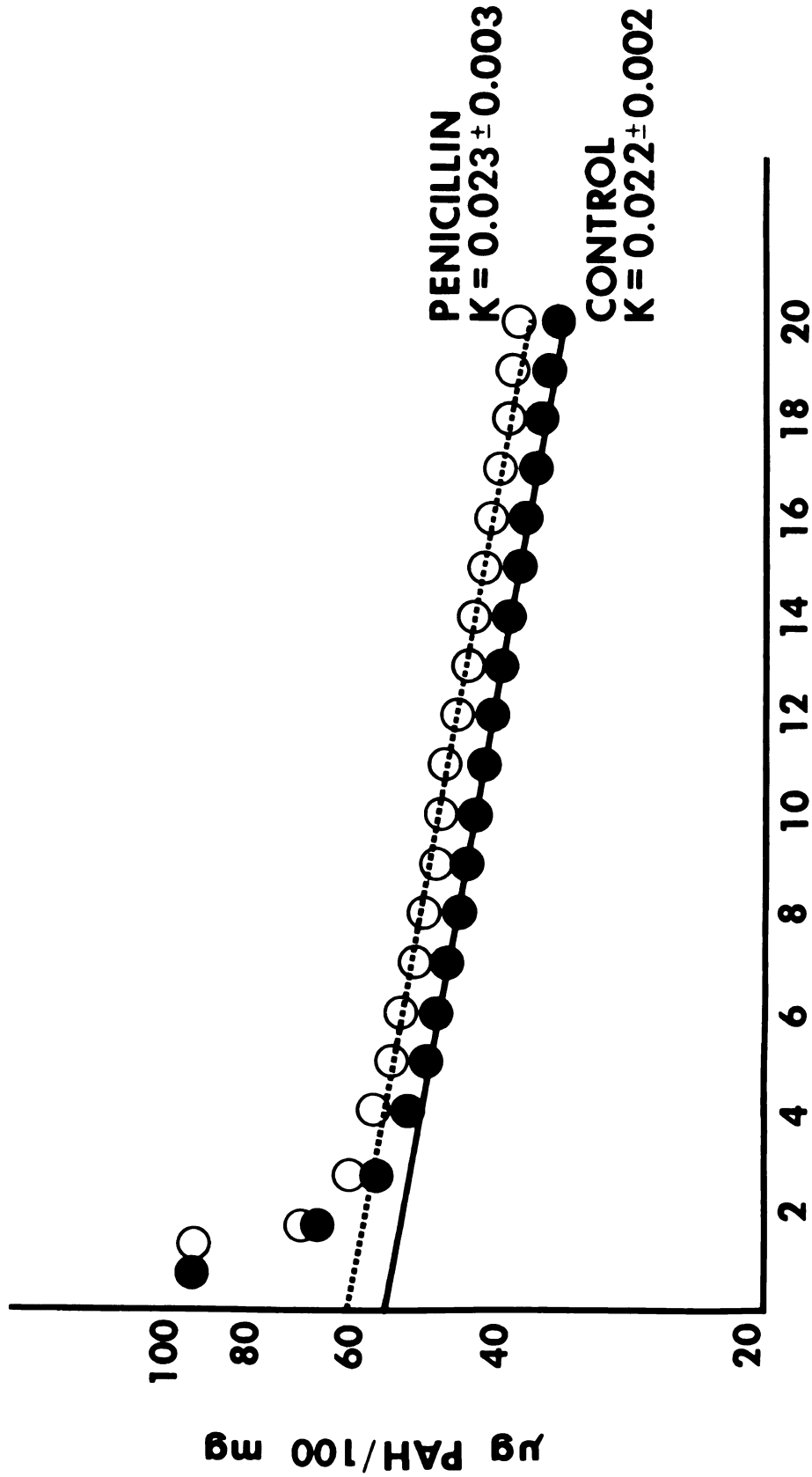

PENICILLIN
K = 0.023 ± 0.003
CONTROL
K = 0.022 ± 0.002
μg PAH/100 mg
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
MINUTES

Figure 8

Figure 9. Double reciprocal plot of p-aminohippuric acid (PAH) uptake in separated proximal tubules from 2-week control and penicillin-pretreated rabbits. At 11 days of age, treatment was begun. Ninety thousand International Units procaine penicillin G was administered twice daily for 2 days. Animals were killed 24 hours after the final injection. In each experiment, tubules were preincubated for 15 minutes without PAH. PAH was then added to produce concentrations in the medium of 1, 4, or 8 x 10^{-4} M and the tubules further incubated for 15 minutes. Each point represents mean $\pm$ S.E. of pups from 3 litters.

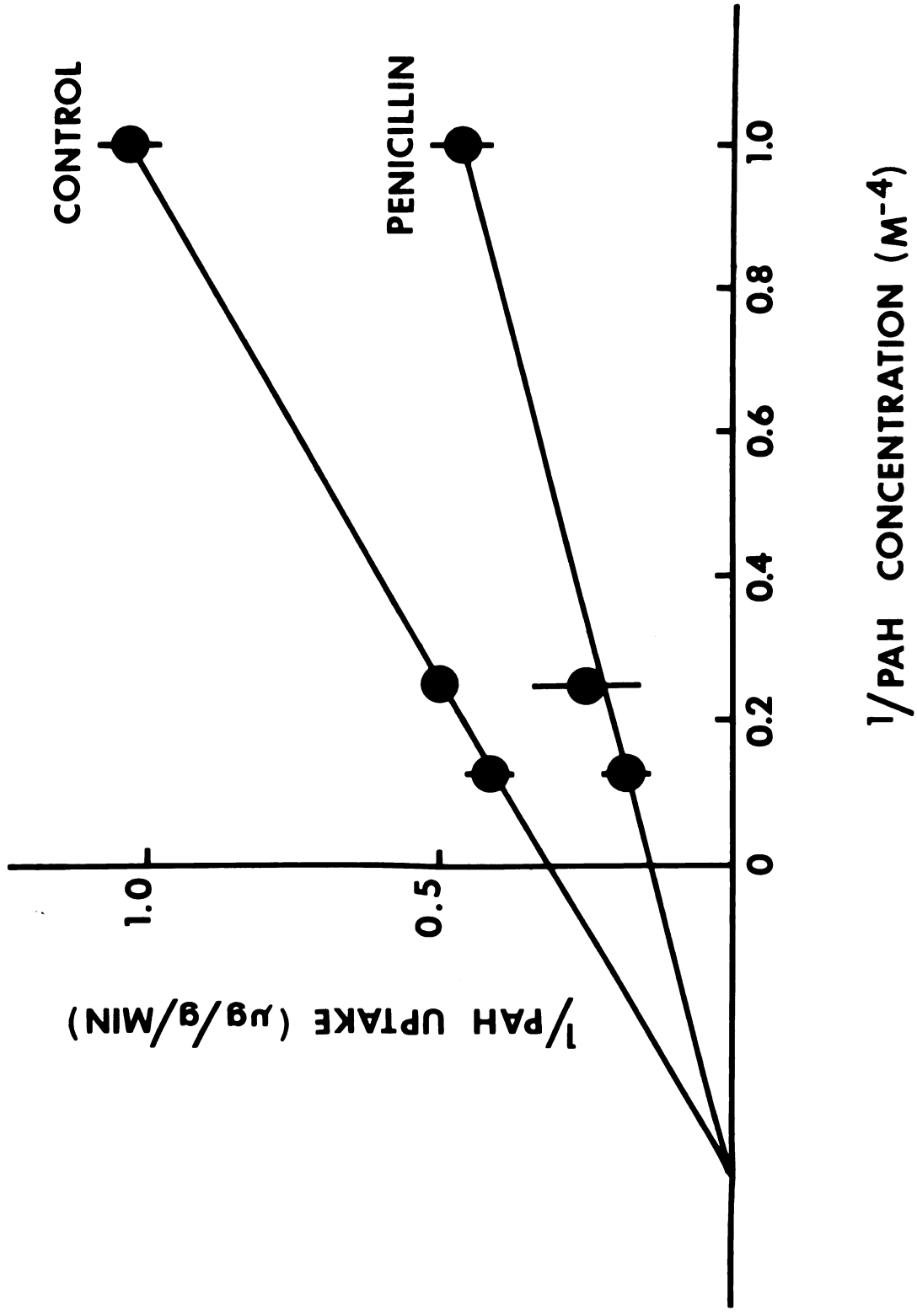

CONTROL
PENICILLIN
1.0
0.5
0
0.2
0.4
0.6
0.8
1.0
1/PAH UPTAKE (μg/g/MIN)
1/PAH CONCENTRATION (M-4)

Figure 9

Figure 10. Effect of cycloheximide on the penicillin-induced enhancement of PAH transport by rabbit renal cortical slices. Animals were treated with penicillin (90,000 I.U. s.c.) and/or cycloheximide (0.18 mg i.p.) twice over a 24-hour period. Beginning on the 12th day of life, rabbits were killed 24 hours after the second injection. Each bar represents the mean (± S.E.) obtained from 3 litters.

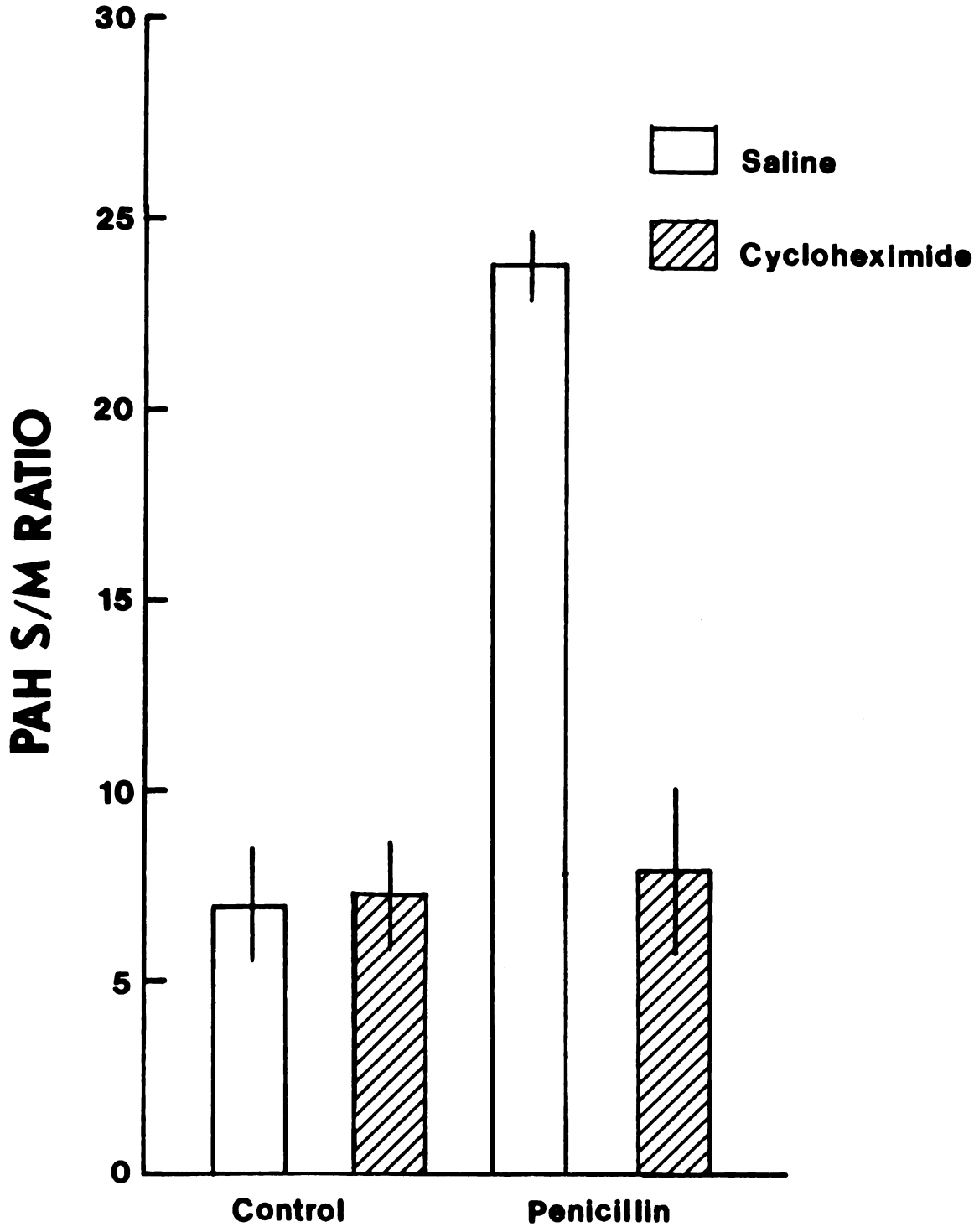

30
25
20
15
10
5
0
PAH S/M RATIO
Saline
Cycloheximide
Control
Penicillin

Figure 10

Figure 11. Electron micrograph of separated proximal tubules from (a) adult and (b) 2-week rabbit (x6020). Both tissues are structurally similar. Differences in structure include a more convoluted brush border (BB), a greater complexity and interdigitation of basilar infoldings (BI) and a thicker basement membrane (BM) in the adult. Due to the lesser complexity of BI, less compartmentation and alignment of mitochondria are observed in tissue from young animals. Other structures are comparable.

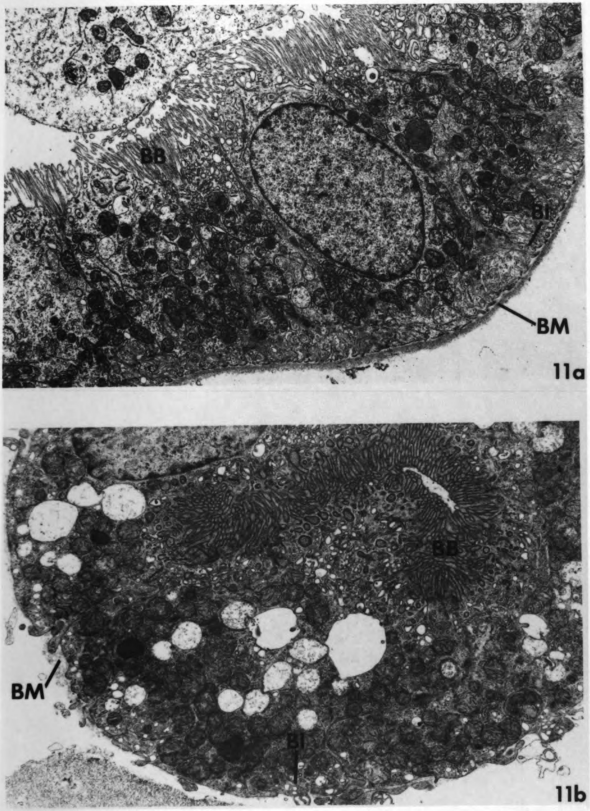
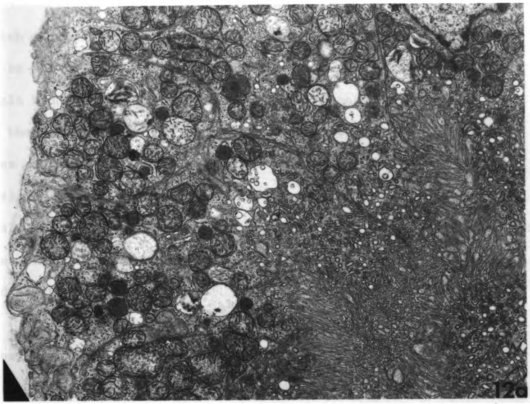


Figure 11

Figure 12. Electron micrograph of separated proximal tubules from 2-week rabbits (x6400). (a) Saline control, (b) penicillin treated. Animals received 90,000 I.U. of procaine penicillin twice daily for 2 days and were sacrificed 24 hours after the final injection. PAH transport capacity was enhanced with no alterations in tubular ultrastructure.



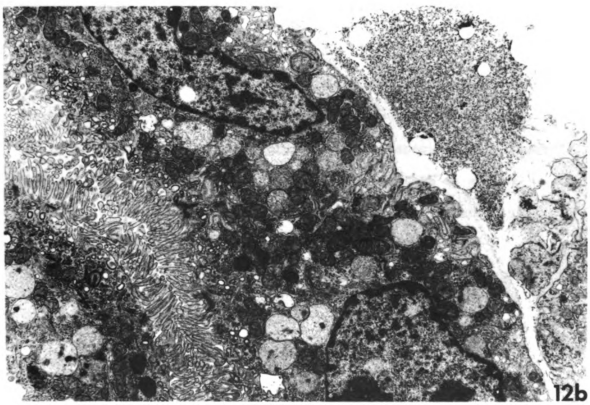


Figure 12

which were irregularly preserved in both groups, were not as thick as in adult animals. There was no difference between newborn and adult kidney in the degree of disruption of basement membranes due to the enzymic digestion. Because basilar membrane infoldings were less complex in the newborn, mitochondria were not aligned in basilar compartments as in the adult tubule; they appeared to be unaltered in size. Other structures, including endoplasmic reticulum, free ribosomes, and dense bodies were comparable in both newborns and adults.

Electron micrographs of specimens from control and penicillin-treated animals showed no difference in cellular structure (Figure 12). The density of brush border and of apical vacuoles, the size and number of lysosomal vacuoles, and the configuration of basilar membranes were comparable in proximal tubules of both groups.

5. Na, K-ATPase

Na, K-ATPase activity in crude homogenates of renal cortex (Figure 13) was less in the newborn than the adult (0.32 $\pm$ 0.08 and 0.69 $\pm$ 0.08 µmole PO_4/mg protein/10 min, respectively). Treatment of 2-week animals with penicillin had no effect on enzyme activity (0.30 $\pm$ 0.05 µmole PO_4/mg protein/10 min). Similarly, treatment had no effect on magnesium-dependent ATPase.

Studies on Possible Correlations Between PAH Transport Capacity and GSH S-Transferase

1. Quantification of enzyme activity

The concentration of aryltransferase substrates in the incubation medium was limited by low water solubility. Dinitrochlorobenzene

Figure 13. Effect of penicillin pretreatment and maturation on renal cortical Na^+, K^+-activated ATPase determined from a crude cortical homogenate. Beginning on day 11, 90,000 I.U. procaine penicillin G was administered twice daily for 2 days. Animals were sacrificed 24 hours after the final injection. Each bar represents mean $\pm$ S.E. of 4 determinations.

