ENDOGENOUS FATTY ACIDS DO NOT INHIBIT RENAL TRANSPORT OF ORGANIC ACIDS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology & Toxicology

1979

ABSTRACT

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A large number of observations suggest that a link exists between renal nonesterified fatty acids (NEFA) and the transport of organic anions. Recently, the mechanism of enhanced renal transport of PAH after penicillin treatment of the neonate was postulated to be reduced inhibition of transport subsequent to reduction of the cellular content of NEFA by penicillin.

Analysis of this problem required the resolution of three questions. First, are NEFA transported into the cell of the proximal tubule by the organic anion transport system? Second, do NEFA, in physiological concentrations, act as inhibitors of organic anion transport? Third, is the alteration in lipid metabolism observed after penicillin treatment responsible for the alteration in transport capacity?

Palmitic acid effectively inhibited PAH transport in vitro. However, enhanced organic anion transport

capacity was not associated either with increased incorporation of labeled fatty acid into tissue total lipid nor was it associated with increased extraction of label from the medium. Thus it appears that NEFA do not enter the renal cell primarily by the organic anion transport system.

Alterations in transport capacity were not associated with alterations in tissue NEFA. Penicillin treatment enhanced transport but did not decrease cellular NEFA and, in fact, increased serum NEFA. Fasting did not significantly alter renal NEFA content although transport was decreased. Renal NEFA content was higher in adult kidneys than it was in immature rabbit kidneys although adults had a greater transport capacity. Finally incubation conditions under which fatty acids decrease transport were associated with tissue NEFA concentration markedly above the endogenous concentration of NEFA in the kidney. Thus, NEFA are not likely to exert an inhibitory effect on organic anion transport at physiological concentrations.

Penicillin induced alterations in lipid metabolism were not observed in glomeruli, indicating the dependence of altered metabolism on organic anion transport. However, penicillin treatment enhanced triglyceride synthesis and decreased incorporation of labeled fatty acid into tissue

NEFA without a concommitant change in transport capacity. Nicotinic acid treatment of the adult rabbit produced a similar change in metabolism but failed to enhance transport capacity. PAH, probenecid, and nicotinic acid produced similar *in vitro* effects. Furthermore changes in renal triglyceride and serum NEFA produced by penicillin are similar to the reported effects of nicotinic acid. These results suggest that penicillin induced changes in renal metabolism of NEFA are not related to alterations in transport and that penicillin may be acting in a manner similar to nicotinic acid in producing altered lipid metabolism.

These results, therefore, are not consistent with the conclusion that penicillin pretreatment of the neonate enhances organic anion transport by decreasing the endogenous inhibition of NEFA.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. J. B. Hook for his counsel, encouragement and constructive criticism during the course of this investigation. I would also like to thank Drs. T. M. Brody, J. Bennett and M. Bailie, M.D. for their assistance in preparing this thesis.

My wife, Ellen, deserves special recognition for her patience, love, encouragement and motivation without which I would not have been successful.

I have also received excellent technical assistance and would like to express my appreciation to Mr. T. Steele and Ms. S. Mehl whose efforts greatly furthered the research.

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INTRODUCTION

The report by Marshall and Vickers (1923) of secretion of phenolsulphonephthalein by the dog kidney was one of the earliest demonstrations of the secretion of an organic anion (Weiner, 1973). It was, however, the discovery of the secretion of penicillin, coupled with its limited supply and its clinical value, which provided the initial impetus for intensive study of the renal organic anion transport system (Beyer et al., 1944; Beyer 1950). The subsequent realization that a wide variety of organic anions are secreted by the mammalian kidney has sustained interest in this system as a determinant of organic anion pharmakokinetics.

The renal organic anion transport system may be involved in processes in addition to excretion of drugs and chemicals. For instance, the anion secretory system may play a role in the renal utilization of metabolic fuels such as alpha-ketoglutarate and nonesterified fatty acids (Cross and Taggart, 1950; Cohen and Wittman, 1963; Barac-Nieto and Cohen, 1968; Selleck and Cohen, 1965). The action of several diuretics upon the kidney appears to depend upon

their secretion (Bowman, 1975; Duggan, 1966). Similarly, the nephrotoxicity of cephaloridine is also dependent upon its cellular accumulation by the organic anion transport system (Tune and Fernholt, 1973; Tune, 1972). The apparent secretion of prostaglandins, coupled with their potent renal actions, suggests that their effects may, in part, be dependent upon secretion by the transport system (Bito and Baroody, 1978). Recent studies also indicate that a frequent early sign of renal toxicity is enhanced or decreased organic anion transport (Hirsch, 1976). While the mechanisms underlying these alterations remain obscure, it seems apparent that an understanding of the mechanism of transport would also yield significant insight into the mechanisms of renal toxicity. These observations indicate that an increased understanding of the mechanism of renal organic anion transport will be of broad interest to clinical, industrial, and regulatory pharmacologists.

Extensive study of renal organic anion transport has led to an extensive body of knowledge and several different hypotheses have been presented concerning the mechanism of transport. A relatively recent approach to this problem has been to use the immature animal as a tool to evaluate the mechanisms of transport.