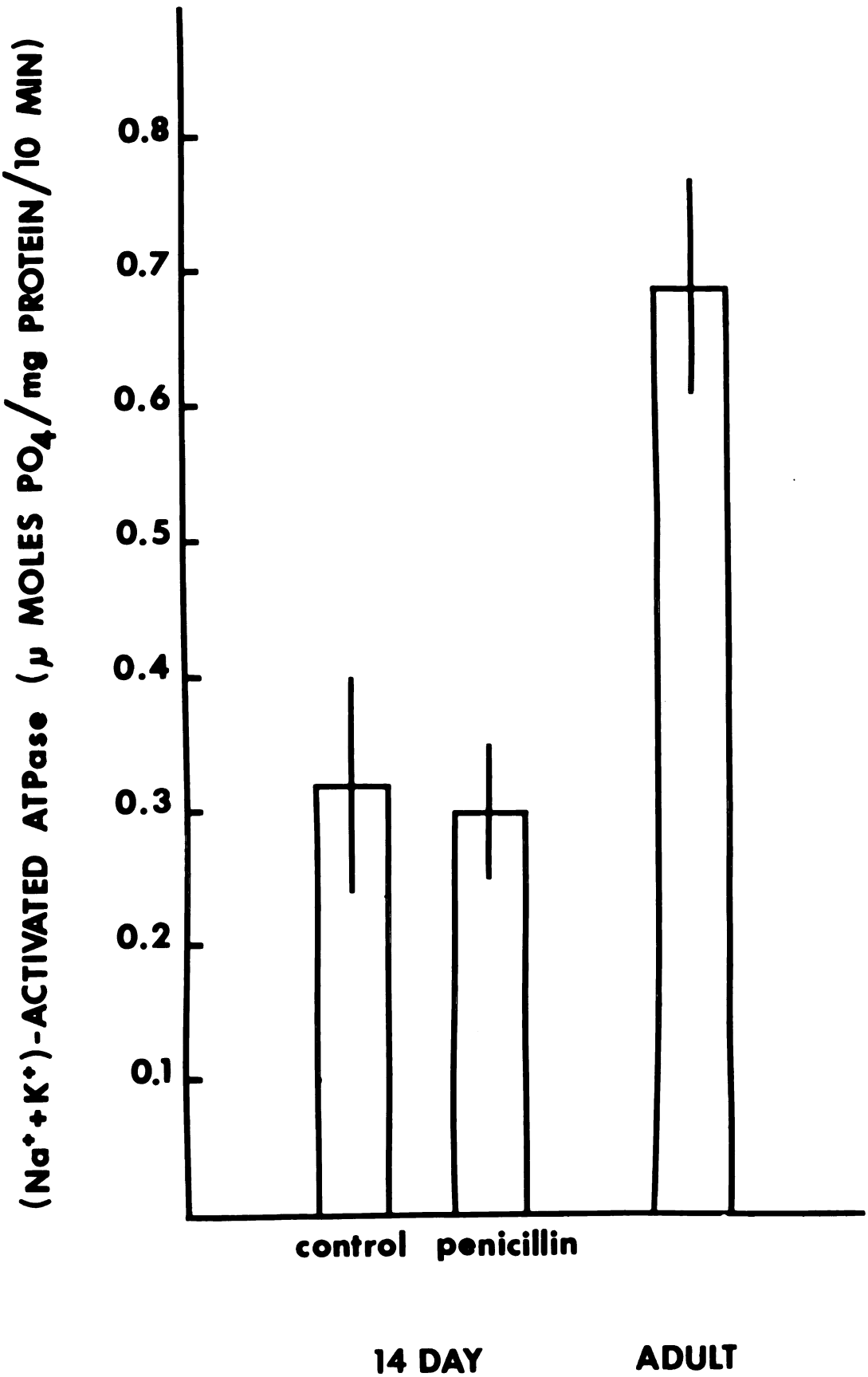

(Na⁺+K⁺)-ACTIVATED ATPase (μ MOLES PO4/mg PROTEIN/10 MIN)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
control
penicillin
14 DAY
ADULT

Figure 13

concentration in the medium exceeded Km values for each of the glutathione S-transferase enzymes. Dichloronitrobenzene concentration was slightly less than the reported Km values for the transferase enzymes (Habig et al., 1974b). Increased optical density of the enzyme incubation mixture was linearly related to time during the 15 minute incubation period using both substrates. The rate of increase in optical density was directly proportional to protein concentration. Addition of TCA or boiling destroyed enzyme activity and reduced changes in optical density to values observed in buffer blanks.

2. Effect of age

PAH S/M ratio was less in renal cortical slices from newborn rats and rabbits than in adults and increased with age. GSH S-aryltransferase activity using both dichloronitrobenzene (Figures 14 and 15) and dinitrochlorobenzene was also less in the newborn of both species, but development of enzyme activity and transport capacity were asynchronous. At 1 week of age GSH S-aryltransferase activity using dichloronitrobenzene as substrate was not significantly different than in kidney from adult rat while transport capacity was less (Figure 14). In rabbits, dichloronitrobenzene conjugating capacity in kidney cytosol from 3-day and 2-week pups was not different, though transport was significantly less in the 3-day animal (Figure 15). Enzyme activity and transport capacity were greater in 4-week animals than in either 3-day or 2-week pups.

Figure 14. Effect of age on accumulation of PAH by rat renal cortical slices and on GSH S-aryltransferase activity in 100,000 x g supernatant of kidney homogenates. Each bar represents the mean ± S.E. from at least 4 experiments.

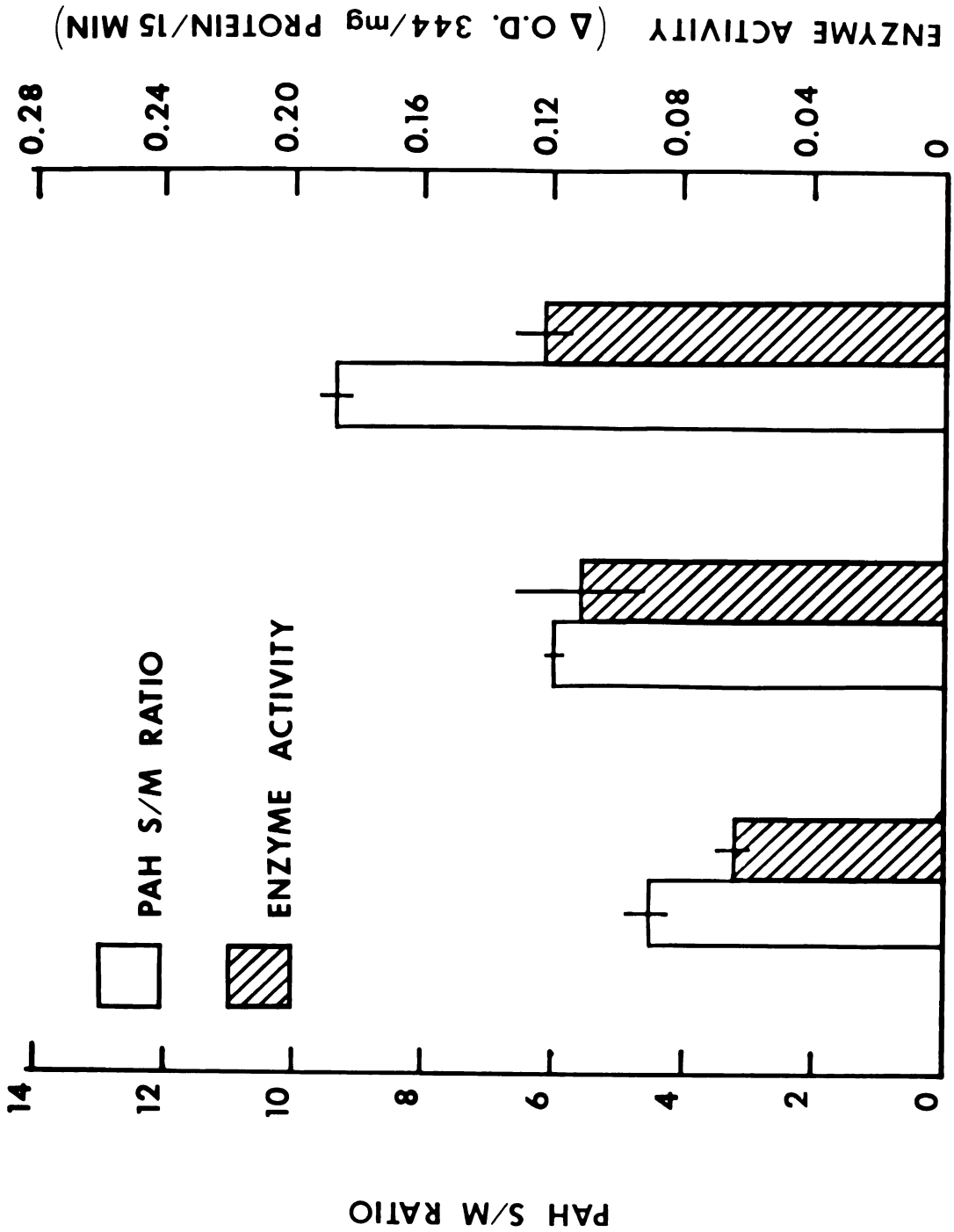

PAH S/M RATIO
0
2
4
6
8
10
12
14
ENZYME ACTIVITY (Δ O.D. 344/mg PROTEIN/15 MIN)
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
PAH S/M RATIO
ENZYME ACTIVITY
1 DAY
1 WEEK
ADULT
AGE

Figure 14

Figure 15. Effect of age on accumulation of PAH by rabbit renal cortical slices and on GSH S-aryltransferase activity in 100,000 x g supernatant of kidney homogenates. Each bar represents the mean $\pm$ S.E. determined in 4 litters.

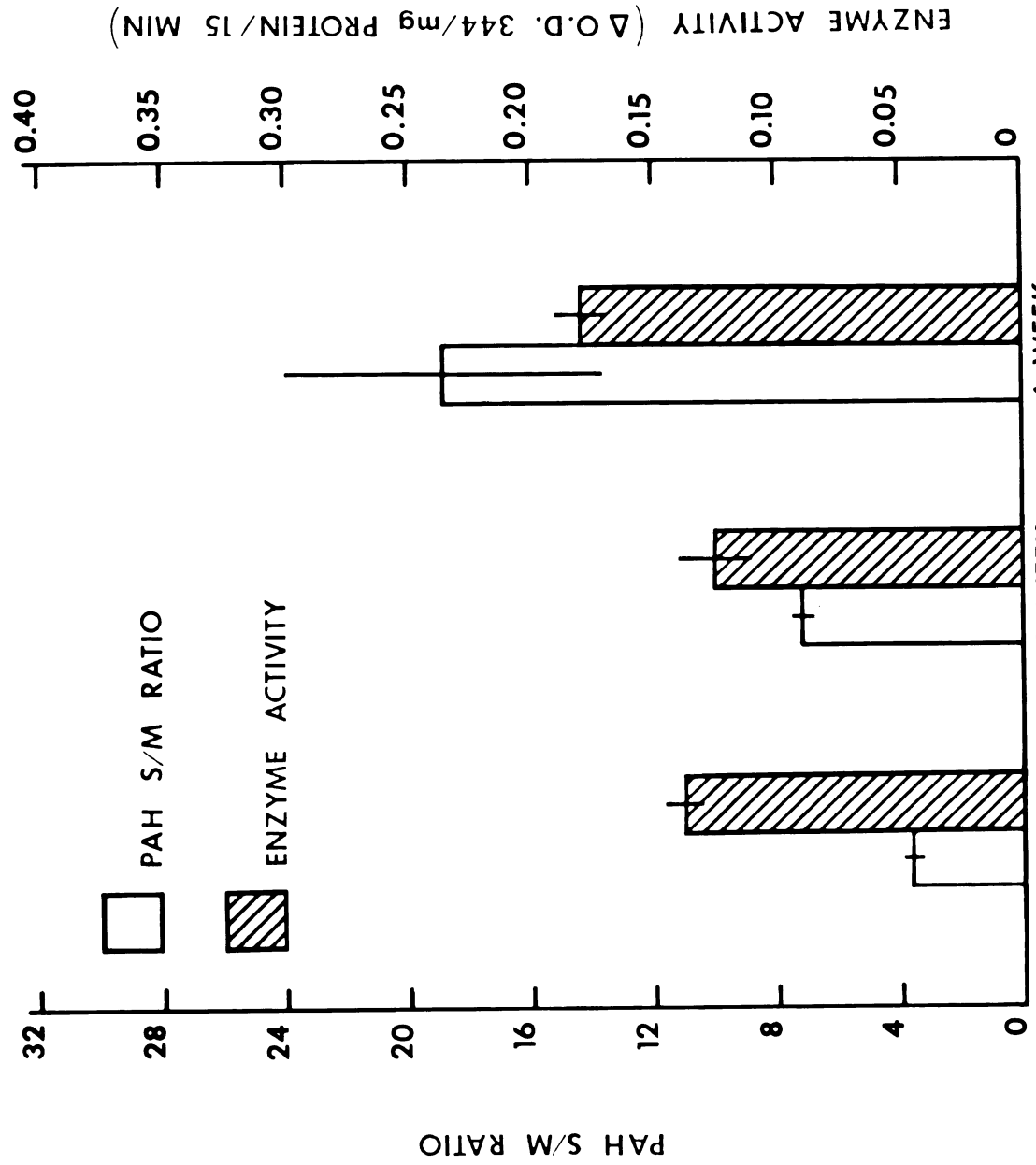

PAH S/M RATIO
0
4
8
12
16
20
24
28
32
ENZYME ACTIVITY (Δ O.D. 344/mg PROTEIN/15 MIN)
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
PAH S/M RATIO
ENZYME ACTIVITY
1 DAY
2 WEEK
4 WEEK
AGE

Figure 15

3. Effect of 3-methylcholanthrene (3-MC)

PAH transport capacity was slightly, though not significantly, reduced in weanling rats pretreated with 25 mg/kg 3-MC when compared to corn oil controls (Figure 16). Kidney weight to body weight ratios, a measure of toxicity, were not altered by treatment. Dinitrochlorobenzene conjugation in kidney cytosol from treated animals was significantly increased following 3-MC (Figure 16).

3-MC pretreatment of 2-week rabbits resulted in a dose dependent increase in PAH S/M ratio. Maximal enhancement of S/M ratio was observed following 40 mg/kg 3-MC (Figure 17). Kidney weight to body weight ratio, one measure of toxicity, was not altered by 3-MC (Figure 17). GSH S-aryltransferase activity in kidneys from treated animals was similarly increased with dose. Maximal enhancement of enzyme activity was observed following 20 mg/kg 3-MC (Figure 18).

4. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

In preliminary studies PAH S/M was decreased when compared to control after 7 days in animals which had been treated with 1 or 5 µg TCDD intragastrically (Table 1). Ten micrograms per kilogram TCDD had no effect on PAH S/M 3 or 7 days after treatment (Table 2). PAH S/M ratio in animals which had received 25 µg/kg TCDD intraperitoneally 7 days earlier was decreased (Table 2). There was no significant difference in percent extracellular water between control and treated at either dose and, therefore, the effect of TCDD reflected a true decrease in transport capacity. Though the maximal accumulation of PAH in all cases was less in perpendicular slices than in parallel, the relationship between control and treated within

Figure 16. Effect of 3-MC on accumulation of PAH by rat renal cortical slices and on GSH S-aryltransferase activity in 100,000 x g supernatant of kidney homogenates. Weanling rats were administered 25 mg/kg 3-MC intraperitoneally once a day for 3 days and sacrificed 24 hours after the final injection. Each bar represents the mean $\pm$ S.E. of 5 determinations.

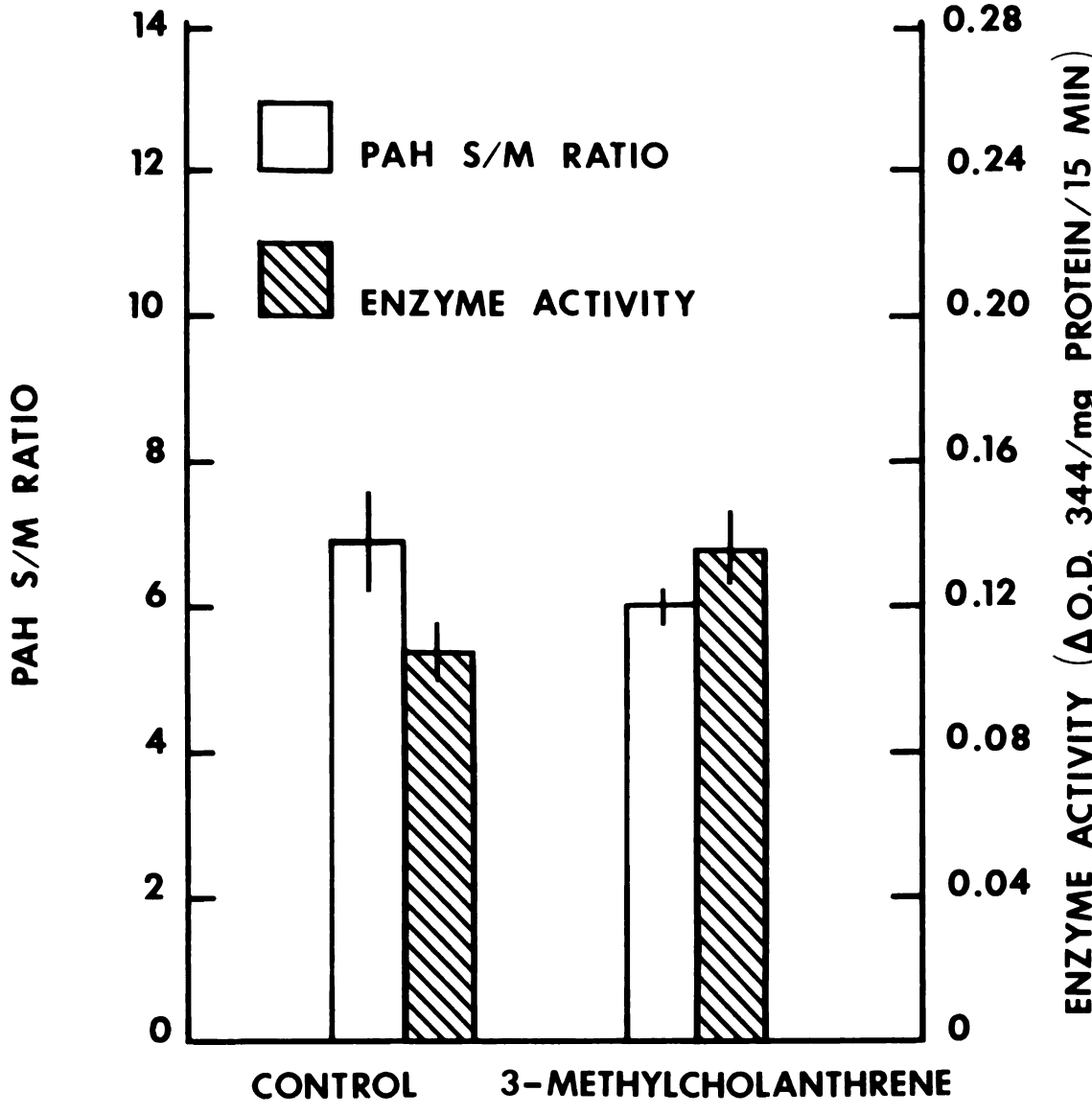

14
12
10
8
6
4
2
0
PAH S/M RATIO
PAH S/M RATIO
ENZYME ACTIVITY
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
ENZYME ACTIVITY (ΔO.D. 344/mg PROTEIN/15 MIN)
CONTROL
3-METHYLCHOLANTHRENE

Figure 16

Figure 17. Effect of 3-MC on PAH accumulation by rabbit renal cortical slices and kidney wt/body wt ratio. Beginning on day 11, animals were treated with 10, 20, 30 or 40 mg/kg 3-MC twice daily for 3 days and sacrificed 24 hours after the final injection. Controls received vehicle. Each bar represents the mean $\pm$ S.E. of 4 or 5 litters.

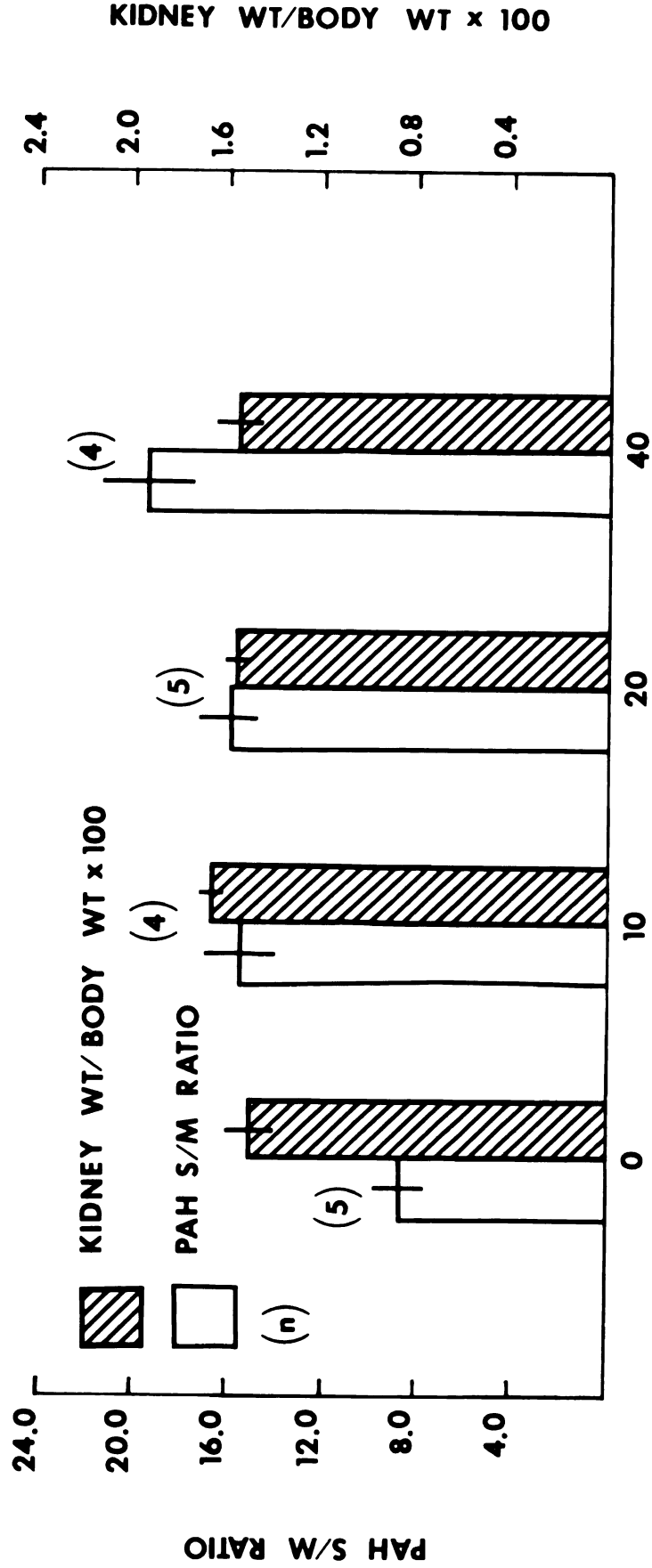

KIDNEY WT/BODY WT x 100
2.4
2.0
1.6
1.2
0.8
0.4
(4)
(5)
(4)
(5)
40
20
10
0
KIDNEY WT/ BODY WT x 100
PAH S/M RATIO
(n)
24.0
20.0
16.0
12.0
8.0
4.0
PAH S/M RATIO
3-METHYLCHOLANTHRENE (mg/kg)

Figure 17

Figure 18. Effect of 3-MC on GSH S-aryltransferase activity in 100,000 x g supernatant of rabbit kidney homogenates. See Figure 8 for treatment details. Each bar represents the mean ± S.E. of 3 litters.

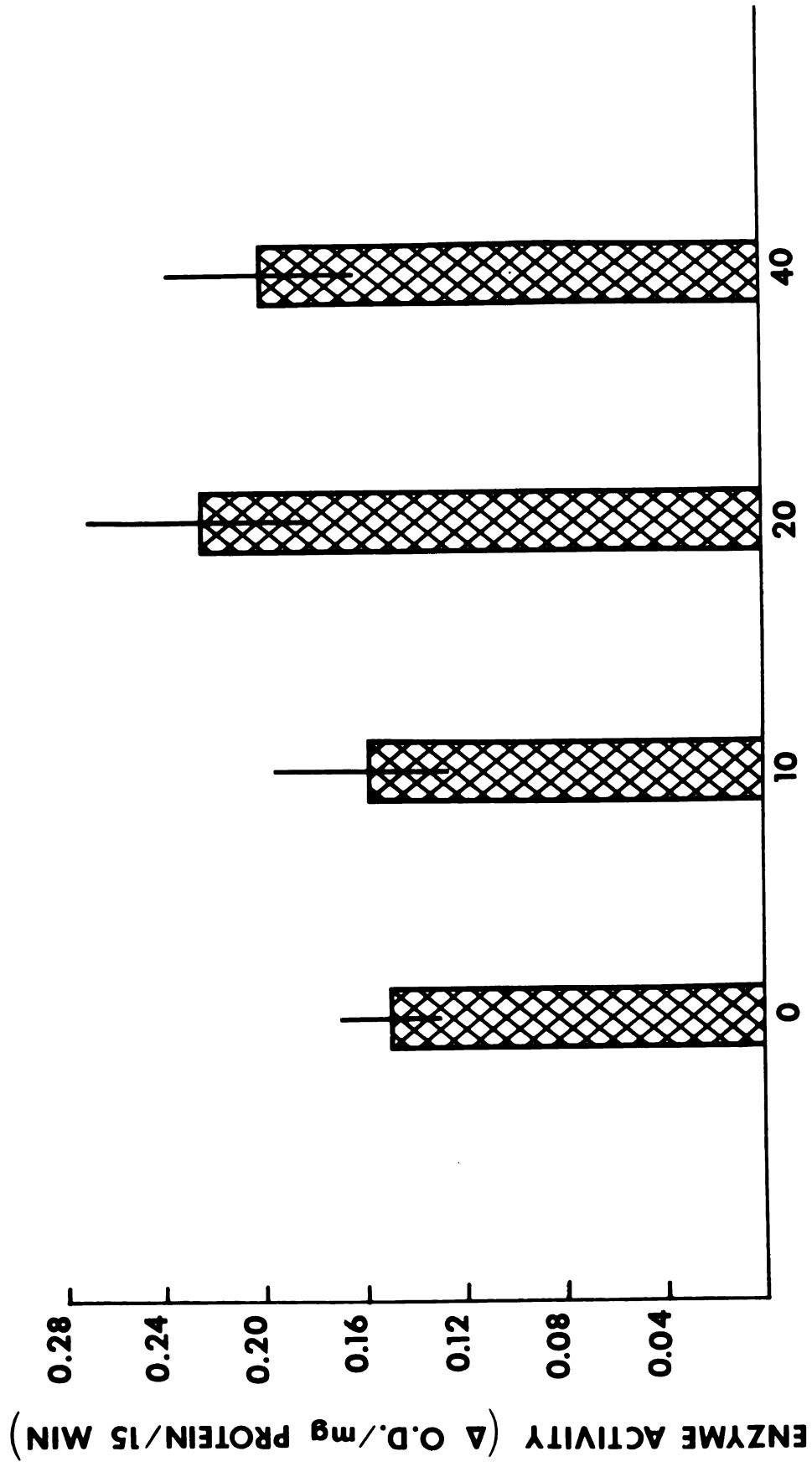

ENZYME ACTIVITY (Δ O.D./mg PROTEIN/15 MIN)
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
10
20
40
3-METHYLCHOLANTHRENE (mg/kg)

Figure 18

Table 1. Effect of TCDD on accumulation of PAH by slices of renal cortex from adult rats[a]

Treatment	n[b]	PAH S/M Ratio	
		Control	TCDD Treated
1 μg	4	11.0 ± 0.3	9.6 ± 0.4
5 μg	2	11.0 ± 0.9	9.2 ± 0.8

[a]Animals were treated with 1 or 5 μg TCDD, intragastrically. Controls received vehicle. After 7 days animals were sacrificed and PAH transport capacity in slices of renal cortex determined. Each value represents the mean (± S.E.).

[b]n = number of animals.

Table 2. Effect of TCDD on accumulation of PAH and NMN by slices of renal cortex from adult rats[a]

Treatment	Duration (days)	S/M Ratio			
		PAH		NMN	
		Parallel	Perpendicular	Parallel	Perpendicular
Control	3	13.8	8.5	9.1	5.3
10 μg/kg TCDD		14.2	9.3	7.5	4.5
Control	7	13.7	7.8	8.6	5.4
10 μg/kg TCDD		13.2	6.4	6.5	3.8
Control	7	12.8	6.3	7.9	4.3
25 μg/kg TCDD		11.4	5.6	5.1	3.9

[a]Animals were treated with 10 or 25 μg/kg TCDD, intraperitoneally. Controls received vehicle. Three and 7 days later animals were sacrificed. Slices of renal cortex were taken in both a horizontal and parallel plane to the major renal axis. Slices from 3 control and 3 treated rats were pooled before incubation. Therefore, each number represents the mean value of a duplicate incubation of tissue from its respective pool.

a type of slice remained. NMN accumulation appeared to be decreased at all doses of TCDD (Table 2).

5. Effect of chronic metabolic acidosis

Chronic ammonium chloride acidosis increased kidney weight to body weight ratio. PAH S/M ratio in slices from acidotic animals was significantly decreased whereas GSH S-aryltransferase activity was increased at least 8-fold using both enzyme substrates (Figure 19).

6. Effect of substrate stimulation by penicillin

Penicillin pretreatment significantly increased PAH S/M ratios as did 3-MC but had no effect on GSH S-aryltransferase activity using either enzyme substrate (Figure 20).

7. Sex difference

GSH S-aryltransferase activity was 10-fold higher in kidneys from adult mice than adult rats. PAH S/M ratio was significantly greater in male mice than in females. Enzyme activity determined using dichloronitrobenzene was not significantly different between sexes (Figure 21).

8. Effect of uninephrectomy

Accumulation of PAH by renal cortical slices was significantly increased in the remaining kidney of uninephrectomized rats 48 hours following surgery. GSH S-aryltransferase activity was not significantly increased using either dichloronitrobenzene (Figure 22) or dinitrochlorobenzene substrates.

Figure 19. Effect of chronic ammonium chloride acidosis on accumulation of PAH by rat renal cortical slices and GSH S-aryltransferase activity in 100,000 x g supernatant of kidney homogenates. Rats were maintained for 7 days on 0.28 M NH_4Cl as sole drinking fluid. Controls were allowed tap water. Each bar represents the mean $\pm$ S.E. of 8 determinations.

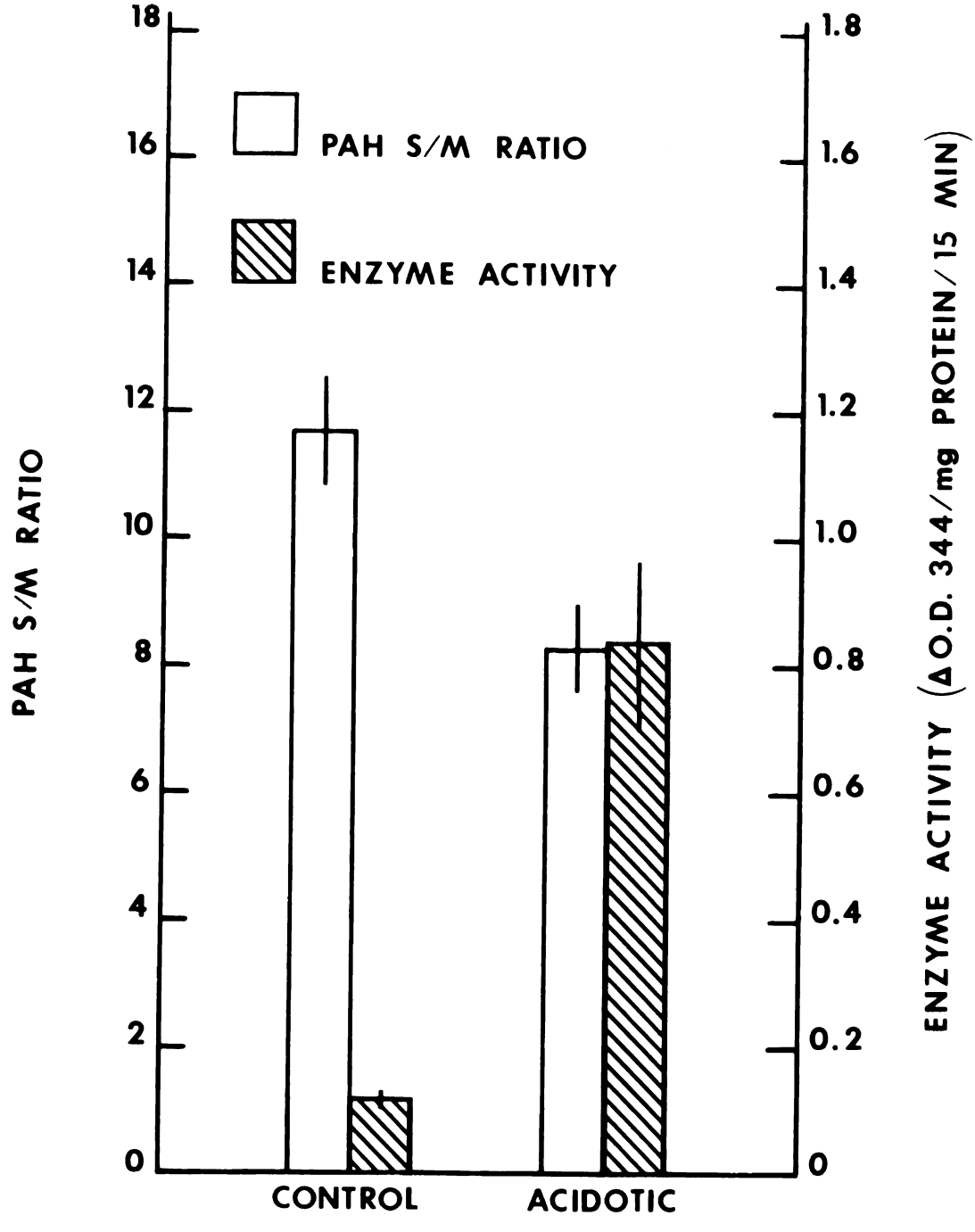

PAH S/M RATIO
ENZYME ACTIVITY
PAH S/M RATIO
ENZYME ACTIVITY (ΔO.D. 344/mg PROTEIN/15 MIN)
18
16
14
12
10
8
6
4
2
0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
CONTROL
ACIDOTIC

Figure 19

Figure 20. Effect of penicillin on PAH accumulation by rabbit renal cortical slices and GSH S-aryltransferase activity in 100,000 x g supernatant of kidney homogenates. Beginning on day 12, animals were treated with 90,000 I.U. procaine penicillin G s.c. twice daily for 2 days. Animals were sacrificed 24 hours after the final injection. Each bar represents the mean $\pm$ S.E. of 6 litters.

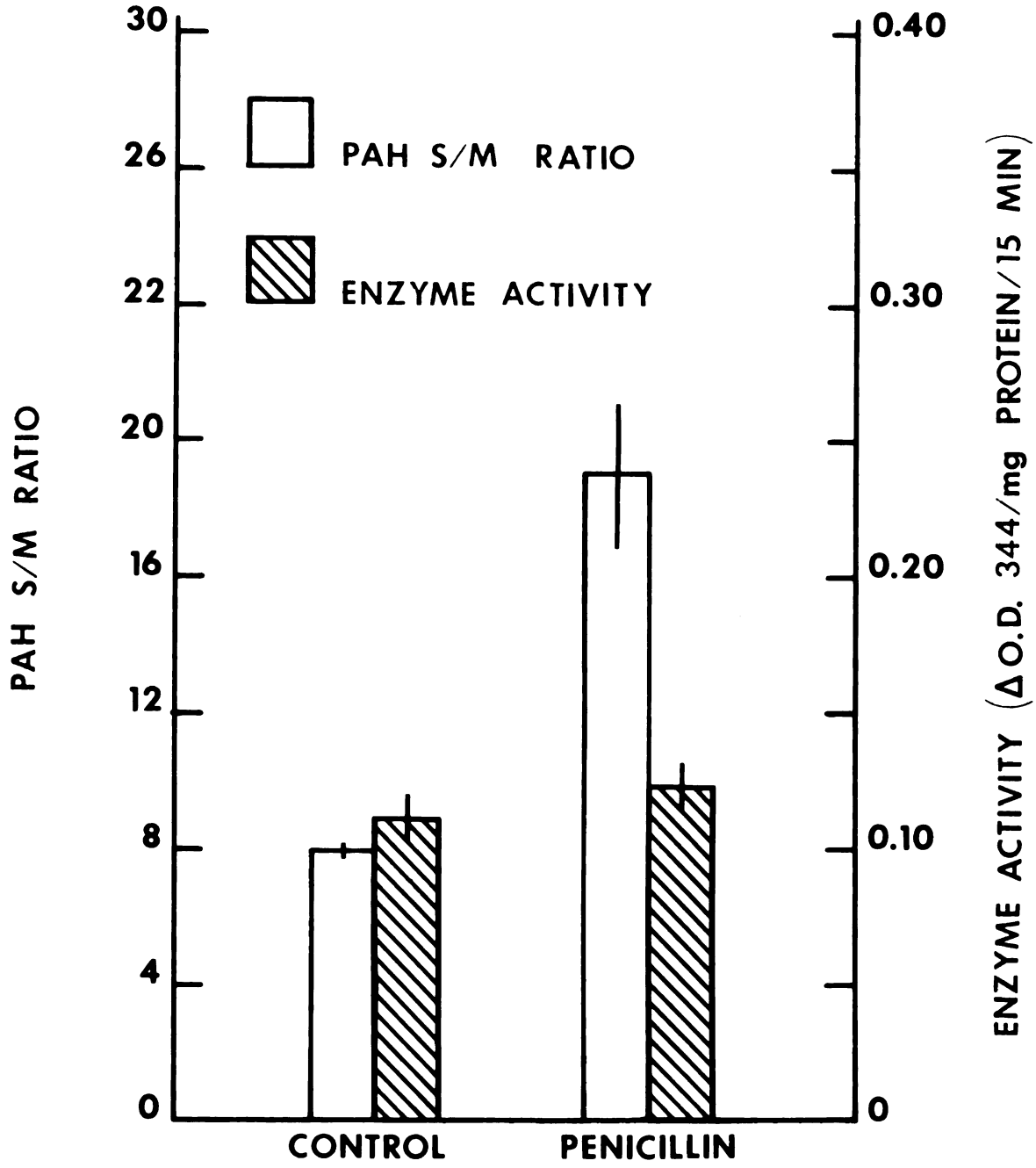

30
26
22
20
16
12
8
4
0
PAH S/M RATIO
PAH S/M RATIO
ENZYME ACTIVITY
0.40
0.30
0.20
0.10
0
ENZYME ACTIVITY (ΔO.D. 344/mg PROTEIN/15 MIN)
CONTROL
PENICILLIN

Figure 20

Figure 21. Accumulation of PAH by renal cortical slices and GSH S-aryltransferase activity in 100,000 x g supernatant from adult male and female mouse kidneys. Each bar represents the mean $\pm$ S.E. of at least 6 determinations.