Use of the immature animal as a model for the analysis of renal mechanisms is based on the observation that the functional capacity of the neonate is less than in adults. Renal blood flow (Rakusan and Marcinek, 1973; Gruskin et al., 1970; Jose et al., 1971; Aschinberg et al., 1975), glomerular filtration rate (Horster and Valtin, 1971; Spitzer and Edelmann, 1971; Aperia and Kerin, 1975), concentrating ability (Sakai and Endov, 1971; Stanier, 1972), and several biochemical measures including ammoniagenesis (Cort and McCance, 1954; Goldstein, 1970), $Na^+ - K^+ -$ ATP ase (Burich, 1974; Schmidt and Horster, 1977), and the transport of organic anions and cations (Horster and Valtin, 1971; Horster and Lewy, 1970; Lewy and Grosser, 1974; Rennick, et al., 1961) are not fully developed in the newborn of most species. Various investigators have quantified alterations in specific renal functions with maturation in attempts to elucidate basic mechanisms of several renal functions (Ullrich, 1976; Schriver et al., 1976; Reynolds and Segal, 1976). Unfortunately, analysis of the biochemical correlates of a functional change during development is frequently complicated by biochemical changes associated with the maturation of other functional parameters. Other investigators have used the immaturity of the kidney to evaluate the

role of a specific renal mechanism in the response to functional loading. For example, saline loading of the immature animal has been used to evaluate the role of alterations in renal blood flow in the renal excretion of excess salt and water (Bengele and Solomon, 1974; Aperia et al., 1975; Arandt, 1978). Finally other investigators have attempted to selectively alter the rate of maturation of specific renal functions by stressing the function physiologically or pharmacologically. This approach can be a powerful tool in the analysis of the biochemical correlates of a specific renal function.

Extensive studies have elucidated three basic types of renal response to functional or pharmacological stimulation. Several physiological functions are resistant to stimulation because of the structural immaturity of the kidney (McCrory, 1972; McCance and Widdowson, 1958). Other functions such as ammoniagenesis (Benyajati and Goldstein, 1975), and some drug metabolizing enzymes (Kluwe et al., 1978) are inducible in the immature animal and are also inducible in the adult. Finally, some functions appear to be inducible only in the immature animal. The administration of cortisol to neonatal rats evoked premature increases of renal glucose-6-phosphatase, and renal aspartate amino

transferase to adult levels, while adults were unresponsive to cortisol treatment (Greengard, 1975). Similarly, and of particular interest to this discussion, renal organic anion transport was inducible in neonates but not in adults by treatment with penicillin or 3 methylcholanthrene (Hirsch and Hook, 1969; Kluwe et al., 1978). While it is unclear why the adult is unresponsive, these results suggest that the neonatal animal offers unique advantages for the analysis of the biochemical correlates of altered organic anion transport.

An overview of the renal organic anion transport system

The excretion of a compound in excess of that attributable to glomerular filtration, implies the net transfer (secretion) of that compound across the tubule from the plasma into the urine. Traditionally, the secretory transport of organic anions has been visualized as a two step process: accumulation into the cell followed by passive diffusion into the urine. The primary role of accumulation into the cell in the transport process allows the use of *in vitro* preparations for investigations of the mechanism by which organic anions are transported. Indeed,

numerous investigators have demonstrated that the ability of renal cortical tissue to accumulate organic anions *in vitro* under a variety of conditions is qualitatively analogous to similar *in vivo* determinations of renal secretion (Weiner, 1973). The overall secretion of organic anions occurs against a concentration gradient (Cross and Taggart, 1950; Foulkes and Miller, 1959; Tune et al., 1969; Carrasquer and Wilczewski, 1971). Transport is dependent on metabolic energy since cold and a nitrogen atmosphere effectively inhibit transport (Cross and Taggart, 1950; Farah and Rennick, 1956). A wide variety of metabolic inhibitors are also effective inhibitors of transport (Maxild, 1973; Mudge and Taggart, 1950; Cross and Taggart, 1950; Shideman and Rene, 1957; Shideman et al., 1952; Farah et al., 1953; Farah and Rennick, 1956; Maxild and Møller; 1969).

The transport system is not only dependent on metabolic energy but is also sensitive to metabolic modulation. Transport is sensitive to pH, K^+ and Na+ and to the presence of a wide variety of metabolic substrates, such as acetate (Cross and Taggart, 1950; Taggart, et al., 1953; Gerencser et al., 1973; Copenhaver, et al., 1965). Unfortunately, the mechanisms by which modulation occurs remain, for the most part unresolved (Podevin and Boumendil-Podevin,

1977; Chung et al., 1970; Weiner, 1973; Kippen and Klinenberg, 1978; Hewitt and Hook, 1978).

The secretion of organic anions has been localized within the kidney to the proximal tubule by a wide variety of techniques including micropuncture (Baines et al., 1968; Cortney et al., 1965; Richards et al., 1938), perfusion of isolated nephron segments (Tune et al., 1969), stop flow (Kessler et al., 1959; Malvin et al., 1958), and autoradiography (Kinter et al., 1960; Wedeen and Jernow, 1968). Most recently, the transport of organic anions has been localized primarily to the S2 segment of the proximal tubule by a combined ultrastructural and isolated perfused segment procedure (Woodhall et al., 1978). Significantly, both the S1 and S3 segments were also capable of secretion but at a rate 23% of that observed in the S2 segment, indicating that while transport was substantially localized in the S2 segment, the transport system is a function of the entire proximal tubule.

A cellular receptor has been implicated in the transport mechanism since the transport system appears to be saturable, susceptible to competitive inhibition, and shows some structural selectivity. In vivo the secretion of organic anions is characterized by a tubular maximum

implying saturation (Weiner, 1973). Competition is indicated since similarly secreted anions such as probenecid, p-aminohippurate or penicillin can reduce the secretion of each other (Beyer et al., 1944; Beyer et al., 1947). Similarly, evidence for saturation and inhibition can also be obtained in vitro (Weiner, 1973). Analysis based upon Michaelis-Menten kinetics suggests that the inhibition is competitive (Huang and Lin, 1965; Park et al., 1971). The transport system also appears to demonstrate some selectivity since organic cations do not affect transport, nor are all anions secreted (Weiner, 1973; Despopoulas, 1965; Nielsen and Rassmussen, 1975).