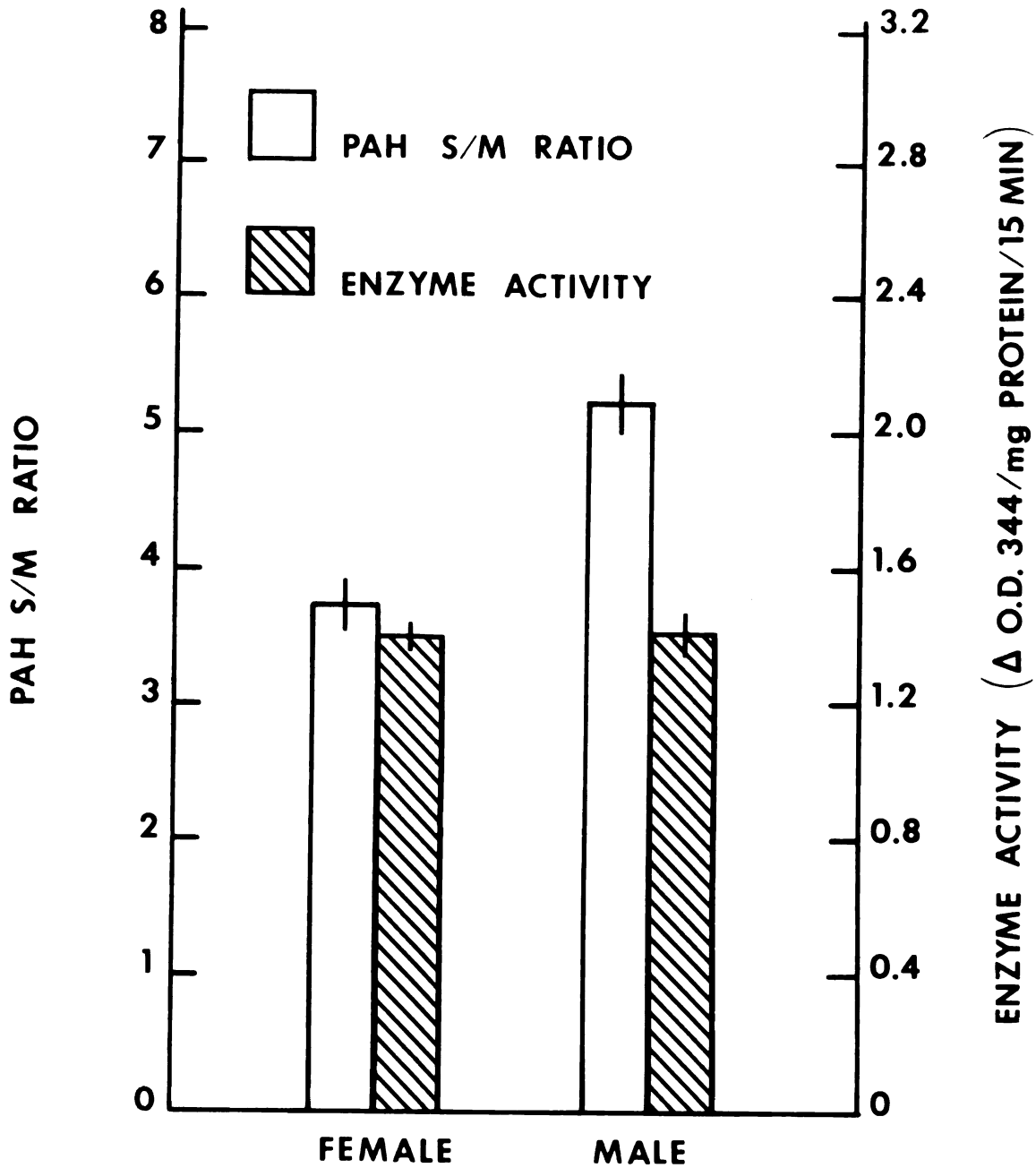

PAH S/M RATIO
ENZYME ACTIVITY
PAH S/M RATIO
ENZYME ACTIVITY (Δ O.D. 344/mg PROTEIN/15 MIN)
0
1
2
3
4
5
6
7
8
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
FEMALE
MALE

Figure 21

Figure 22. Effect of uninephrectomy on accumulation of PAH by rat renal cortical slices and on GSH S-aryltransferase activity in 100,000 x g supernatant of kidney homogenates. The left kidney was removed through a flank incision and animals sacrificed 48 hours following surgery. Controls were sham operated. Each bar represents the mean $\pm$ S.E. of 6 determinations.

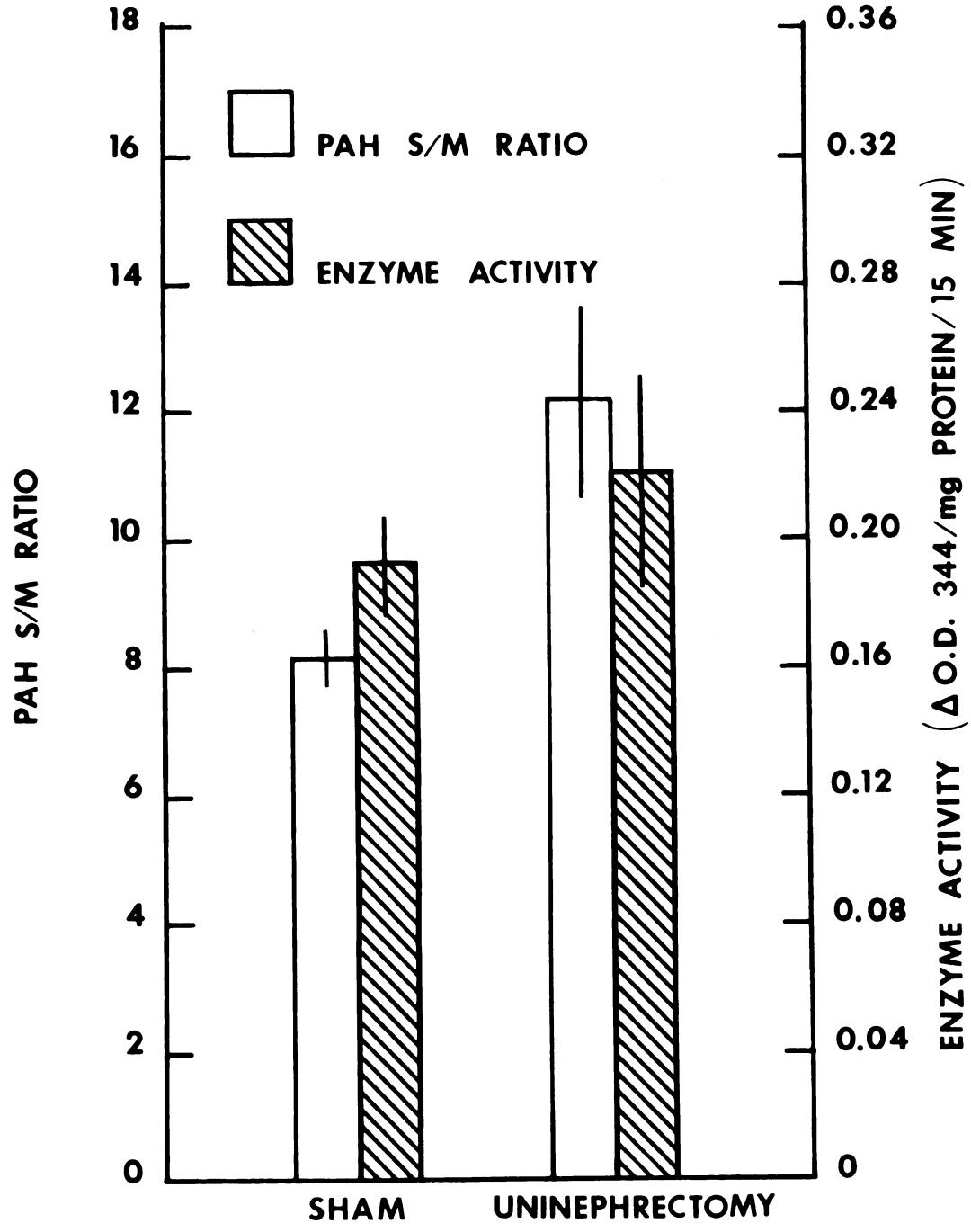

PAH S/M RATIO
ENZYME ACTIVITY
PAH S/M RATIO
ENZYME ACTIVITY (Δ O.D. 344/mg PROTEIN/15 MIN)
18
16
14
12
10
8
6
4
2
0
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
SHAM
UNINEPHRECTOMY

Figure 22

9. PAH transport capacity following GSH depletion

L-Methionine-SR-sulfoximine significantly decreased GSH concentrations in the kidneys of adult male rats (Figure 23). There was no regeneration of GSH in slices from sulfoximine treated rats during incubation *in vitro*. GSH concentration in renal cortical slices taken from control animals was not significantly decreased during the incubation period. PAH S/M ratio was not significantly decreased by sulfoximine pretreatment (Figure 23).

10. Effect of age and penicillin pretreatment on penicillin binding to soluble proteins

Sephadex filtration of soluble proteins from adult renal cortical homogenates resulted in elution of 4 major protein peaks (Figure 24). GSH S-aryltransferase specific activity was greatest in protein eluted with the third peak (sample number 70-80). Two peaks of radioactivity were observed, one associated with the protein peak not containing enzyme activity and the second corresponding to the GSH S-transferase containing peak (Figure 24). Protein profiles of 2-week-old rabbit renal cortical soluble proteins consisted of only 3 major peaks which eluted in similar volumes compared to the adult (Figure 25). Two radioactivity peaks were observed, one corresponding to samples containing no enzyme activity and the second to the major enzyme containing samples. Penicillin pretreatment did not alter either protein or radioactivity elution profiles (Figure 26). Binding was estimated as CPM/mg protein using maximal protein and radioactivity values within the GSH S-transferase containing peak. In the adult, binding was approximately 600 CPM/mg protein (Figure 24), whereas in young animals binding was approximately 400 CPM/mg

Figure 23. Effect of L-methionine-SR-sulfoximine treatment on GSH concentration and accumulation of PAH by renal cortical slices. Rats were treated with 1.85 mmoles/kg sulfoximine and sacrificed 2 hours after injection. Reduced glutathione was assayed in slices before and after 60-minute incubation. Each bar represents the mean $\pm$ S.E. of 6 determinations.

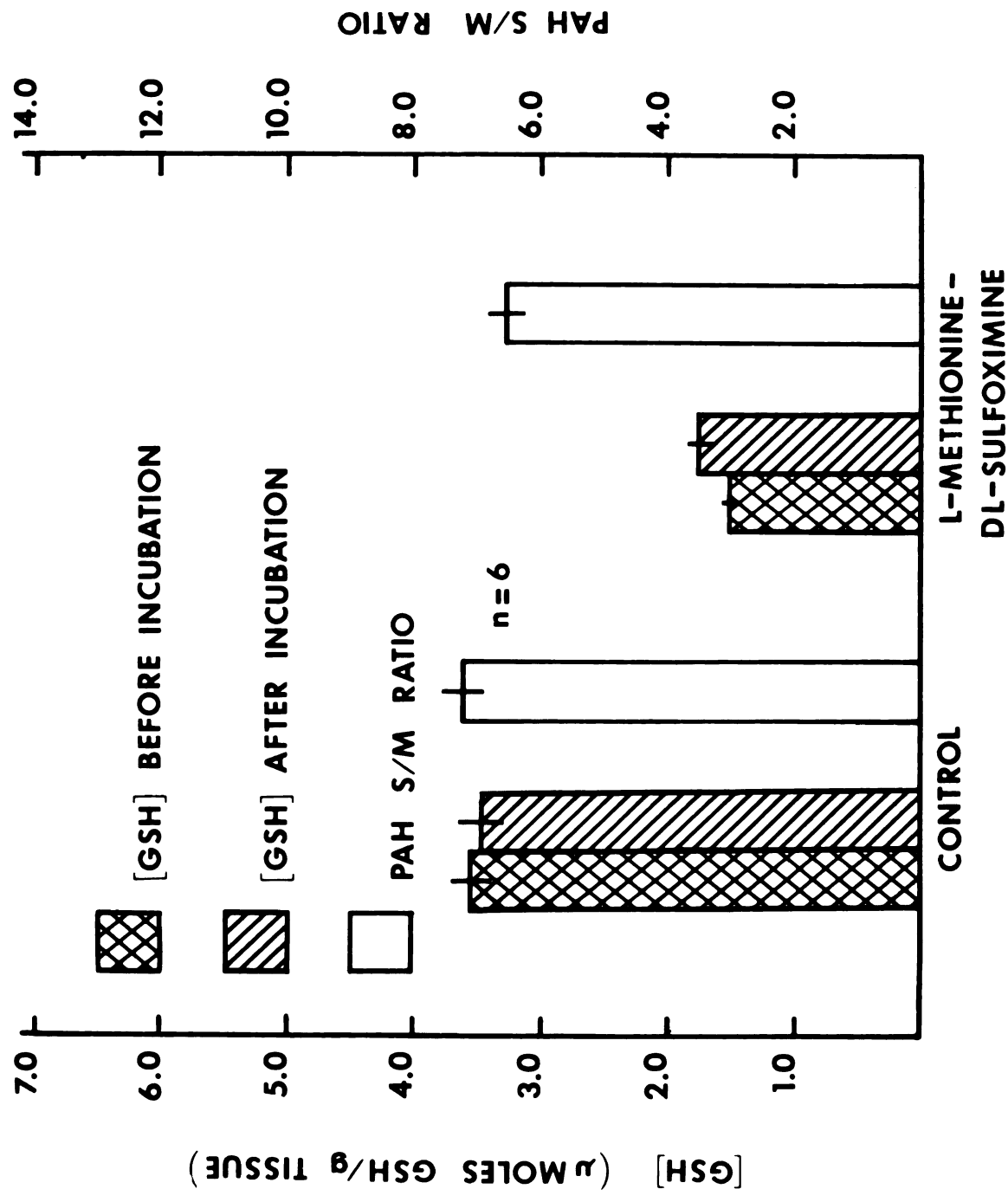

[GSH] (μMOLES GSH/g TISSUE)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PAH S/M RATIO
14.0
12.0
10.0
8.0
6.0
4.0
2.0
[GSH] BEFORE INCUBATION
[GSH] AFTER INCUBATION
PAH S/M RATIO
n=6
CONTROL
L-METHIONINE-
DL-SULFOXIMINE

Figure 23

Figure 24. Binding of ^{14}C-benzyl penicillin to 100,000 x g supernatant protein from adult female rabbit renal cortical homogenates. Fifty milligrams soluble protein, 0.88 µmole benzyl penicillin (0.57 µCi/µmole) and 1 mM reduced glutathione in a total volume of 5 ml 0.25 M sucrose-0.01 M KH_2PO_4 (pH 7.4) were applied to a Sephadex G-100 (3 x 100 cm) column and eluted with 0.01 M KH_2PO_4 (pH 7.4). Protein (closed circles) and radioactivity (open circles) were determined in eluant fractions. Glutathione S-transferase concentration in selected fractions was estimated by measuring glutathione S-aryltransferase activity using dinitrochlorobenzene as substrate and represented as ΔOD_{340}/mg protein/15 min (↔). The figure is representative of results obtained from 3 determinations.

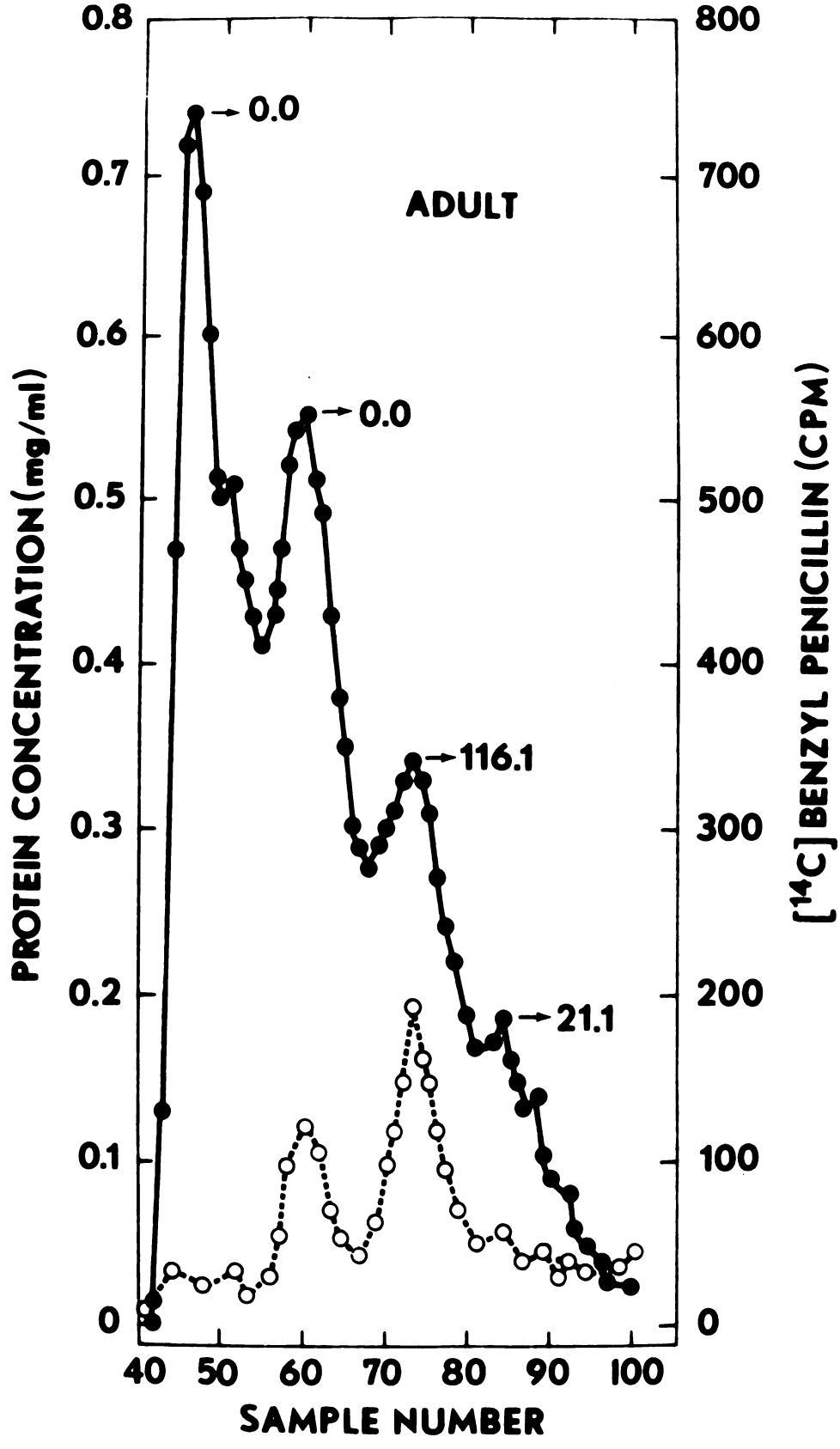

ADULT
0.0
0.0
116.1
21.1
PROTEIN CONCENTRATION (mg/ml)
[14C] BENZYL PENICILLIN (CPM)
SAMPLE NUMBER
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
800
700
600
500
400
300
200
100
0
40
50
60
70
80
90
100

Figure 24

Figure 25. Binding of ^{14}C-benzyl penicillin to 100,000 x g supernatant protein from 2-week saline control rabbits. Other details as in Figure 24. The figure is representative of results obtained from 3 litters.

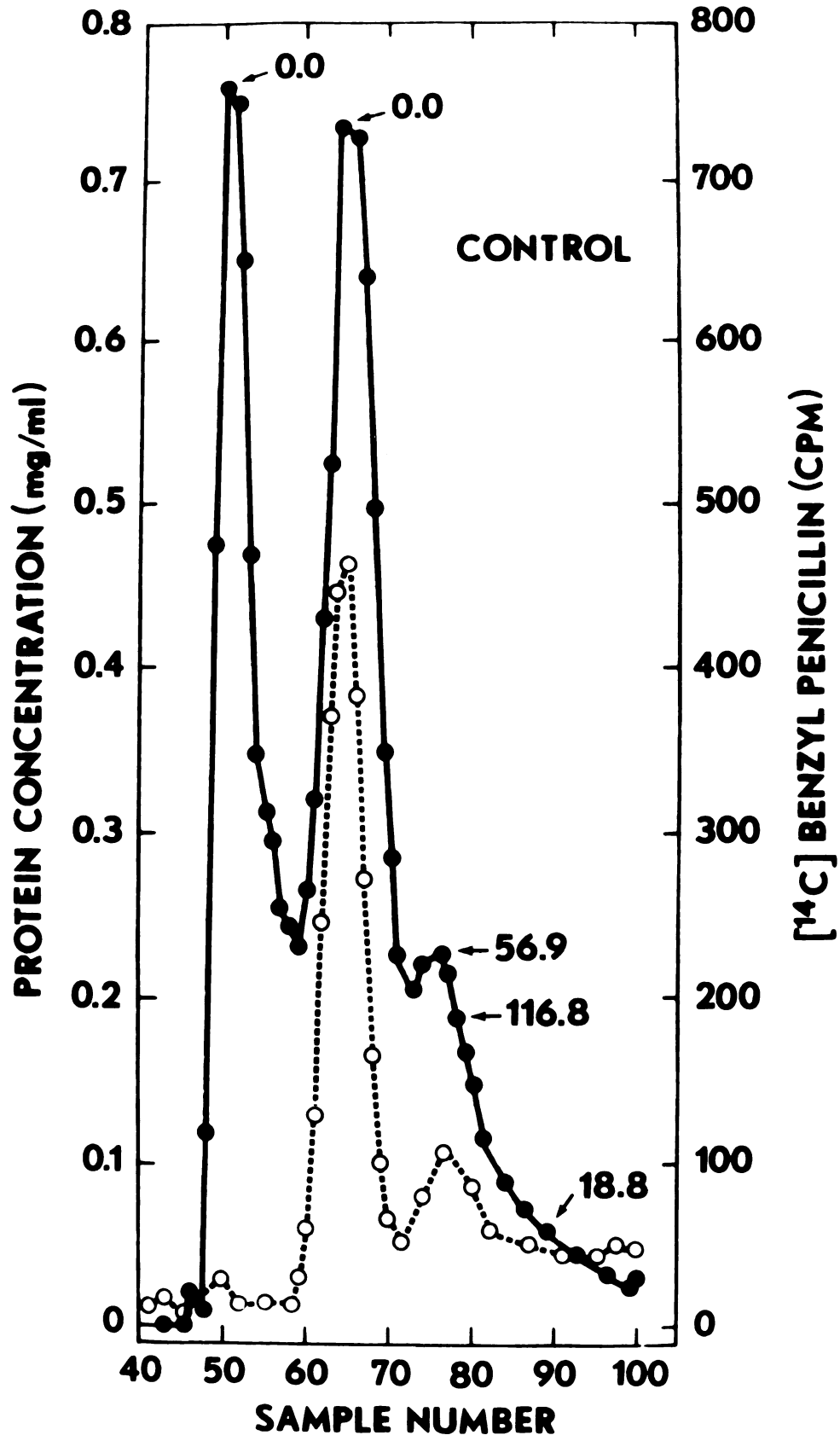

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
800
700
600
500
400
300
200
100
0
0.0
0.0
CONTROL
56.9
116.8
18.8
40
50
60
70
80
90
100
SAMPLE NUMBER
PROTEIN CONCENTRATION (mg/ml)
[14C] BENZYL PENICILLIN (CPM)

Figure 25

Figure 26. Binding of ^{14}C-benzyl penicillin to 100,000 x g supernatant protein from 2-week penicillin treated rabbits. Other details as in Figure 24. The figure is representative of results obtained from 3 litters.

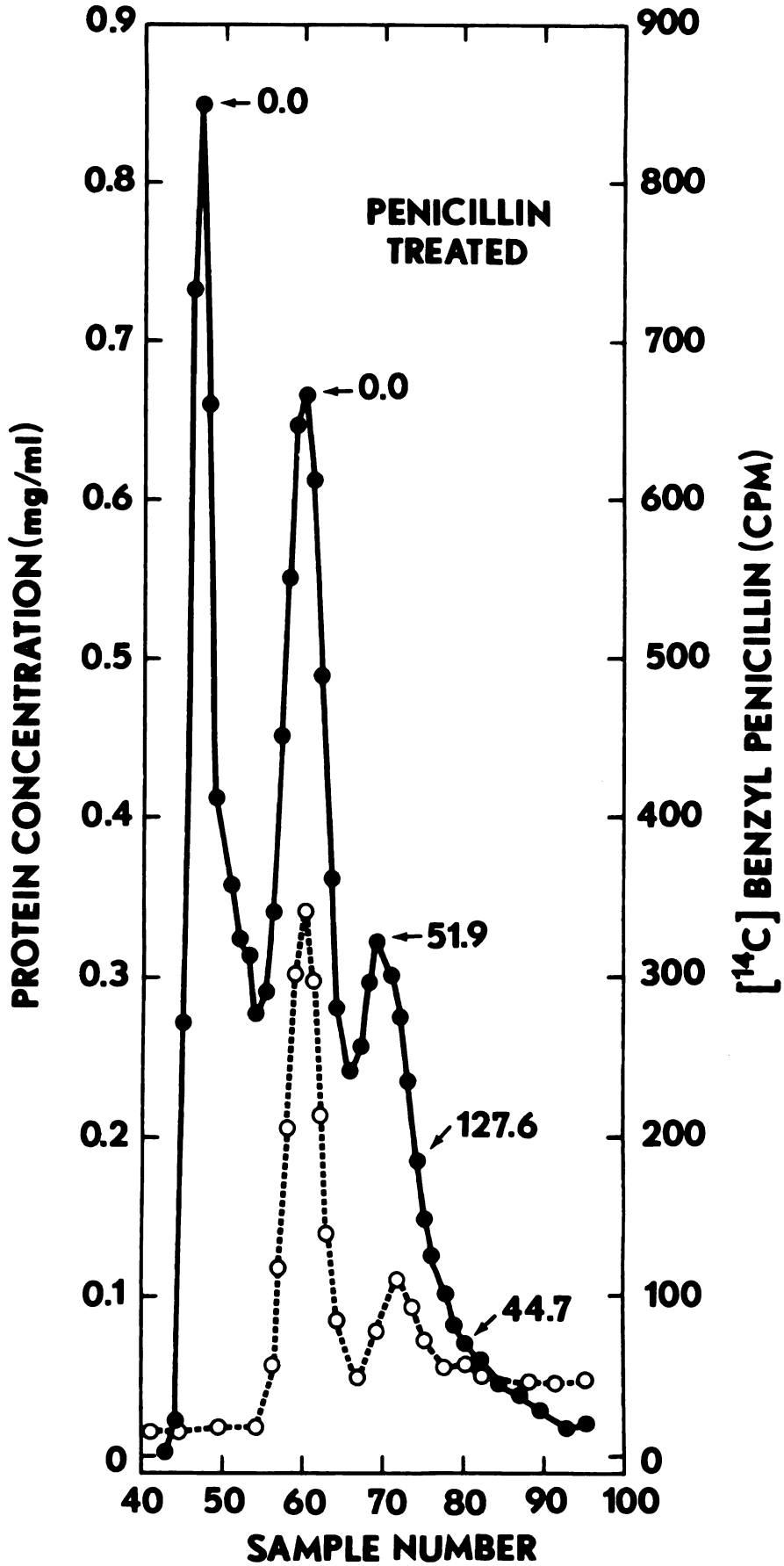

PENICILLIN
TREATED
0.0
0.0
51.9
127.6
44.7
PROTEIN CONCENTRATION (mg/ml)
[14C] BENZYL PENICILLIN (CPM)
SAMPLE NUMBER
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
900
800
700
600
500
400
300
200
100
0
40
50
60
70
80
90
100

Figure 26

protein (Figure 25). Penicillin treatment had no effect on binding capacity (Figure 26).

Incorporation of Amino Acids and Protein Synthesis Following Substrate Stimulation by Penicillin

1. Incorporation of ^{14}C L-leucine *in vitro*

The incorporation of ^{14}C L-leucine into renal cortical slice protein was not significantly different from control following substrate stimulation by penicillin whether factored by mg protein or µg DNA (Table 3). Though intracellular leucine pools were not measured, penicillin had no effect on leucine S/M ratio and probably therefore had little effect on availability of labeled precursors. Penicillin pretreatment also had no effect on DNA/protein, RNA/protein or RNA/DNA ratios in 2-week rabbits (Table 4), though transport capacity was increased several-fold. Incorporation of label into separated proximal tubular protein was not different from control when determined 3 hours after the final injection of penicillin (Table 5). Uptake was slightly increased after 24 hours.

2. Incorporation of ^{14}C L-leucine *in vivo*

Incorporation of label was slightly greater in 100,000 x g pellet protein from animals pretreated with 3 injections of penicillin (Table 6). The difference, however, was not statistically significant. Uptake into total renal cortical protein of identically treated animals was not different from control (Table 6).

Incorporation of label into 100,000 x g pellet protein following a single injection of penicillin was generally greater than control but subject to a high degree of variability (Figure 27).

Table 3. Effect of penicillin treatment of 2-week-old rabbits on incorporation of ^{14}C L-leucine by renal cortical slices[a]

	n[b]	Control	Treated
DPM/mg protein	5	771 ± 75	697 ± 70
DPM/µg DNA	2	28 ± 4	26 ± 4

[a]Four injections 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Animals were sacrificed 24 hours after the final injection. Renal cortical slides were incubated in medium containing 2.7×10^4 M leucine (sp. act. 0.14 µCi/umole). Each value represents the mean (± S.E.).

[b]n = number of litters.

Table 4. Effect of age and substrate stimulation of organic anion transport by penicillin on renal cortical protein and nucleic acid composition in rabbits[a]

Age	Treatment	DNA/protein	RNA/protein	RNA/DNA
Adult	---	0.007±0.001	0.007±0.000	1.051±0.145
2 week	Saline	0.028±0.008	0.020±0.004	0.798±0.136
2 week	Penicillin	0.025±0.007	0.020±0.005	0.831±0.158

[a]Adult female rabbits were not treated. Four injections 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Animals were sacrificed 24 hours after the final injection. RNA, DNA and protein were assayed in renal cortical homogenates. Each value represents the mean (± S.E.) of 3 determinations.

Table 5. Effect of penicillin treatment of 2-week-old rabbits on incorporation of ^{14}C L-leucine by separated proximal tubules[a]

Hours[b]	n[c]	DPM/mg Protein	
		Control	Treated
3	6	1526 ± 197	1487 ± 155
24	4	801 ± 117	894 ± 110

[a]Four injections 90,000 I.U. procaine penicillin G were administered (twice daily for 2 days). Animals were sacrificed 3 or 24 hours after the final injection. Separated proximal tubules were incubated in medium containing 1.26×10^{-4} M leucine (sp. act. 0.27 μCi/umole). Each value represents the mean (± S.E.).

[b]Hours between final injection and sacrifice.

[c]n = number of litters.

Table 6. Effect of penicillin treatment of 2-week-old rabbits on incorporation of ^{14}C L-leucine *in vivo*[a]

	n[b]	DPM/mg Protein	
		Control	Treated
100,000 x g pellet	4	698 ± 153	838 ± 174
Renal cortical protein	5	538 ± 116	515 ± 66

[a]Three injections 90,000 I.U. procaine penicillin G were administered at approximately 12-hour intervals. Sixteen hours after the final injection 8 μCi/kg ^{14}C L-leucine were administered i.v. Uptake into 100,000 x g pellet was measured after 15 minutes exposure, while uptake into total renal cortical protein was determined 60 minutes after pulsing. Each value represents the mean (± S.E.).

[b]n = number of litters.

Figure 27. Uptake of ^{14}C L-leucine in soluble and microsomal protein of renal cortical homogenates from control and penicillin treated 2-week rabbits. Littermates were treated with either a single injection of 90,000 I.U. procaine penicillin G or saline for control. Animals were anesthetized 16 hours after treatment and injected intravenously with 8 μCi/kg (sp. act. 302 μCi/μmole) ^{14}C L-leucine. After 15 minutes animals were sacrificed and renal cortex homogenized in 0.25 M sucrose-0.01 M KH_2PO_4 (pH 7.4). The 100,000 x g pellet and supernatant fractions were assayed for protein and radioactivity. Individual data from litters was plotted. Each pair of points represents the average value obtained for control and penicillin treated pups within a litter.

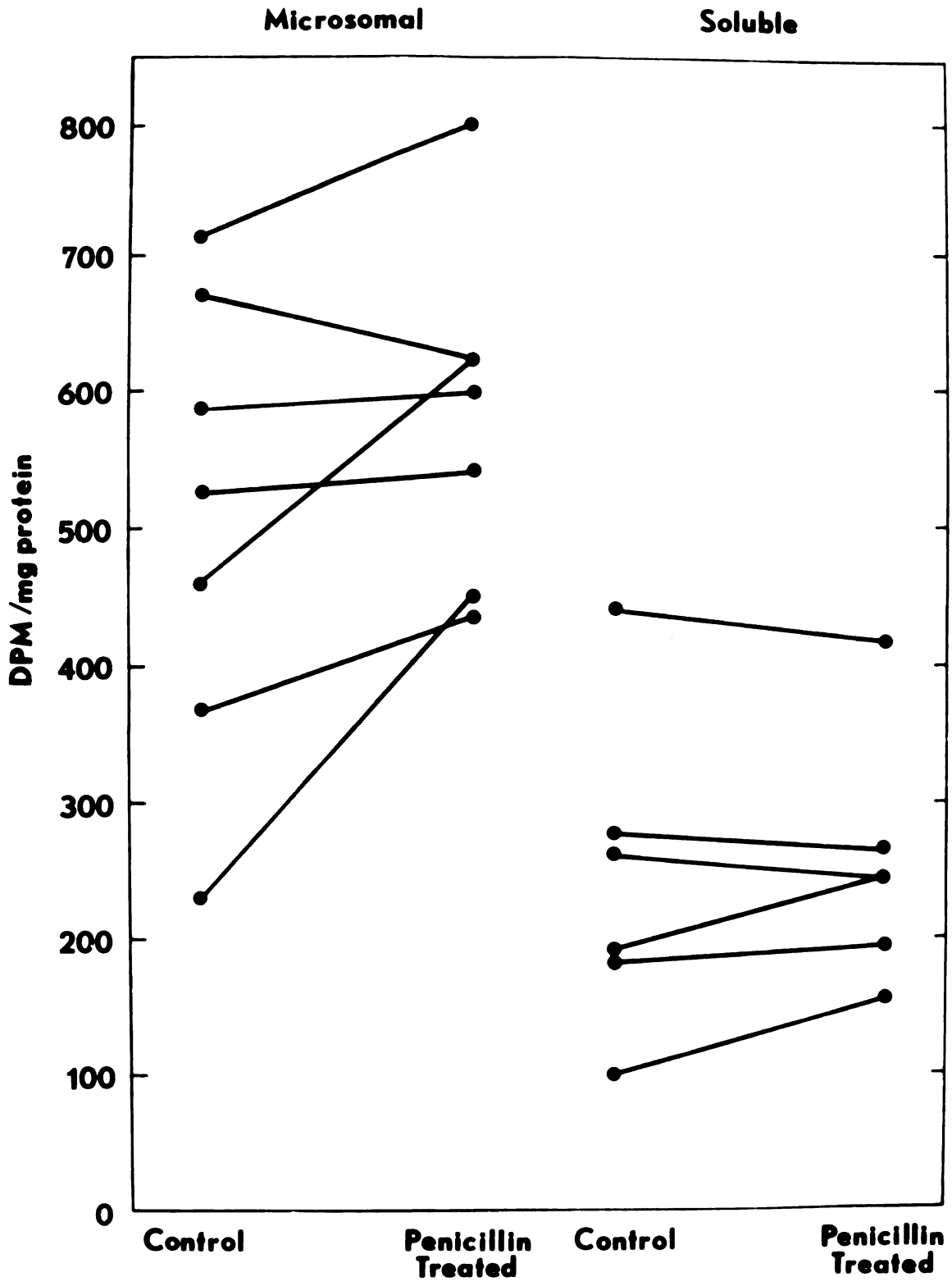

Microsomal
Soluble
800
700
600
500
400
300
200
100
0
DPM /mg protein
Control
Penicillin Treated
Control
Penicillin Treated

Figure 27

There did not appear to be a consistent effect of penicillin pre-treatment on incorporation into soluble proteins (Figure 27).

3. Leucine pool size

The specific activity of acid soluble leucine pools in renal cortical homogenates was not significantly different from control after 1, 2 or 3 injections of 90,000 I.U. procaine penicillin (Table 7). Amino acid incorporation data then would most likely reflect actual rates of protein synthesis in the cell.

Table 7. Effect of penicillin on leucine pool size in renal cortex from 2-week-old rabbits[a]

Number of injections	n[b]	DPM/nmole	
		Control	Treated
1	3	154 ± 28	127 ± 46
2	3	89 ± 26	107 ± 10
3	3	99 ± 11	111 ± 25

[a]One, 2 or 3 injections 90,000 I.U. procaine penicillin G were administered at approximately 12-hour intervals. Sixteen hours after the final injection 8.0 μCi/kg ^{14}C L-leucine were administered intravenously and animals sacrificed after 15 minutes. Each value represents the mean (± S.E.).