While these results clearly imply the presence of a cellular receptor, the site of the receptor remains obscure. Two prominent hypothesis have been advanced. Foulkes and Miller (1959) visualized an intracellular receptor while Tune et al., (1969) have emphasized the presence of a peritubular membrane carrier.

Cellular receptor sites

The demonstration by Foulkes and Miller (1959) of two kinetic components in the efflux of the prototype

organic anion p-aminohippurate (PAH) from renal cortical slices led to the suggestion that two intracellular pools existed for transported organic anions. They suggested that the rapid component of efflux represented freely diffusable intracellular anion, while the slower component represented a subsequent intracellular concentrating or binding step. Several investigators have confirmed that two kinetic components exist in the renal influx or efflux of organic anions, but have differed in their interpretation of the meaning of the two components. Sheikh and Møller (1970) determined that the influx of PAH into isolated tubule suspensions had two kinetic components. They interpreted the initial rapid component as the concentrating step since concentrations greater than the medium were rapidly achieved. The second component was suggested to represent subsequent passive intracellular binding or compartmentalization. Anderson et al. (1969) demonstrated that the accumulation of nitrofurantoin also had an active and passive component but concluded that the two components were arranged in parallel rather than in series. Burg and Orloff (1969) suggested that the two components of efflux were also arranged in parallel and that the rapid

component reflected efflux from injured cells while the slow component reflected efflux from uninjured cells.

In support of the possibility of intracellular binding several investigators have suggested that intracellular binding does occur for a variety of transported organic anions (Duggan, 1966; Berndt, 1966; Berndt, 1967; Berndt and Grote, 1968; Despopolous, 1965; Despopolous and Segerfeldt, 1964; Forster and Hong, 1958). Furthermore, Kirsch et al. (1975) have also demonstrated the presence of low molecular weight binding proteins which can bind organic anions specifically and reversibly. Moreover, the failure to demonstrate intracellular binding of PAH (Weiner, 1973) could be due to the inadequacy of current methodology (Farah et al., 1963). These results suggest that intracellular binding could be involved in organic anion transport. However, the failure to demonstrate intracellular binding of PAH could also imply that intracellular binding is not a component of the transport system, a position which has been taken by several investigators (Foulkes, 1963; Tune et al., 1969).

An alternative to intracellular binding was proposed based upon measurements of transport in the isolated perfused tubule (Tune et al., 1969). When PAH was supplied to the tubule in the bathing media it was concentrated in the

cell. If, however, PAH was supplied from the tubule lumen, it was not concentrated in the tubule. Tune et al., (1969) proposed a model of PAH transport in which intracellular binding was not involved. They suggested that the energy dependent concentration step occurred at a peritubular site via a membrane carrier. Subsequent efflux out of the cell occurred via passive diffusion along a concentration gradient. Furthermore, since the peritubular membrane was less permeable to efflux than the luminal membrane, net secretion was assured once the organic anion was concentrated in the cell. They, however, did not suggest a mechanism to account for the difference in permeability. Furthermore, while a peritubular membrane carrier probably does exist, the Tune model is subject to other criticisms. First, several studies have failed to elucidate a mechanism by which transport is linked to cellular metabolism at a peritubular membrane site. Second, the conclusion that intracellular binding does not occur is based upon the observation that PAH was not concentrated inside the cell when it was supplied from the lumen. The use of back flow to test for intracellular binding assumes that the luminal membrane is permeable to anions presented from the luminal surface. The back flow test also assumes that intracellular binding

will occur without prior interaction with a peritubular site. Thus if the luminal membrane were relatively impermeable to PAH or if the binding of PAH in the cell required a prior, perhaps catalytic, interaction with the peritubular carrier, then despite the involvement of intracellular binding in the transport of organic anions, luminally presented organic anions would not be concentrated.

Peritubular membrane carriers

Confirmation of the existence of a peritubular membrane carrier has been provided by several investigators. Fujimoto and his colleagues (Watrous et al., 1970; Hakim et al., 1970) demonstrated that the secretion of infused morphine ethereal sulphate (MES) and 5-hydroxyindolacetic acid (5-HIAA) could be inhibited by probenecid, indicating that these organic anions were secreted by the renal organic anion transport system. However, excretion of 5-HIAA and MES generated intrarenally via the metabolism of the organic cations serotonin and morphine, was not affected by probenecid. These results suggest that the interaction between the organic anions occurred prior to their entry into cellular metabolic pools. The authors concluded that a

peritubular membrane interaction consistent with a membrane carrier was responsible for the effect of probenecid on the excretion of MES and 5-HIAA. In further support of the concept of a peritubular carrier, Hong et al. (1978) observed that the "nonpermeating" organic anion, isothiocyano-2, 2'-disulfonic stilbene (SITS), inhibited PAH transport in an apparently competitive manner.

Berne and Kinne (1976) demonstrated that PAH penetration into basal-lateral membrane vesicles was susceptible to probenecid inhibition. Ross and his colleagues (Holohan et al., 1976; Kinsella et al., 1977) demonstrated that penetration of PAH into basal-lateral membrane vesicles was both saturable and susceptible to competitive inhibition and was not sensitive to the presence of organic cations. Thus, it appears that a peritubular membrane carrier, as suggested by Tune et al. (1969), does exist. If, however, the rest of the Tune model is correct, then it is difficult to defend the earlier concept of the involvement of intracellular binding in the transport of organic anions. Several observations, however, indicate that the Tune model may have to be revised, since it appears that the coupling of cellular energy with the transport system may not occur at the

peritubular membrane, and that a carrier mechanism may be involved in the efflux of organic anions from the cell.