[b]n = number of litters.

Separation of Protein Fractions of Renal Cortical Homogenates Following Substrate Stimulation by Penicillin and *in vivo* Labeling

1. Sephadex filtration

Protein elution profiles consisted of 3 major peaks (Figure 28). GSH S-transferase activity was eluted in the third peak (#70-80),

Figure 28. Sephadex G-100 column chromatography of 100,000 x g supernatant protein from 2-week control and penicillin treated rabbits following *in vivo* pulse labeling with ^{14}C L-leucine. Littermates were treated with a single injection of 90,000 I.U. procaine penicillin G or saline for control. Animals were anesthetized 16 hours after treatment and 3 intravenous injections of 8 μCi/kg (sp. act. 302 μCi/μmole) ^{14}C L-leucine administered at 20-minute intervals. Twenty minutes after the final injection, animals were sacrificed and 100,000 x g supernatant protein from renal cortical homogenates eluted from a Sephadex G-100 (3 x 100 cm) column. Elution samples were assayed for protein (closed circles) and radioactivity (open circles). The figure is representative of results obtained from 2 litters.

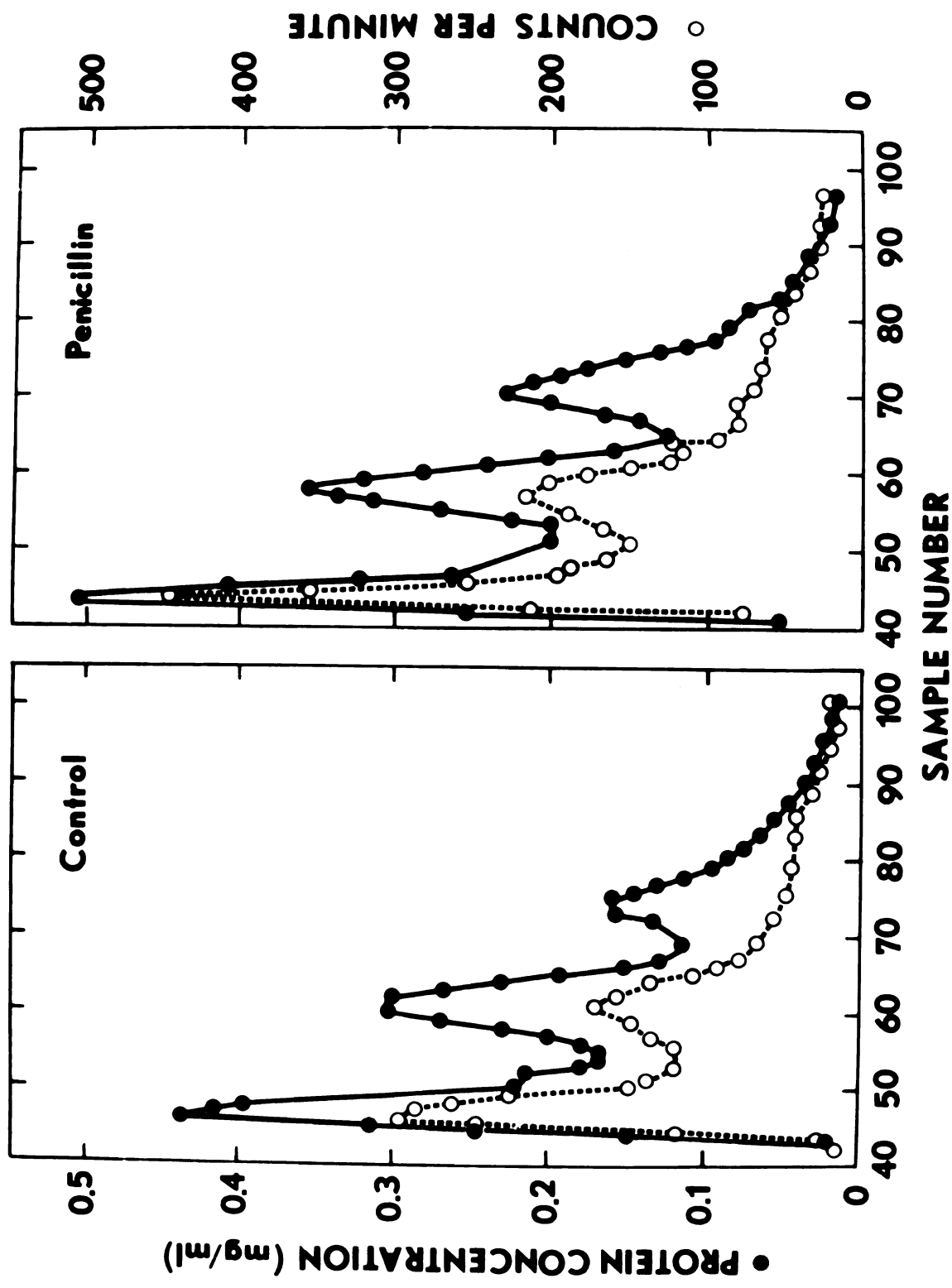

Control
Penicillin
• PROTEIN CONCENTRATION (mg/ml)
○ COUNTS PER MINUTE
SAMPLE NUMBER
0.5
0.4
0.3
0.2
0.1
0
500
400
300
200
100
0
40
50
60
70
80
90
100

Figure 28

which contained organic anion binding capacity. Two peaks were observed in the radioactivity elution profiles corresponding to protein maximums containing no GSH S-transferase activity (Figure 28). A peak in radioactivity was not observed to be associated with the eluant fractions containing GSH S-transferase activity in either control or penicillin treated animals.

2. Gel electrophoresis

Protein banding patterns in Coomassie Brilliant Blue stained gels prepared from the solubilized 100,000 x g pellet of control and penicillin treated rabbits were visually identical (Figure 29). Radioactivity in gel slices was so low that quantification of label incorporation was not attempted.

Figure 29. SDS polyacrylamide gel electrophoresis of renal cortical microsomal proteins from control and penicillin treated 2-week rabbits following *in vivo* pulse labeling with ^{14}C L-leucine. Littermates were treated with a single injection of 90,000 I.U. procaine penicillin G or saline for control. Animals were anesthetized 16 hours after treatment and 3 intravenous injections of 8 μCi/kg (sp. act. 302 μCi/μmole) ^{14}C L-leucine administered at 20-minute intervals. Twenty minutes after the final injection, animals were sacrificed and 100,000 x g pellet protein from renal cortical homogenates electrophoresed. Gels were stained using Coomassie Brilliant Blue and destained in 33% methanol-10% TCA overnight. A, control; B, penicillin.

Figure 29

DISCUSSION

The uptake of p-aminohippurate (PAH) by renal cortical slices has been widely employed for study of PAH transport. Though such data appear to be representative of function in the intact animal (Berndt, 1976), numerous complicating factors are introduced by the artificial nature of the preparation. Even the thinnest of renal cortical slices consists of several cell layers. Therefore, all of the tubular cells are not contiguous with the incubation medium and the outermost layers may act as a barrier between the medium and the inner core of tissue. Wedeen and Weiner (1973a,b) demonstrated that the fraction of the slice that participates in uptake is dependent upon the concentration of substrate, time of incubation, temperature, presence of inhibitors and slice thickness. These investigators reported that the maximum depth of penetration for PAH was 0.3 mm from each surface at 25°C in Cross and Taggart (1950) medium containing 6.7×10^{-5} M PAH and 10 mM acetate. Slices prepared in this laboratory are 0.3 to 0.5 mm thick (Hook and Bostwick, unpublished results) and for equilibrium studies are incubated in an identical medium containing a slightly higher concentration of PAH (7.4×10^{-5} M). Therefore, availability of PAH to tubular cells is probably not a limiting factor. Because PAH uptake is extremely sensitive to oxygen deprivation (Beyer, 1950; Cross and Taggart, 1950; Taggart, 1958), these data suggest that

oxygen availability is also not limiting. Furthermore, renal cortical slices are capable of regenerating ATP and maintaining electrolyte concentration gradients (Mudge, 1951; Ross and Weiner, 1972; Whittam and Davies, 1954). Therefore, slices appear to provide a suitable model for study of cellular transport function.

The S/M ratio averages tissue uptake in regions where accumulation is absent either as a result of decreased cellular viability or the presence of non-transporting tissue. Renal cortical slices consist of distal tubular and connective tissue structures in addition to proximal tubular cells where concentrative uptake of PAH occurs (Wedeen and Weiner, 1973a). Because uptake is factored by tissue weight, slice to medium concentration ratios represent an underestimate of actual transport capacity in functioning cells. Tissue concentrations in regions participating in concentrative uptake may differ from those calculated by the whole slice technique (Wedeen and Weiner, 1973b, 1974). Furthermore, alterations in PAH S/M ratio could result from a redistribution of PAH within the slice. Inhibitors of PAH accumulation such as iodoacetamide, dinitrophenol and cyanide apparently produce a portion of their effect by causing a redistribution of PAH within tissue slices (Wedeen and Weiner, 1974). Iodoacetamide and dinitrophenol decrease concentrative uptake at the slice surface but have no effect on deeper cortical cells. Cyanide inhibits concentrative uptake and penetration of PAH throughout the slice. The S/M ratio does not reflect redistribution phenomena. Theoretically, then, low accumulation of PAH by renal cortical slices from newborn animals might be due to the increased amount of non-transporting undifferentiated tissue in the

slice (Hirsch et al., 1971) and the enhanced PAH S/M ratio following substrate pretreatment to redistribution. PAH accumulation using separated proximal tubules which are devoid of non-transporting tissue, however, is also less in the newborn and is enhanced by substrate pretreatment (Ecker and Hook, 1974a). Similarly, PAH transport capacity measured *in vivo* as clearance, extraction and plasma elimination is immature in young animals (Bond et al., 1976; Horster and Lewy, 1970; Pegg and Hook, 1975) and is increased by penicillin pretreatment (Bond et al., 1976; Kaplan et al., 1975; Lewy and Grosser, 1974). Therefore, PAH transport capacity quantified as an S/M ratio is qualitatively similar to PAH uptake data obtained using suspensions of separated proximal tubules and *in vivo*. It would appear, then, that the major determinant of S/M ratio was the capacity of tubular cells to actively accumulate PAH and does, therefore, reflect the activity of the transport system.

The rate of maturation of organic anion transport may be selectively enhanced by pretreatment of newborn animals with substrate. In early studies, Hirsch and Hook (1970a,b,c) employed a single effective dosage regimen of penicillin to newborn animals. If substrate stimulation was to be used as a tool to uncover basic mechanisms of renal organic anion transport, the effect of dosage and treatment schedules had to be further defined. The maximal increase in PAH S/M ratio was observed after pretreatment with 90,000 I.U. procaine penicillin G twice daily for 2 days (Figure 1). Larger doses of penicillin over extended periods of time did not result in further enhancement of the ability of newborn renal cortical slices to accumulate PAH (Figures 2 and 3). The net increase in transport capacity

was greatest when animals were killed 24 hours after the final penicillin injection (Figure 4). Though several organic anions are capable of stimulating transport capacity, penicillin, which is transported as efficiently as PAH (Weiner, 1973), has proven to be the most effective. In the majority of studies penicillin was administered as the procaine salt in order that absorption from subcutaneous injection sites would be retarded and plasma concentrations maintained for longer periods of time. Procaine is an organic base and has no effect on organic anion transport capacity (Hirsch and Hook, unpublished results).

Penicillin is a competitive inhibitor of PAH transport (Beyer et al., 1944; Hirsch and Hook, 1970c). Residual drug in renal cortical tissue during incubation might decrease PAH uptake. Of major concern in these studies was whether penicillin concentration in tissue was sufficiently reduced 24 hours after termination of treatment to alleviate interference with PAH transport. Bond et al. (1976) reported that 48 hours were required for plasma penicillin to decrease to concentrations which were unlikely to interfere with PAH transport *in vivo*. These investigators, however, used dogs and administered larger total doses of penicillin during treatment. To estimate residual penicillin in rabbit renal cortex, a mixture of procaine penicillin and ^{14}C-benzyl penicillin was administered as the final dose in the treatment regimen. After 24 hours there was no detectable radioactivity in renal cortex. Because labeled penicillin was injected only with the final dose, the renal disposition of drug could possibly have been different than for total drug resulting in an underestimate of tissue content. However, during

preparation of slices and storage in ice cold saline prior to incubation, a significant portion of any residual penicillin would probably be leached from the tissue. In any regard, the data suggest that the stimulating effect of penicillin on organic anion transport capacity is maximally expressed 24 hours after the fourth 90,000 I.U. injection.

During normal development PAH S/M ratio increases from low values at birth to a peak at 4 weeks (Hirsch and Hook, 1970a). This peak is not exceeded after penicillin pretreatment (Hirsch and Hook, 1969, 1970a). Thus, treatment of newborn with substrates of the organic anion transport system results in earlier maturation but does not increase maximal intrinsic transport capacity. In a further attempt to increase maximal transport capacity, animals were exposed to penicillin both pre- and postnatally. When compared to saline controls, PAH S/M ratio in pups from penicillin treated does was significantly increased immediately following birth (Hirsch and Hook, 1970c). Large doses of penicillin were then administered postnatally to these animals with stimulated transport capacity. The ability of renal cortical slices to accumulate PAH following postnatal substrate challenge was enhanced such that PAH S/M ratio at 3 days, 1 and 2 weeks of age was not significantly different from the 4-week peak (Figure 6). At 4 weeks postnatal penicillin had no effect on organic anion transport capacity (Figure 6). Thus, even with combined prenatal and postnatal treatment, the maximal PAH S/M ratio seen at 4 weeks was not exceeded. These data could be interpreted to indicate that a maximal number of transport sites exist within the kidney and that these are maximally functional at 4 weeks

of age. Penicillin treatment then results in full expression of transport potential.

PAH S/M ratio is measured in a steady state when tissue concentration is sufficiently high that the rate of passive efflux is equivalent to the rate of active influx of PAH. Superficially, it would appear that an increase in PAH S/M reflected an increased transport capacity. However, enhanced accumulation of PAH following penicillin might result from increased uptake, decreased efflux or some combination resulting in altered intracellular concentration. Hirsch and Hook (1970c) suggested that the action of penicillin on PAH accumulation was the result of an increased entry into the cell. To quantify the effect of penicillin on uptake of PAH, renal cortical slices were preincubated for 30 minutes in oxygenated buffer. Ross and Weiner (1972) demonstrated that freshly prepared renal cortical slices exhibited decreased adenylate energy charges, probably due to hypoxia during preparation. Aerobic incubation for 30 minutes allowed the slices to regain normal adenylate energy charges and presumably normal levels of tissue constituents necessary for maximal rates of transport (Ross and Weiner, 1972). Previous kinetic analysis (Ecker and Hook, 1974b; Kim et al., 1972) did not include preincubation and were in reality measuring transport under less than optimal conditions. After preincubation, rates of PAH uptake were estimated for only 15 minutes so that influx would most likely be the major component of PAH movement. Finally, tissue slices incubated under 100% nitrogen developed PAH S/M ratios of 1 (Cross and Taggart, 1950). PAH uptake under nitrogen was therefore taken as representative of that portion of uptake resulting from passive inward

diffusion and was subtracted from total uptake to result in an estimate of the active, oxygen requiring component of uptake. The stimulating effect of penicillin pretreatment on uptake of PAH was confined to the active portion of the process (Figure 7). Passive properties of the cell membranes were apparently not altered by treatment because influx of PAH under nitrogen (Figure 7) and efflux determined as runout from preloaded slices was unaffected (Figure 8). Therefore, these data suggested that the action of penicillin to increase transport capacity resulted from a stimulating effect on the energy requiring mechanism involved in translocation of organic anions from extracellular to intracellular space.

Although physical distortion in the transport process using slices is probably inconsequential in equilibrium studies, it may be very significant in estimating initial rates of uptake or in analyzing the direction of movement of substrate between the incubation medium and the intracellular space. Penetration and distribution of substrate in the slice are critical and may become rate limiting (Wedeen and Weiner, 1973b, 1974). Barriers to diffusion of constituents in the incubation medium are absent when using suspensions of separated proximal tubules. This preparation actively transports organic anions, uptake is more rapid and equilibrium is approached sooner than in slices (Burg and Orloff, 1962; Ecker and Hook, 1974a). Therefore, separated proximal tubules appeared to be a better preparation with which to study initial rate kinetics (Huang and Lin, 1965; Park et al., 1971; Sheikh and Moller, 1970).

The apparent theoretical maximal velocity of PAH uptake in separated proximal tubular cells was increased following penicillin

pretreatment, while there was no change in the apparent affinity of the carrier for substrate (Figure 9). Conclusions from this analysis, however, must be drawn with caution because, unlike cell-free enzyme systems, transport processes in tubular cells are complex and probably do not follow classical Michaelis Menten kinetics (Christensen, 1969; Weiner, 1973). It was concluded, however, that the change in the transport system was quantitative (theoretical maximum transport velocity) rather than qualitative (affinity). Similar alterations in kinetic parameters of the transport system were observed during development. Kim et al. (1972) and Ecker and Hook (1974b) determined that the theoretical maximum velocity of PAH uptake in renal cortical slices from rats and rabbits increased with age and that the apparent affinity of the carrier for substrate was not changed. The similarities between substrate stimulation and development provide further support for the hypothesis that a primary factor in development of transport capacity is endogenous substrate load and, in addition, suggest that alterations in transport after penicillin pretreatment are analogous to those occurring during development, both involving increased activity of the active transport system.

The postulated carrier for organic anions has not been isolated or identified. Therefore, the possibility must be considered that penicillin is acting on a process indirectly related to transport, for example energy transfer. As are many other transport systems, organic anion secretion is dependent upon the presence of sodium (Gerencser and Hong, 1975; Gerencser et al., 1973). To determine whether this aspect of function was altered by penicillin,

Na, K-ATPase activity was measured. The activity of Na, K-ATPase, a basal cell membrane marker (Busse et al., 1975; Post and Sen, 1967; Silverman and Black, 1975) was less in separated proximal tubule homogenates from newborn than adult rabbits (Figure 13), which is consistent with increased cell size and differentiation during development (Hirsch et al., 1971). Penicillin pretreatment did not alter enzyme activity (Figure 13), suggesting that enhanced transport capacity was not the result of increased cell surface area and passive membrane flux of PAH or availability of PAH to transport sites. Furthermore, it would appear that substrate stimulation did not occur as the result of increased energy transfer through Na, K-ATPase. The possibility of a separate organic anion stimulated ATPase was investigated by incubating aliquots of tubular homogenates with a wide range of PAH in the medium but no activity was observed (unpublished results). Therefore, these data support the hypothesis that the effect of penicillin is specific for the transport system.

Agents such as 3-methylcholanthrene and phenobarbital increase the capacity of hepatic drug-metabolizing enzyme systems to metabolize drugs due to increased synthesis of enzyme molecules (proteins) (Conney, 1967). Inhibitors of protein synthesis such as puromycin block substrate stimulation in the liver (Conney and Gilman, 1963). Hirsch and Hook (1970b) observed increased incorporation of ^{14}C-leucine into renal cortical slice homogenates after penicillin, suggesting increased protein synthesis. Cycloheximide, a potent inhibitor of protein synthesis (Ennis and Lubin, 1964; Young et al., 1963), blocked enhancement of PAH S/M ratio (Hirsch and Hook, 1970b).