Energy coupling to transport

Transport of organic anions does not appear to be directly dependent on the concentration of cellular adenosine triphosphate (ATP). Maxild (1973) used the uncoupling agents, 2, 4-dinitrophenol and carbonylcyanide-m-chlorophenylhydrazone and found that at concentrations which decreased transport 50%, little of any change in cellular ATP was observed. If, however, transport was significantly inhibited, the addition of 10 mM acetate would enhance transport to values comparable to those observed in the absence of the uncoupling agent and the presence of acetate. Moreover, the addition of acetate rather than increasing cellular ATP actually appears to decrease cellular ATP (Gerencser et al., 1977). These results suggest that cellular transport of organic anions is not directly driven by cellular ATP and that the decrease in transport is not due to competition for the peritubular carrier between the uncoupling agent and PAH.

Numerous investigators have demonstrated that the transport of organic anions is dependent on the cation sodium (Chung et al., 1970; Gerencser et al., 1973). Sodium dependent carrier mediated transport, as has been described for the renal transport of sugars and amino acids, can be regenerated by the application of a sodium gradient across brush border vesicles (Kinne, 1976; Ullrich, 1976). Podevin et al. (1978) have recently demonstrated that PAH can be concentrated in sodium depleted renal cortical slices by application of a NaCl gradient, suggesting that organic anion transport is, in part, dependent on a sodium gradient. However, neither Berne and Kinne (1976) nor Kinsella et al. (1977) were able to demonstrate sodium linked co-transport in brush border or basal-lateral membrane vesicles. These results suggest that the dependency of transport on sodium is not explained as simple sodium driven co-transport. Hewitt (1977) has suggested that the lack of "energized" transport in vesicles was consistent with the transport process being coupled with an intracellular step. In any case, the relationship between the peritubular carrier, the sodium dependency of transport and the coupling of transport with cellular energy remains unresolved.

Luminal membrane carriers

The efflux of organic anions from the cell cannot be explained as simple passive diffusion as suggested in the Tune model. Several lines of evidence suggest that a membrane carrier exists in the luminal membrane for the efflux of organic anions from the cell. The mechanism by which organic anions leave the cell is clearly different from the mechanism by which they enter the cell. Developmental studies of transport in renal cortical slices demonstrate that a different developmental pattern exists for efflux and influx. Measurements of initial uptake rates show a peak uptake rate at 4 weeks in rabbit kidneys which subsequently declines to adult values. Efflux exhibits a gradual increase in the rate of efflux as the animal matures (Ecker and Hook, 1974; Cole et al., 1978). Furthermore, penicillin induced stimulation of PAH transport selectively affects the initial uptake rate without altering efflux time constants (Pegg et al., 1975). Other investigators confirm the distinction between efflux and influx and further imply that efflux is carrier mediated.

Early studies of the effect of organic anions on the efflux or runout of PAH from renal cortical slices

preloaded with PAH demonstrated that low concentrations of PAH in the bathing medium enhanced the runout of PAH from the slice. Higher concentrations of PAH in the medium, however, decreased the runout of intracellular PAH (Farah et al., 1963; Ross and Farah, 1966). Runout may represent the sum of diffusion out of the cell plus a component of active reuptake of organic anion which had diffused out of the cell (Farah et al., 1963). The increase in runout at low concentrations of PAH in the medium is thus consistent with competitive inhibition of a high affinity, carrier mediated uptake mechanism. Inhibition of runout at higher medium concentrations of PAH is similarly suggestive of inhibition of a carrier mediated efflux mechanism.

Use of the renal cortical slice as a model of the efflux of organic anions from the cell across the luminal membrane into the urine has been criticized since the tubular lumens appear to be for the most part collapsed (Bojesen and Leyssac, 1965). This result suggests that organic anion could escape from the slice across the luminal membrane only with difficulty. Efflux from the slice is thus likely to represent actions occurring primarily at the peritubular membrane. Several studies, however, suggest that similar considerations apply to the luminal membrane.

A peritubular efflux carrier is suggested by the observation of an apparent secretion of organic anions across the luminal membrane in the tubule of certain teleost fishes (Forster and Hong, 1958; Hong and Forster, 1959; Puck et al., 1952). Similarly, autoradiographic studies of renal cortical slices incubated in the presence of H^3-hippuran, demonstrated high luminal concentrations of label (Wedeen and Weiner, 1973; Wedeen and Vyas, 1978). If phlorizin is added to the incubation medium, the steady state concentrations of PAH (PAH S/M) are increased, but the distribution of label to the lumens is inhibited. This observation suggests a selective effect on efflux. Foulkes (1977) found that low doses of probenecid increased the time required for a pulse of PAH to reach the urine. Furthermore, the pulse occurred after a pulse of simultaneously injected inulin. These results indicate that secretion of PAH had been blocked and that the filtered PAH had distributed to a cellular compartment after filtration. If a larger dose of probenecid was given, the transit time was reduced to that of inulin indicating that the intracellular distribution of filtered PAH across the luminal membrane had been blocked by inhibition of a luminal carrier. Phenolsulphonthalein binding to a microsomal fraction indicates the presence of

two binding sites, one sensitive to competitive inhibition by 2, 4-dinitrophenol and a second, higher affinity site sensitive to 2,4-dinitrophenol and probenecid (Evelhoff et al., 1976). While the high affinity site appears to correlate with the peritubular carrier in basal-lateral membrane vesicles (Bernet and Kinne, 1976; Evelhoff et al., 1976), the role of the low affinity site remains obscure. Evelhoff et al. (1976) suggested that it represented a weak intracellular binding site. But clearly, a second binding site is also consistent with the concept of two membrane carriers. Kinsella et al. (1977) have confirmed that saturable and inhibitable uptake of PAH into brush border vesicles exists. Berne and Kinne (1976) also observed a weak probenecid effect on PAH influx into brush border vesicles at a concentration of probenecid which markedly reduced influx into basal-lateral vesicles, indicating that if a carrier is involved, it is of a lower affinity than the carrier in the peritubular membrane or that it is similar to the low affinity binding site observed by Evelhoff et al. (1976) which was inhibitable by 2,4-dinitrophenol but not by probenecid.