Cycloheximide, however, also depressed control PAH S/M as either the result of transport protein turnover without replacement or systemic toxicity. Hirsch and Hook (1970b) treated the animals with 6 injections of cycloheximide over a 3-day period. Rapid protein synthesis in developing animals would cause them to be especially susceptible to the toxic side effects of inhibitors of protein synthesis. The dose of cycloheximide used in the current study was increased to 0.4 mg/kg and the duration of treatment reduced to 2 injections during a 24-hour period. In adult rats 0.5 mg/kg cycloheximide was observed to significantly decrease renal protein synthetic rate *in vivo* (Rothblum et al., 1976). Increased susceptibility of young animals to the effects of drugs would then suggest that the present dose of cycloheximide was also effective in inhibiting protein synthesis. Stimulation of PAH transport by penicillin was completely blocked by cycloheximide with no decrease in control values (Figure 10). These data suggest that substrate stimulation is dependent upon an intact protein synthetic mechanism and support the hypothesis that transport is increased as the result of synthesis of a protein component of the transport system.

The theoretical maximum velocity of PAH uptake is increased following penicillin pretreatment (Figure 9) and stimulation is blocked by concurrent treatment with cycloheximide (Figure 10). Similar observations have been made following stimulation in other active transport systems. Reynolds et al. (1974) observed that amino acid transport in newborn rat kidney cortex was increased by preincubation at 37°C, while slices from adult rats did not respond. Transport stimulation was blocked by cycloheximide and the authors

suggested that transport of new carrier proteins was involved. Hexose starvation of chick embryo fibroblasts in culture produced by growth in glucose free medium resulted in increased 2-deoxyglucose accumulation following resuspension in sugar containing medium (Kletzien and Perdue, 1975). Enhanced uptake was characterized by an increase in the maximal velocity of transport and was blocked by cycloheximide. Results of both investigations were interpreted to indicate an increased number of transport sites (Kletzien and Perdue, 1975; Reynolds et al., 1974). These observations involved regulatory mechanisms but similar results were obtained following hormone stimulation. Tews et al. (1975) reported that glucagon pretreatment of rats stimulated amino acid transport capacity in liver slices by a mechanism sensitive to cycloheximide inhibition. The rate of stimulation decay was used as an estimate of turnover rate and it was concluded that the half life of the induced protein was extremely short ($\approx$ 1 hr). These investigators suggested that stimulation might be the result of transport activation rather than increased synthesis of a critical protein; however, the inhibitory effect of cycloheximide was taken as evidence that activation probably was not the mechanism. The possibility of a protein or peptide modulator or an alteration in transport systems without increased carrier synthesis (i.e., transport efficiency) was not considered in these studies. Therefore, for these investigators to hypothesize that enhanced transport capacity resulted from increased synthesis of transport carrier is attractive, but with the available data, perhaps oversimplistic.

One of the initial objectives of this investigation was to characterize and perhaps isolate penicillin induced components of the organic anion transport system. Though conclusive data were not available, it was considered that enhanced transport capacity following penicillin might occur as the result of increased transport carrier synthesis (Hirsch et al., 1970b). Since a prerequisite of carrier mediation is a reversible interaction between substrate and transport protein, binding data may provide specific evidence for quantification and localization of components of the transport system. Substrate binding without correlation to transport is not, however, absolute proof of carrier function, though the two are often taken as synonymous. Using binding as the criteria for localization of transport sites, two possibilities exist, one soluble (renal ligandin) (Kirsch et al., 1975b) and the second particulate (microsomal) (Holohan et al., 1975). Therefore, studies were undertaken to investigate the effects of substrate stimulation on particulate and soluble components of proximal tubular cells and to determine their relation to organic anion transport capacity.

Enhanced hepatic drug metabolizing capacity resulting from phenobarbital induced increases in synthesis of microsomal drug metabolizing enzymes is characterized ultrastructurally by a proliferation of smooth endoplasmic reticulum (Chiesara et al., 1967; Conney et al., 1967). Though penicillin pretreatment had no effect on proximal tubular cell morphology by light microscopy (Hirsch et al., 1971), if the organic anion transport system is membrane bound as several investigators suggest (Holohan et al., 1975), and if substrate stimulation occurs through increased synthesis of protein

components of the transport system (Hirsch and Hook, 1970b), alterations in tubular ultrastructure might be observed following penicillin pretreatment. Therefore, separated proximal tubules from adult and control and penicillin treated 2-week rabbits were prepared, coded and sent to the microscopy laboratory in a double blind fashion. Several electron microscopy samples were randomly sectioned from each tubule pellet, analyzed and conclusions tabulated prior to breaking the code.

The major criticism resulting from use of separated proximal tubules prepared by collagenase digestion of renal cortex is basement membrane erosion caused by the proteolytic action of the enzyme. Viability of tubular cells does not appear to be severely affected in that they are metabolically responsive, continue to consume oxygen and maintain normal electrolyte concentration gradients (Ecker and Hook, 1974a; Guder and Rupprecht, 1975; Guder et al., 1971). In this study, collagenase concentration in the medium was adjusted to produce adequate yields of tubules with the least possible amount of structural damage. Though some basement membrane disruption was inevitable, intracellular organelles were mostly intact and structures normal (Figure 11). In addition, there was not a differential effect of collagenase on adult and 2-week tissue or on control and penicillin treated 2-week tissue that might explain differences in transport capacity with age and treatment.

Penicillin had no effect on intracellular morphology when compared to saline control (Figure 12). Though transport measurements suggest that penicillin increases the rate of maturation through a mechanism similar to the normal development, there was no conversion

of 2-week tubular cells to more "adult"-like forms. The increase in basilar plasma membrane surface area (cell size, membrane infolding) with age and the lack of a penicillin effect on membranes in 2-week animals is consistent with the results obtained when Na, K-ATPase was measured (Figure 13). Though the significantly lesser enzyme activity in newborn might be primarily explained due to immaturity of the enzyme system in kidney (Davis and Dixon, 1971) rather than decreased basal membrane area, the lack of altered enzyme activity following penicillin would more likely be indicative of constant membrane area in the cell. A necessary assumption is, of course, that penicillin itself has no specific effect on the Na, K-ATPase enzyme. Therefore, it was concluded that the effect of penicillin on organic anion transport was either too subtle to be observed ultrastructurally or that the sensitive component was cytosolic and not membrane bound. These data do not, however, exclude the possibility that the transport system is associated with membranes or that penicillin acts to increase transport through a protein synthetic mechanism because, for example, 3-methylcholanthrene increases synthesis of microsomal drug metabolizing enzymes in the liver but induction is specific enough that gross alterations in endoplasmic reticulum are not observed (Fouts and Rogers, 1965). Alternatively, however, one might conclude that the transport acceptor is not membrane bound and, therefore, if induced would not be observed ultrastructurally. Indeed, recent evidence suggests that a soluble enzyme with characteristics similar to the hepatic organic anion binding protein, ligandin, binds PAH and may therefore be implicated in renal transport (Kirsch et al., 1975b).

Ligandin, a cytosolic organic anion binding protein isolated from homogenates of rat and human liver, has been implicated in transfer of organic anions from plasma into parenchymal liver cells (Levi et al., 1969; Reyes et al., 1971). Inducers of drug metabolizing enzymes such as phenobarbital increase hepatic ligandin concentration as well as uptake and binding of transported organic anions (Reyes et al., 1969, 1971). These data are consistent with the hypothesis that ligandin acts as a primary acceptor protein during transport in liver. However, a role of ligandin as an obligatory rate limiting step for organic anion transport in the liver has been questioned. Klaasen (1975) investigated the relationship between hepatic ligandin and biliary excretion of sulfobromophthalein (BSP) and ouabain. Hepatic clearance of both BSP and ouabain was increased by inducers of drug metabolizing enzymes in the liver. However, there was poor correlation between induction of ligandin and enhanced biliary excretion of BSP. Though plasma disappearance and biliary excretion of ouabain was increased following treatment, ouabain, a neutral compound, was not bound by ligandin. Therefore, the role of ligandin in increased biliary excretion of drugs following treatment with microsomal enzyme inducers was questioned (Klaasen, 1975). Similarities between renal and hepatic ligandin (Fleischner et al., 1972; Kirsch et al., 1975a,b) might then suggest that the role of the renal protein in organic anion transport should also be investigated.

Ligandin is physically and immunologically similar to GSH S-transferase B, one of a class of enzymes which conjugate various aryl, alkyl, aralkyl and epoxy compounds to reduced glutathione

(Habig et al., 1974b). Enzymes of this class possess overlapping catalytic specificity (Habig et al., 1974a,b). GSH S-transferases A, B and C are capable of conjugating GSH to several aryl anions. Habig et al. (1974b) determined that the specific activities of transferases A and C using the substrate dichloronitrobenzene were 3 orders of magnitude greater than observed for transferase B. Using dinitrochlorobenzene as substrate, conjugating activity was similar among the 3 transferases but several-fold greater than when dichloronitrobenzene was used. Extrapolation from enzyme activity using dichloronitrobenzene to ligandin concentration is therefore difficult. However, data presently available do not exclude the possibility that other transferases function in organic anion binding and therefore transport phenomena (Kaplowitz et al., 1975; Ketley et al., 1975). Because hepatic and renal glutathione transferases are structurally and functionally similar (Fleischner et al., 1972; Kirsch et al., 1975a,b), it might be predicted that there was also binding overlap in the kidney. Therefore, in the present investigation both aryl substrates were tested in several instances. Specific alterations in GSH S-transferase B concentration unrelated to total transferase activity would most likely be observed using dinitrochlorobenzene. Results obtained using both substrates were qualitatively similar, suggesting that when a change in enzyme activity occurred, total transferase, not specifically GSH S-transferase B activity, was changed.

Organic anion transport capacity in newborn rats and rabbits is less than in the adult (Figures 14 and 15). During the neonatal period transport increases. The pattern of transport development,

however, is different than that for GSH S-aryltransferase activity (Figures 14 and 15). Assuming that measurement of enzyme activity is indicative of enzyme concentration, the data suggest that development of transport capacity and enzyme activity do not occur synchronously and therefore may not be interdependent. Enzyme concentration in renal homogenates was increased by chronic metabolic acidosis, a non-specific stimulus to kidney growth (Figure 19) and 3-MC pretreatment (Figure 16), but there was no stimulating effect on transport capacity. To determine whether stimulation of transport was lost by measuring steady state accumulation of substrate following 3-MC, the rate of PAH uptake into rat renal cortical slices was measured. There was no effect of 3-MC on either rate of uptake or steady state accumulation of PAH (unpublished results). TCDD, another microsomal enzyme inducing agent, was reported by Kirsch et al. (1975b) to increase ligandin concentration and *in vivo* excretion of organic anions in the kidney. TCDD also had no stimulating effect on transport of PAH *in vitro* (Tables 1 and 2). Therefore, during development and following treatments which enhanced enzyme activity, a lack of interdependence was observed between GSH S-transferases and PAH S/M ratio.

Penicillin increased transport capacity in 2-week-old rabbits but had no effect on GSH S-aryltransferase activity (Figure 20). Kidney weight to body weight ratios in penicillin treated animals were not increased and therefore the absence of enhanced enzyme activity probably cannot be attributed to a dilutional effect resulting from non-specific increases in total renal protein. 3-MC, a polycyclic hydrocarbon shown to increase GSH S-aryltransferase

activity in rat kidneys, enhanced transport capacity in kidneys of 2-week rabbits (Figure 17). Since 3-MC is not an organic anion, the increase in transport cannot be attributed to substrate stimulation. GSH S-aryltransferase activity was also increased in these animals (Figure 18). However, 3-MC induces other drug metabolizing enzymes in the kidney (Conney, 1967; Gelboin and Blackburn, 1964) and the enhanced transport need not be a direct result of increased GSH S-transferase concentration.

The increase in organic anion transport capacity following uninephrectomy may constitute a special case of substrate stimulation (Goldberg et al., 1970). Most of the organic acids found in the urine are derived from bacterial metabolism in the gut (Goldberg et al., 1970). The majority of these compounds are derived from dietary precursors of either aromatic or non-aromatic form (Armstrong et al., 1955; Asatoor, 1965). Serum from azotemic nephrectomized rats was reported to depress uptake of iodohippurate in renal cortical slices, probably due to the presence of organic anions in the plasma resulting in competitive inhibition of hippurate transport (Orringer et al., 1971). Uninephrectomy then results in an increased substrate load on the remaining kidney. Goldberg et al. (1970) reported that administration of neomycin sulfate following uninephrectomy prevented the transient increase in transport capacity normally observed, presumably by sterilizing the gut and preventing absorption of organic anions responsible for the functional demand on residual tissue. The compensating kidney is characterized by hypertrophy and hyperplasia of renal tubular cells. The major portion of the response occurs through increased size and protein and RNA content of cells

(Goldberg et al., 1970). Increased organic anion transport capacity, however, is observed prior to measurable alterations in cell constituents and is therefore probably specific to the transport system (Goldberg et al., 1970). Though enhanced PAH S/M ratio was observed following uninephrectomy, in this investigation there was no significant increase in GSH S-aryltransferase activity (Figure 22).

Enzyme activity and transport capacity were determined in male and female mice to investigate the correlation without exogenous manipulation. Ecker and Hook (unpublished observation) had previously observed a significantly greater ability of renal cortical slices from adult male mice to accumulate PAH than slices from female mice. Greater transport capacity in male mice, however, was not accompanied by increased GSH S-aryltransferase activity (Figure 21). On the other hand, Clifton et al. (1975b) determined that aryltransferase activity was greater in the male than female rats, which is consistent with the observation of Bowman and Hook (1972) that transport capacity for PAH was also greater in the male.

Active reabsorptive systems for amino acids and sugars in proximal tubular cells appear to be dependent upon cellular GSH concentrations (Hewitt et al., 1974; Pillion and Leibach, 1975). Accumulation of various amino acids and the sugar α-methyl-D-glucoside by renal cortical slices is inhibited by diamide, a thiol oxidizing agent which depletes tissue GSH. GSH oxidation by diamide, however, is reversible and tissue concentrations are regenerated *in vitro* after approximately 30 minutes (Hewitt et al., 1974). The convulsant L-methionine-SR-sulfoximine, when administered to intact animals, irreversibly inhibits the GSH synthesizing enzyme, gamma glutamyl

cysteine synthetase (Palekar et al., 1975). Ketley et al. (1975) reported that binding of substrate to glutathione S-transferases was increased approximately five-fold by glutathione. Thus, if PAH transport were dependent upon GSH S-transferases, depletion of tissue GSH might decrease transport of organic anions. Tissue GSH concentrations in adult male rats were decreased approximately 50% by sulfoximine pretreatment, but there was no significant effect on transport function (Figure 23). These data might suggest that renal organic anion transport was not dependent upon GSH. However, the assumption must be made that the remaining pool of GSH was not sufficient to support transport at control values.

The relative lack of correlation between GSH S-transferase activity and PAH transport capacity suggests that GSH S-transferase enzymes either do not function in transport or do not constitute the major rate limiting component of the renal organic anion transport system. The role of intracellular binding to cytoplasmic (Kirsch et al., 1975b) or particulate (Holohan et al., 1975) proteins in concentrative uptake of organic anions, however, has not been resolved. The evidence is clearer for compounds such as phenol red, which are accumulated intracellularly to concentrations greater than in the bathing medium under anoxic conditions when the active transport system is completely inhibited (Sheikh, 1972). In this instance, the only mechanism of uptake can be intracellular binding. PAH, however, is not accumulated under nitrogen except for that quantity which enters the cell by passive diffusion resulting in S/M ratios of unity (Cross and Taggart, 1950). Therefore, one might conclude that PAH binding is not a major determinant of cellular

uptake. However, additional evidence is available. Miatello et al. (1966), using autoradiography and electron microscopy, observed that PAH existed free in the cytoplasmic matrix and was only sparingly associated with endoplasmic reticulum and mitochondria. Farah et al. (1963) and Welch and Bush (1970), from studies of PAH efflux by preloaded renal cortical slices, concluded that two intracellular compartments of PAH existed, one small and capable of rapid equilibration with extracellular space and the second larger, less readily exchangeable with free PAH pools and responsible for concentrative uptake in the cell. Once across the membrane from extra- to intracellular water these investigators suggest that organic anions enter a free pool from which they are sequestered by the slowly exchanging pool. Maximal uptake of organic anions is then a function of the number of available sites on the cell components which constitute the slowly exchanging pool. From the data of Miatello et al. (1966), it would appear this pool was cytosolic and therefore supports the soluble organic anion binding protein hypothesis of Kirsch et al. (1975b). To explore the relationship between transport capacity in kidney and soluble binding protein, the effect of age and substrate stimulation was investigated.

The method of binding quantification used by Arias and co-workers (Kirsch et al., 1975b) and duplicated in this laboratory involves elution of a protein-substrate mixture from a 3 x 100 cm Sephadex G-100 column. Elution of free ligand is retarded relative to the larger protein-ligand complex. However, because of the time required (14-18 hours) and the length of the column, it is possible that significant alterations in binding equilibrium occur during elution,

making interpretation of data difficult. These studies, however, constitute the only direct evidence of cytosolic protein binding in renal cortical tissue (Kirsch et al., 1975b). Using this technique, it was determined that binding of penicillin to the GSH S-transferase containing protein peak of 100,000 x g supernatants was less in tissue from newborn rabbits than adult (Figures 24 and 25), which is consistent with the immaturity of PAH transport capacity and enzyme activity in newborns (Figures 14 and 15). Binding was not, however, affected by penicillin pretreatment, which produced an at least 2-fold stimulation of transport capacity (Figures 20 and 26). Similarly, penicillin pretreatment had no effect on uptake of PAH under 100% nitrogen atmosphere (Figure 7), suggesting that enhanced transport capacity following substrate stimulation was not the result of increased intracellular binding. These data provide further evidence to support the hypothesis that the action of penicillin is specific and confined to the active uptake process.