The possibility of carrier mediated diffusion across the luminal membrane suggests that the membrane could

exhibit differential permeability. A carrier could have a higher affinity for organic anions when it is orientated toward the inside of the cell than it would have when orientated toward the outside of the cell. This effect would make the luminal membrane relatively more permeable to anions inside the cell compared to the anions outside the cell. This possibility suggests that measurement of permeability of the luminal membrane based on efflux from the cell as performed by Tune et al. (1969) may not reflect the permeability of the brush border membrane to influx of PAH into the cell across the luminal membrane as was assumed by Tune et al. (1969). The failure to observe significant intracellular concentration of PAH when PAH was supplied from the luminal surface may therefore be a manifestation of low permeability of the luminal membrane to influx into the cell. Unfortunately, comparative measurements of influx and efflux from brush border vesicles have not been reported. The Tune model for organic anion transport does not rule out the possibility for the involvement of intracellular binding in the transport of organic anions and may have to be expanded to account for a carrier for efflux of anions as well as an influx carrier.

Mechanisms of penicillin enhanced transport

Renal organic anion transport can be enhanced by two principle manipulations: Acetate in the medium and penicillin pretreatment of neonates. Several mechanisms have been proposed to account for the enhanced transport observed after acetate (Cross and Taggart, 1950; Schachter et al., 1955; Cohen and Randall, 1964; Weiner, 1973; Hewitt et al., 1976). It appears that the mechanism for penicillin enhanced transport is distinct from the acetate enhanced transport. Acetate enhanced transport is effective in neonates and in adults while penicillin is effective only in neonates (Kim et al., 1972; Kim and Hook, 1972; Pegg and Hook, 1975). Furthermore, acetate enhances transport in naive and penicillin pretreated neonates alike (Hirsch and Hook, 1970; Pegg and Hook, 1975). The addition of acetate produces an immediate *in vitro* effect (Hewitt et al., 1976). In contrast penicillin induced enhancement of transport requires pretreatment several hours prior to evaluation for expression of enhanced transport (Pegg and Hook, 1975). Finally, while penicillin specifically enhances PAH transport and does not enhance urate transport (Hewitt and Hook,

1978), acetate and other metabolites enhance urate and PAH transport (Kippen and Klinenberg, 1978).

Penicillin treatment, despite its ability to increase kidney weight, does not appear to increase transport by a nonspecific enhancement of growth. Acid loading increased kidney weight without enhancing anion transport (Pegg and Hook, 1977). Penicillin treatment also failed to enhance cation transport (Hirsch and Hook, 1970a), or the transport of several organic anions which appear to be secreted by the kidney (Hewitt et al., 1977). Penicillin treatment also failed to alter the histological (Hirsch et al., 1971) or ultrastructural (Pegg et al., 1976) appearance of renal tissue. Recently, the transport of PAH in control and penicillin pretreated neonates was evaluated with the isolated perfused tubule technique pioneered by Tune et al. (1969). In this preparation, transport was increased per mm of tubule confirming that penicillin enhanced transport by an intrinsic change in the transport system (Schwartz et al., 1978). The lack of effect on cation transport or urate transport also suggests that penicillin treatment does not increase transport by a generalized increase in cellular energy.

Kinetic analysis

Several attempts to define the phenomenon of penicillin stimulation of organic anion transport by the use of kinetic analysis have been made and have met with mixed success. Analysis based on Michaelis-Menten kinetics of the initial uptake of PAH into isolated proximal tubule suspensions indicated that stimulation was associated with a change in the apparent maximal velocity of transport without a change in the apparent affinity of the transport system for the organic anion (Pegg et al., 1975). If, however, the transport was evaluated using renal cortical slices, then a change in the apparent affinity was observed instead of a change in maximal velocity (Pegg, 1976). The complexity of the renal cortical slice or the tubule preparations makes kinetic analysis of transport a hazardous approach (Christensen, 1969; Weiner, 1973). The reason for the different results with these two methods remain unresolved, but their variance serves to underscore the hazard of analyzing the biochemical mechanism of transport using kinetic analysis in complex systems such as the slice or isolated tubule.

Protein synthesis

Several studies have implicated the involvement of protein synthesis in the stimulation of transport by penicillin. Inhibition of protein synthesis with cycloheximide appeared to block penicillin stimulated transport (Hirsch and Hook, 1970b; Pegg and Hook, 1975). However, cycloheximide has several effects other than inhibition of protein synthesis (Jomain-Baum and Hanson, 1975). Furthermore, subsequent investigators have been unable to repeat the blocking effects of cycloheximide without significant signs of toxicity (Hewitt, unpublished observation; Stroo, unpublished observation). These results suggest that the earlier observations with cycloheximide may have been manifestations of renal toxicity. Unfortunately, the previous studies did not include simultaneous determinations of oxygen consumption nor of the function of other cellular transport processes. Hirsch and Hook (1970b) observed an increase in the incorporation of amino acids into TCA precipitable protein after penicillin treatment, an observation which is consistent with increased protein synthesis. However, the increase in kidney weight associated with penicillin treatment suggests that the increased synthesis

might be due to enhanced growth rather than a manifestation of the increased synthesis of a specific protein of the transport system. In contrast, Pegg (1976) failed to observe a significant increase in protein synthesis although a trend toward increased incorporation into microsomal protein was observed. These results are thus suggestive of the involvement of protein synthesis in the mechanism of penicillin enhanced transport, but fail to conclusively demonstrate its involvement.

Intracellular binding

The demonstration of an organic anion binding protein in the renal cortical cytosol (Kirsch et al., 1975), and the apparent correlation of hepatic organic anion transport with the binding protein, ligandin or Y protein (Levi et al., 1969; Reyes et al., 1969; Reyes et al., 1971), suggested that penicillin might produce enhanced transport by increasing intracellular binding to ligandin (Pegg and Hook, 1977). However, penicillin treatment failed to alter ligandin content (Pegg and Hook, 1977). Furthermore, a recent developmental study revealed no clear correlation between development of the renal organic anion transport

system and renal ligandin activity (Cole et al., 1978). It is interesting to note that the role of ligandin in the hepatic transport of organic anions has also been questioned (Klassen, 1975). These results thus fail to demonstrate a role for intracellular binding to ligandin in the renal transport of organic anions.

An alternative mechanism involving intracellular binding visualized penicillin treatment as disinhibiting the renal organic anion transport system. Hewitt and Hook (1978) suggested that nonesterified fatty acids (NEFA) inhibited organic anion transport by binding to intracellular binding sites which could bind PAH and which are involved in the transport of PAH. The binding of the NEFA thus inhibited renal organic anion transport. Several observations support this hypothesis. Several subtrates of the renal organic anion transport system including probenecid, inhibit the renal utilization of exogenous NEFA in vivo (Barac-Nieto and Cohen, 1968; Dies et al., 1970) and decrease oxidation of palmitate in vitro (Pakarinen, 1970; Heinemann et al., 1975). Barac-Nieto (1970) demonstrated that palmitate could inhibit transport of PAH in an apparently competitive manner. The addition of carnitine relieved this inhibition, suggesting that the interaction involved increased

mitochondrial use of palmitate, since the primary action of carnitine is to enhance the permeability of the mitochondria to fatty acids (Bressler, 1970). Pakarinen (1970) similarly suggested that probenecid altered lipid metabolism from an intracellular site since he failed to observe any change in incorporation of a labeled fatty acid into the tissue total lipid fraction. Consistent with the suggestion that fatty acids may be important modulators of transport is the report that fasting increases renal fatty acid content and plasma fatty acid concentration, and also decreases renal organic anion transport (Dahlquist et al., 1972; Chorvathova et al., 1974; Hook and Munro, 1968; Fox et al., 1976). It is thus interesting to note that the newborn animal is also hyperlipemic in comparison to the adult (Palkovic et al., 1976; Battaglia and Mesckin, 1978). These results suggest that the decreased transport in the fasted animal and in the neonate may be the result of inhibition of transport by intracellular NEFA.

Metabolic studies further support this hypothesis. Alpha-bromopalmitate inhibits fatty acid oxidation <u>in vitro</u> and decreases the incorporation of labeled fatty acid into tissue triglyceride while increasing the incorporation of label into the tissue NEFA fraction (Trimble

et al., 1977; Ockner and Manning, 1976). Alpha-brompalmitate also markedly inhibits organic anion transport at a concentration which produces minimal effects on oxygen consumption (Maxild, 1970). In contrast, the enhanced transport associated with penicillin treatment of neonates is associated with decreased incorporation of label into tissue NEFA and increased incorporation into tissue triglyceride (Hewitt and Hook, 1978). These results suggest that transport can be modulated by the renal content of NEFA and the content of NEFA can be regulated by renal metabolism. Hewitt and Hook (1978) supported the hypothesis that the altered metabolism of fatty acids was related to the enhanced transport of PAH by demonstrating that penicillin treatment of adults and iodipamide treatment of neonates, failed to alter transport and also failed to alter metabolism. These results suggest that the alteration in metabolism is a specific manifestation of altered organic anion transport, and not a non-specific effect of organic anion treatment.

While altered lipid metabolism does appear to be associated with altered organic anion transport, contrary to earlier expectations, NEFA do not appear to be extracted from the plasma by the renal organic anion transport system. Hewitt and Hook (1978) observed that penicillin stimulation

of transport failed to significantly increase incorporation of labeled NEFA into CO_2 or into tissue total lipid. This would appear to indicate that NEFA are not substrates of the renal organic anion transport system and is consistent with the suggestion that the interaction of the transport system with lipid metabolism is at an intracellular site.

Several problems remain to be resolved before the relationship between renal lipid metabolism and renal organic anion transport can be evaluated. Several investigators have reported that they failed to observe significant inhibition of PAH transport by NEFA (Maxild, 1970; Kippen and Klinenberg, 1978). This result suggests that the failure of Hewitt and Hook (1978) to observe increased utilization of palmitate may have been due to the use of inappropriate conditions for demonstrating palmitate uptake by the transport system. Alternatively, the failure to observe increased utilization may have been due to the several metabolic fates available to NEFA. While Hewitt and Hook (1978) failed to observe increased oxidation or incorporation of labeled fatty acid into total lipid they did observe a tendency for both parameters to increase. This observation suggests that while the individual fates for NEFA metabolism were not increased, the possibility exists

that the sum of those fates would indicate a significant increase.