PAH S/M ratio is significantly enhanced following penicillin pretreatment of newborn rabbits (Figure 1) as a direct result of an increased rate of active PAH uptake (Figures 7 and 9). The sensitivity to cycloheximide inhibition (Figure 10) and increased incorporation of labeled amino acids into renal cortical protein following substrate stimulation in rats (Hirsch and Hook, 1970b) suggest the effect is exerted through altered synthesis of transport proteins. Therefore, it was considered that in rabbits, which are more susceptible to substrate stimulation, an effect of greater magnitude might be observed. From available data, the half life of the induced components of the transport system in rabbits is approximately 20

hours (Figure 4). Therefore, the rate constant for degradation, defined as Ke = ln $2/T_{1/2}$, is 0.034 hr^{-1}. Assuming that transport data may be handled in a manner analogous to enzyme systems, and that transport capacity is a function of the turnover of a rate limiting protein component of the system, the basal rate of synthesis of this component may be calculated from the equation S = PK where S = rate of synthesis and P = content per unit weight (Berlin and Shimke, 1965). Assigning a basal S/M ratio of 7 to the system, S = 0.24 units/hr^{-1}. Following induction S/M ratio increases to approximately 25. If the rate of degradation does not change the rate of synthesis required to maintain this steady state is 0.85 units/hr. Therefore, from these theoretical considerations the rate of synthesis following penicillin pretreatment should be 3.5 times the basal rate. However, though PAH S/M ratio was increased 2- to 3-fold by penicillin pretreatment, there was no stimulating effect on ^{14}C L-leucine incorporation into renal cortical slice protein (Table 2). In addition, though developmental changes in rabbit renal cortical RNA/protein, DNA/protein, and RNA/DNA were evident, there was no effect of penicillin pretreatment on these ratios in the newborn (Table 4). This method of determination of cellular nucleic acid and protein content, however, is relatively insensitive and specific subtle changes might not be observed. Because the specific activity of soluble intracellular leucine pools was not determined, the leucine incorporation data cannot be equated to rates of protein synthesis. Penicillin pretreatment did not, however, alter leucine pool size *in vivo* (Table 7), which might suggest that pool sizes *in vitro* would also not be affected. Riggs

and Walker (1963) observed that active transport of amino acids in Ehrlich ascites tumor cells was related to the rate of incorporation of that amino acid into protein. Increased intracellular concentration of free amino acid enhanced the rate of incorporation into protein and vice versa. Theoretically, penicillin could alter the rate at which amino acids were taken up by tubular cells, leading to false estimates of protein synthetic rate. However, leucine S/M ratio, a measure of transport activity and intracellular concentration, was not affected by penicillin pretreatment. The results are therefore probably not artifacts of a decreased amino acid availability.

The discrepancy between previous work by Hirsch and Hook (1970b) using rats, in which increased amino acid incorporation was observed following penicillin, and the present study may be the result of different techniques of sample preparation. Homogenization of renal cortical tissue in TCA results in precipitation of proteins, nucleic acids and lipids. Therefore, contamination of the pellet with label from amino acyl tRNA complexes as well as metabolized or adsorbed leucine might result. In the present investigation lipids were extracted from TCA insoluble material using ethanol and ethanol-ether, RNA by alkaline digestion and DNA by hot acid digestion (Schneider, 1957). Furthermore, it must be considered that mechanisms of stimulation in the rat and rabbit differ.

Suspensions of separated proximal tubules may more closely approximate conditions *in vivo* because each cell is constantly bathed in medium and not dependent upon diffusion through exterior cell layers for oxygenation (Burg and Orloff, 1962). As a result,

subtle changes in cell function which may not be observed in slices due to the heterogeneous population of cells (interior versus exterior) could possibly be more evident using separated tubules. The incorporation of labeled L-leucine into protein, however, was not significantly different from control when determined 3 or 24 hours after the final injection of penicillin (Table 5), though a slight increase was observed after 24 hours. Preparations of separated proximal tubules using collagenase digestion of renal cortex has been criticized in that a significant degree of basement membrane disruption is produced as the result of enzyme digestion. Though ultrastructural analysis has indicated that intracellular organelles are intact (Figure 11), the possibility of impaired function cannot be liminated. To minimize contributions due to tissue disruption and less than optimal conditions for protein synthesis, amino acid incorporation was also quantified *in vivo*.

Isolation of binding components of the renal organic anion transport system has been hindered by failure to develop an effective, irreversible inhibition of transport. Recently, however, Ross and co-workers (Holohan et al., 1975) quantified a specific, competitive binding of several transported organic anions to subcellular fractions of renal cortical homogenates. The 100,000 x g pellet was observed to possess the greatest capacity to bind organic anions. These data suggest that the binding component of transport is also localized to the particulate (microsomal) fraction of renal homogenates (Holohan et al., 1975). Therefore, the effect of penicillin pretreatment on amino acid uptake into 100,000 x g pellet protein was investigated. Fifteen minutes after intravenous injection of ^{14}C

L-leucine, label content of 100,000 x g pellet protein from penicillin treated animals was slightly increased (Table 6). When incorporation in whole cell protein was determined, there was no effect of penicillin. Intuitively, it would appear logical to conclude that because the proximal tubule is capable of such a large number of transport and metabolic functions (Weiner, 1973; Wesson, 1969), the organic acid transport system might constitute a relatively small percentage of total cellular protein. The effect of penicillin on transport is specific, i.e., organic base transport is not altered and it would be expected that any changes in protein synthesis would also be for specific proteins in a manner similar to the effect of 3-methylcholanthrene on hepatic drug metabolism (Fouts and Rogers, 1965). Unlike phenobarbital, which induces synthesis of a large array of drug metabolizing enzymes (Conney, 1967), 3-methylcholanthrene has an inducing effect on a limited number of enzyme systems and therefore does not produce ultrastructural alterations in liver cell morphology as does phenobarbital. Therefore, to insure the highest possible sensitivity in detection of altered protein synthetic rates in kidneys after penicillin, measurement of uptake into subcellular fractions was used. Furthermore, because data suggest that the transport acceptor may be either soluble (Kirsch et al., 1975b) or particulate (Holohan et al., 1975), both 100,000 x g supernatant and pellet material were assayed for radioactivity following labeling.

Stimulation of transport capacity was fully developed after only two injections of 90,000 I.U. procaine penicillin G (Figure 3). Up to 8 injections over 4 days produced no further stimulation (Figure 3). Prior measurements of amino acid incorporation were

made after 3 or 4 injections of penicillin (Tables 3, 5 and 6). If the presence of excess substrate results in an immediate, rapid increase in transport capacity as the data suggest (Figure 3), synthesis of an induced protein component might also be rapidly increased and followed by a slower rate of synthesis sufficient to maintain the new, higher steady state as long as substrate is present. Diminution of substrate load then removes the stimulus required to maintain enhanced transport capacity. Numerous examples of similar substrate induced adaptations are available (Knox et al., 1956). An effect of substrate stimulation on amino acid incorporation might then not be detected when using longer treatment schedules. To investigate early stimulating effects of penicillin on transport capacity, animals were administered only a single injection. PAH S/M ratio was significantly increased 8 hours after treatment and stimulation of transport was maximal after 24 hours (Figure 5). Further measurements of the effect of penicillin on amino acid incorporation were therefore conducted during this early phase of stimulation.

Incorporation of ^{14}C L-leucine into 100,000 x g pellet protein 16 hours after a single injection of penicillin was variable but increased in all but one determination (Figure 27). There was not, however, a statistically significant difference in mean control or penicillin treated values. No consistent change in labeling of soluble proteins was observed.

Within the past several years the importance of amino acid pool size (specific activity) in determinations of protein synthetic rates has become increasingly evident (Alpers and Thier, 1972;

Bergen, 1975; Regier and Kafatos, 1971; Ross et al., 1973). The specific activity of labeled amino acids in protein is a reflection of the specific activity of intracellular soluble amino acids available for protein synthesis. Certain experimental manipulations (Regier and Kafatos, 1971; Ross et al., 1973) and endogenous functions such as metabolism (Bergen, 1975) alter amino acid pools resulting in erroneous interpretation of amino acid incorporation data as applied to protein synthesis. Theoretically, if the specific activity of intracellular leucine pools in kidney cortex following penicillin treatment were decreased, actual protein synthetic rates would be higher than estimated from incorporation data. However, 1, 2 or 3 injections of penicillin had no effect on leucine pool size (Table 7), leading to the conclusion that amino acid incorporation presented a true estimate of protein synthetic rate and not an artifact of altered leucine pool size.

Incorporation of labeled leucine into microsomal and cytosolic proteins following penicillin pretreatment was tested as a possible marker to facilitate identification of a protein or group of proteins involved in substrate stimulation of organic anion transport in the kidney. From the previous data (Figure 27), two possibilities are apparent. First, incorporation of label is an accurate measure of the effect of penicillin on macromolecular biochemistry and there is no significant change in protein synthetic rate; and second, penicillin induces synthesis of a limited number of proteins but the magnitude of the effect is diminished by quantification of uptake in whole subcellular fractions. In the latter case, partial purification of soluble or microsomal proteins might remove much of the

contamination arising from other loci of amino acid incorporation and essentially magnify the effect of penicillin on sensitive proteins. Therefore, following penicillin treatment and pulse labeling *in vivo*, 100,000 x g supernatant protein was applied to a Sephadex G-100 column and eluted. Radioactivity and protein were determined in each eluant fraction. The lack of significant amino acid incorporation into protein obtained from samples in the area of the curve containing GSH S-transferase activity made the data difficult to interpret (Figure 28) but was consistent with earlier data (Figure 20), indicating no change in enzyme activity in renal cortical homogenates after penicillin. Because GSH S-transferases comprise 2% of renal supernatant protein (Kirsch et al., 1975b), induction of synthesis in magnitude comparable to stimulation of transport capacity would more than likely be detected. The effect of penicillin on microsomal protein was investigated using SDS polyacrylamide gel electrophoresis. Visual protein banding patterns in gels stained with Coomassie Brilliant Blue were not different from control following penicillin pretreatment (Figure 29). Determination of radioactivity in protein bands by slicing and solubilization in Soluene 100 was impossible due to low levels of label. These data suggest that either protein synthesis is not involved in substrate stimulation of organic anion transport, an unlikely hypothesis in that stimulation is sensitive to cycloheximide inhibition (Figure 10), the methods used lacked sensitivity, or that the mechanism of stimulation does not require increased synthesis of protein component but occurs through some other modification of the transport system which

requires an intact protein synthetic potential but does not require increased synthetic rate.

The mechanism of substrate stimulation of renal organic anion transport capacity has not been firmly established. PAH accumulation in renal cortical tissue is enhanced following penicillin pretreatment due to an increased rate of active uptake (Figures 7 and 9) and is blocked by concurrent treatment with cycloheximide (Figure 10). The requirement of protein synthetic capacity has been taken as evidence that penicillin increases synthesis of a protein component of the transport system (Hirsch and Hook, 1970b). The half life of transport stimulation observed following penicillin was approximately 20 hours. Theoretically, synthesis of a rate limiting component of the system would proceed at 0.24 units/hr in the basal state and 0.85 units/hr in the stimulated state, a 3.5-fold increase. Intuitively, one might conclude from these data that changes in protein synthesis following penicillin pretreatment would be evident using labeled precursors. However, the data presented in this study do not consistently indicate a significant increase in the rate of amino acid incorporated into renal cortical protein (Tables 3, 5 and 6; Figure 27). Because the renal organic anion transport system probably constitutes a relatively small percentage of total cortical protein, it is possible that detection methods were insensitive to changes which were occurring. Of similar probability, however, would be to conclude that protein synthetic rates were not different. If this was the case, what are the possible alternatives?

Enzyme induction occurs through either an increase in the amount of enzyme protein (Conney, 1967) or enhanced efficiency of the enzyme reaction (Griffin and Cox, 1966). Enzyme protein may be increased by either stimulating synthesis (Conney, 1967) or inhibiting degradation (Shimke et al., 1965). Catalytic efficiency, defined as the energy requirement of the transition state, may be enhanced by either an alteration in the conformation of newly synthesized enzyme thereby yielding a more efficient orientation toward substrate (Brockenhoff, 1974) or induced synthesis of another substance, a modifier, which interacts with the enzyme molecule to enhance its activity. The modifier may either activate previously dormant sites or increase the activity of functioning sites. Therefore, several possible alternatives to the "increased synthesis" hypothesis exist. The only alternative for which there is a basis for exclusion is decreased protein degradation. Cycloheximide completely blocked substrate stimulation and had no effect on control PAH S/M (Figure 10). Because of the rapidity at which stimulation of transport becomes evident (Figure 5), in order for this system to be operative, turnover rates would have to be very rapid. Assuming turnover was sufficiently fast to produce stimulation, inhibition of protein synthesis in non-stimulated animals should result in decreased transport capacity which was not observed (Figure 10). Therefore, the other alternatives must be explored.

Cox et al. (1971) reported that alkaline phosphatase activity in HeLa 65 cells was stimulated following addition of cortisol to the culture medium. The maximal velocity of the reaction was increased, but there was no effect on the Km. Induction also was

blocked by cycloheximide. There was not, however, an increased concentration of enzyme protein within the cell. These investigators concluded that the catalytic efficiency of the enzyme reaction had been increased, probably by a peptide or protein modulator. Assuming data obtained from an enzyme reaction can be applied to carrier mediated transport processes, it would appear that substrate stimulation of renal organic anion transport possessed many of the same properties (Figures 1, 9 and 10).

Amino acid incorporation data are not clearly indicative of an increased rate of protein synthesis. How, then, can stimulation occur? One hypothesis might be that the rate of protein synthesis remained relatively constant and that penicillin, through some sort of regulatory system (substrate sensitive), induced the synthesis of a slightly different form of transport protein which was more efficient in translocation of organic anions through cell membranes. The increased efficiency might result from a more favorable orientation to or within the membrane and increased substrate accessibility, or facilitated energy transfer to active components of the transport system. In this manner substrate binding sites need not be altered and a change in apparent affinity not observed. Secondly, penicillin might increase the synthesis of a peptide modulator. Small peptides are not precipitated by TCA and would be lost during the washing procedure. Weber (1963) suggested that acute adaptation of hepatic enzyme activity in immature animals usually involved substrate stimulation of preexisting enzymes. This hypothesis is especially attractive in that transport capacity decreases in rabbits at birth, probably due to the large substrate overload as the

neonatal kidneys assume excretory function previously performed by the placenta (Hook, 1974). During development transport capacity increases, reaching a maximum at 4 weeks of age (Hirsch and Hook, 1970a). The decline in transport from 4 weeks to adult is probably due to an inhibitor associated with environmental changes in weaning (Ecker and Hook, 1974b). Substrate challenge at any age prior to 4 weeks results in full expression of a maximal intrinsic transport capacity normally observed at 4 weeks (Figure 6). These data might then suggest that at birth a full complement of transport sites exists but are not all active due to the presence of endogenous inhibitors such as fatty acids or metabolic intermediates which are unique to the newborn. Penicillin treatment then might result in release of inhibition through synthesis of a modulator and allow full expression of transport potential. The one complicating factor common to each hypothesis, however, is that techniques are not available to quantify components of the transport system. Conclusive evidence must await development of an irreversible inhibitor of organic anion transport.

SUMMARY

Renal organic anion transport capacity in newborn rabbit renal cortical slices was significantly enhanced by pretreatment with penicillin. A dose dependent relationship existed between penicillin and PAH S/M ratio. Maximal accumulation of PAH by slices was observed following treatment with doses of 90,000 I.U. of procaine penicillin G. The stimulating effect of penicillin on transport capacity was fully developed after two injections at 12-hour intervals and was greatest when animals were killed 24 hours after the final injection of the treatment regimen. Therefore, maximal stimulation of renal organic anion transport capacity in rabbits occurred following treatment with 90,000 I.U. procaine penicillin G twice daily for two days. Animals were killed 24 hours after the final injection.

During normal development in rabbits, transport capacity increased rapidly during the third and fourth week after birth until a maximum was reached, after which transport decreased to values observed in adults. Penicillin treatment of animals 3 days, 1 and 2 weeks after birth increased PAH S/M ratio to a level not significantly different from the peak value attained at 4 weeks of age. PAH S/M ratio in slices from penicillin pretreated 4-week animals was not significantly different from saline control. Therefore, these data are interpreted to indicate that a maximal intrinsic

transport capacity exists which is fully expressed at 4 weeks and not surpassed following substrate stimulation.

The capacity of penicillin pretreatment to increase PAH accumulation by slices of newborn rabbit renal cortex was dissected into two components, uptake and runout. The oxygen requiring component of the uptake process was significantly enhanced by penicillin pretreatment, whereas passive influx and runout were unaffected. Due to inherent limitations of the slice technique in the study of rapid membrane flux, the kinetics of PAH uptake were investigated using suspensions of separated proximal tubules. Penicillin pretreatment enhanced the rate of PAH uptake into separated proximal tubules (collagenase digestion) from newborn rabbits. A double reciprocal plot of these data suggested that penicillin increased the maximum velocity of PAH uptake and had no effect on the apparent affinity of the carrier. It was concluded that penicillin pretreatment increased renal cortical accumulation of PAH solely by stimulating the uptake process and that the change in the transport system was quantitative (theoretical maximal transport velocity) rather than qualitative (affinity).

Substrate stimulation of PAH transport by penicillin in rabbits was blocked by concurrent treatment with cycloheximide, a potent inhibitor of protein synthesis. PAH transport is sodium dependent but penicillin pretreatment had no effect on proximal tubular Na, K-ATPase activity. Though structural development of rabbit proximal tubular cells was evident using electron microscopy, no ultrastructural changes could be attributed to penicillin pretreatment. Thus, the effect was either too subtle to be observed or did not involve

particulate proteins. Therefore, substrate stimulation of PAH transport does not occur through alterations in Na, K-ATPase and may involve soluble rather than membrane bound proteins.

Recent evidence suggests that soluble enzymes (GSH S-transferases) with characteristics similar to the hepatic organic anion binding protein, ligandin, bind PAH and may be implicated in renal organic anion transport. GSH S-aryltransferase activity, an estimate of GSH S-transferase concentration, and organic anion transport capacity developed in newborn rats and rabbits at different rates. Enhancement of enzyme activity using TCDD, 3-MC and chronic metabolic acidosis was not accompanied by parallel increases in transport capacity. GSH S-aryltransferase activity in rabbits was not altered by several-fold increases in transport capacity following pretreatment with penicillin, but was increased in parallel with stimulation of transport resulting from 3-MC pretreatment. GSH depletion had no effect on organic anion transport capacity in rats. Binding of ^{14}C-benzyl penicillin to protein in GSH S-transferase containing eluant fractions following Sephadex filtration of 100,000 x g supernatant proteins from newborn rabbit renal cortical homogenates was less than observed in fractions from adults. However, penicillin pretreatment had no stimulating effect on binding. The relative lack of correlation between GSH S-transferase and transport capacity suggests the enzymes may not constitute a major rate limiting component of the renal organic acid transport system.

In vitro ^{14}C-leucine incorporation into renal cortical slice and separated proximal tubular protein was not significantly affected by penicillin pretreatment. *In vivo* incorporation of ^{14}C L-leucine

into 100,000 x g pellet protein from renal cortical homogenates following pulse labeling was slightly increased after penicillin. The effect was not observed when uptake into total renal cortical homogenate was quantified. PAH S/M ratio was significantly increased 8 hours following a single dose of penicillin and the effect was maximal after 24 hours. ^{14}C L-leucine incorporation into 100,000 x g pellet protein was slightly increased after a single injection of penicillin, but the stimulating effect was variable and not statistically significant. There was no consistent effect of penicillin pretreatment on incorporation into soluble proteins. The specific activity of soluble intracellular leucine pools was not significantly altered by penicillin pretreatment. Using Sephadex gel filtration and gel electrophoresis, there did not appear to be localized uptake of label into either 100,000 x g supernatant or pellet proteins. These results suggest that substrate stimulation of renal organic anion transport capacity, though dependent upon protein synthesis, may not occur through increased synthesis of specific transport proteins.

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