The relationship between altered metabolism and altered transport is also subject to criticism. Close examination of the effects of iodipamide on lipid metabolism indicates that while metabolism was not significantly altered, the incorporation of labeled palmitate into triglyceride was quantitatively similar to that observed after penicillin treatment. This result suggests that the failure to achieve significant alterations in metabolism may be due to the use of an inadequate treatment dose. Similarly, since the adult secretory capacity and renal blood flow is greater than the newborn's, a given dose will have a shorter duration of action in the adult. This suggests that the failure to observe an effect in adults may also be due to an inadequate treatment dose. Finally, critical to the hypothesis of disinhibition of transport by reduced renal NEFA content is the demonstration that penicillin treatment reduces renal NEFA content. However, no evidence to support this hypothesis has been obtained.

Triglyceride biosynthesis

The central role played by triglyceride synthesis in the Hewitt and Hook model suggests that it would be pertinent to review the biochemistry of triglyceride synthesis. Triglycerides are synthesized directly from diglycerides by the action of the microsomal enzyme, diacylglycerol acyl transferase (DAGAT) (O'Doherty, 1978). Recent studies using the hepatic enzyme indicate that this reaction is dependent on the presence of an intracellular binding protein; fatty acid binding protein or Z protein (O'Doherty and Kuksis, 1978). Cellular diglycerides are derived from two pathways. The pathway which predominates in the liver but which may also be active in the kidney, converts monoacylglycerol to the diglyceride via the action of a soluble enzyme monoacylglycerol acyltransferase (O'Doherty, 1978; Hübscher, 1961). Diglycerides can also be derived from phosphatidic acid by the action of phosphitidate phosphohydrolase. This pathway is of central importance to the biosynthesis of phospholipids since phosphatidic acid is a common precursor to the phospholipids. The failure to observe any alteration in phospholipid metabolism suggests that the penicillin

induced alteration in metabolism is not in the phosphatidic acid pathway.

An interaction with Z protein is indicated since alpha-bromopalmitate binds to Z protein (Ockner and Manning, 1976), decreases incorporation into triglyceride and increased incorporation into NEFA (Trimble et al., 1977; Ockner and Manning, 1976). Furthermore, the addition of Z protein enhances the synthesis of di-, and triglycerides (Mishkin and Turcotte, 1974; O'Doherty and Kuksis, 1978). These results suggest that penicillin treatment may alter renal lipid metabolism by interaction in the monoacylglycerol pathway or by an interaction with the intracellular binding protein; Z protein.

Objectives

The objective of this study was to clarify the relationship between penicillin enhanced transport and altered lipid metabolism. Several specific questions were evaluated. First, does penicillin enhance renal utilization of NEFA? Second, is the altered metabolism an indication of a specific interaction of lipid metabolism with the transport system? Third, is the altered metabolism a specific indication of

transport? Finally, we sought preliminary evidence for the site at which penicillin alters renal lipid metabolism.

METHODS AND MATERIALS

Litters of 2 week old New Zealand White Rabbits were bred in the departmental animal quarters or purchased from local suppliers.

Young animals remained with their mothers until immediately prior to use. Adult female New Zealand White Rabbits (2-4kg) and adult male Sprague-Dawley Rats (150-300g) were purchased from local suppliers and acclimated to the animal facility prior to use.

Neonates (13-15 days old) were treated with 30,000 I.U. procaine penicillin G (Crystacillin, E.R. Squibb and Sons) by subcutaneous injection. In multiple dose treatment regimens, injections were given at 12 hour intervals. A total of 2 or 4 doses was given. Animals were killed 24 hours after the last dose except as noted in some treatment groups where the animals were killed 12 hours after the last dose.

Nicotinic acid (Sigma Chem. Co.) was dissolved in 0.9% NaCl and the pH adjusted to 7.4 before being injected intraperitoneally. Fasted adult rabbits were killed 24

hours after a single 250 mg/kg dose of nicotinic acid. Fasted adult male Sprague-Dawley rats were treated with 250 mg/kg nicotinic acid at 6 hour intervals for four treatments. Animals were then killed 6-8 hours after the last treatment. Controls were treated with equivalent volumes of 0.9% NaCl.

The in vitro effect of 2 mM PAH, probenecid and nicotinic acid on lipid metabolism was determined following a 15 minute preincubation of tubules in the presence of a 2 mM concentration of the organic anion followed by incubation in the presence of the anion and the standard palmitate containing KRP-BSA medium.

Fasted animals were allowed free access to water but food was removed 48 hours prior to use.

Media

Several media were used to evaluate organic anion transport or lipid metabolism. Transport in slices was determined in the phosphate based medium originally described by Cross and Taggart (1950) containing 7.4×10^{-5} M p-aminohippurate (PAH) and 1×10^{-4} M tetraethylammonium (TEA) to evaluate organic anion and cation transport, respectively. Isolated tubules were prepared in a Krebs-Ringer Phosphate

Buffer (KRP-buffer) medium containing 2.15 g/l $Na_2HPO_4.7H_2O$, 0.267 g/l KH_2PO_4,0.296 g/l $MgSO_4.7H_2O$, 7.614 g/l NaCl, 1.2076 g/l, and 0.147 g/l $CaCl_2$. Anhydrous sodium acetate was also added to yield a concentration of 10 mM. A second medium had an identical electrolyte composition but included approximately 10 mg/ml magnetic iron oxide. The third medium had an identical electrolyte composition as the KRP-buffer medium but also contained 1% defatted Bovine Serum Albumin (BSA). The final medium was identical in electrolyte composition to the KRP-buffer, but also contained 1% BSA and 1 mM sodium palmitate with sufficient 1-C^{14}-palmitic acid to yield a concentration of 0.4μCi/ml and a specific activity of 0.4μCi/μ mole.

Magnetic iron oxide was prepared as described by Cook and Pickering (1958) except that it was stored in 0.9% NaCl. Sodium hydroxide (2.6 gm) and potassium nitrate (20 gm) were dissolved in 100 ml of oxygen-saturated distilled water and then added to 100 ml of oxygen-saturated water containing 0.9% ferrous sulphate. The mixture formed a green-black slurry which was boiled for 20 minutes. The magnetic iron oxide was then separated from the reaction mixture with a strong magnet and the resulting precipitate was washed and precipitated several times by suspension in distilled water

followed by precipitation with the magnet. The precipitate was then stored in 0.9% NaCl.

Defatted BSA was prepared from Sigma fraction V BSA (Sigma Chem. Co.) as described by Chen (1967). A 10% BSA solution was prepared. Activated charcoal (Norit A) was then added to yield a 4% by weight suspension and the mixture adjusted to a pH of 3.00. The mixture was cooled to o°C in an ice bath and stirred for 30 minutes. Then the mixture was transferred to centrifuge tubes and spun at 18,000 rpm. The resulting supernate was separated and recentrifuged at 18,000 rpm. Then, the supernate was adjusted to a pH of 7.4 and the protein content assayed with Biuret reageants.

Sodium palmitate was prepared from palmitic acid (Sigma Chem. Co.) by dissolving the free acid in absolute ethanol followed by the addition of 2.5 mM NaOH. The ethanol was evaporated and the sodium palmitate dissolved in 100 ml of freshly boiled distilled water. Media containing palmitate were prepared by adding warm stock palmitate to the media containing the electrolyte and BSA but without calcium. The mixture was then equilibrated for 30 minutes prior to the addition of labeled palmitic acid. The mixture was allowed to equilibrate for a further 15 minutes. Calcium was then added and the media pH adjusted to 7.4.

Preparation of tubules and glomeruli

The preparation used was essentially that described by Hewitt and Hook (1978) as modified from the method described by Brendel and Meezan (1974).

The animals were killed by a blow to the head. Kidneys in adult animals were perfused via the renal artery. Kidneys in neonates were perfused via the abdominal aorta from below the renal arteries after clamping the abdominal aorta above the renal arteries. The kidneys were initially perfused with KRP-buffer. Subsequently, they were perfused with a medium containing magnetic iron oxide. The kidneys were removed and the renal cortex dissected out and minced. The mince was then homogenized by gentle hand homogenization (5-7 strokes, teflon on glass). The homogenate was filtered through a 253μ nylon sieve and the retained material thoroughly washed with KRP-buffer. The filtrate was saved and subjected to a second filtration through a 63μ nylon sieve. The retained solute was saved and resuspended in KRP-buffer. The glomeruli, containing iron oxide particles trapped in the glomerular capillaries, were then separated from the supernate by exposure to a strong magnet which precipitated the iron containing tissue. The precipitate was

resuspended in KRP-buffer, reprecipitated with the magnet to wash the glomeruli and then resuspended in KRP-buffer containing 1% BSA. The tubules suspended in the supernate were reexposed to the magnet. Any resulting precipitate was discarded and the supernate was saved. The supernate was centrifuged at 1,000 x g for 1 minute. The resulting tissue pellet was then suspended in KRP-buffer containing 1% BSA to a concentration of 1 g/10 ml. Microscopic inspection indicated that tubule preparations were 95-99% pure while glomerular preparations were enriched to 85-90% purity.

Determinations of organic anion transport

Transport in renal cortical slices was determined essentially as described by Hook and Munro (1968). Renal cortical slices were incubated in a phosphate buffer medium containing PAH and C^{14}- TEA, under an oxygen atmosphere for 90 minutes. The slices were removed, weighed, and minced in 3 ml of 10% TCA. A 2 ml aliquot of medium was added to 3 ml of 10% TCA. Both the tissue and the medium sample were adjusted to a final volume of 10 ml. The resulting mixtures were then assayed for PAH by the method of Smith et al., (1945) or for TEA by liquid scintillation spectrometry. The

results were expressed as a slice concentration (mg or dpm / g) to medium concentration (mg or dpm / ml) ratio (S/M ratio).

The ability of proximal tubule suspensions to transport organic anions was determined as described by Hewitt and Hook (1978). Five ml of a KRP-buffer medium containing 1% BSA and 1 g/10ml medium tubules was placed in a 25 ml erlenmeyer flask and incubated under an oxygen atmosphere for 15 minutes at 37 degrees C. Subsequent to this pre-incubation, 50 µl of a 7.4×10^{-3} M PAH stock was added and incubation continued for 30 minutes. One minute prior to termination of incubation 120 µl of 5% inulin was added to the reaction mixture in order to estimate extracellular water in the tubules. The incubation mixture was then transferred to a special centrifuge tube described by Burg and Orloff (1962) and centrifuged at 10,000 rpm for 10 minutes. The resulting supernate was separated from the pellet and a 2 ml aliquot added to 3 ml of 10% TCA and 5 ml of water. The pellet was removed and a wet weight obtained. The pellet was then dried overnight at 50 degrees C and a dry weight subsequently obtained. The pellet was removed and pulverized in a graduated cylinder and the powder was suspended in 5 ml of 3% TCA. The PAH in the medium and pellet was assayed by

the method of Smith et al. (1945). Inulin was assayed by the method of Schreiner (1950) and was used to estimate the contribution of the medium trapped in the pellet to the tissue wet weight and tissue PAH content. The results were expressed as a T/M ratio where T was the concentration of PAH in the intracellular water (mg/ml) and M was the concentration of PAH in the medium (mg/ml).

Palmitate Esterification

The capacity of the tissue to esterify palmitate was determined as described by Hinemann, et al. (1975) as modified by Hewitt and Hook (1978). An aliquot (5-10 ml) of the KRP-buffer - 1% BSA tubule suspension was preincubated in an Erlenmeyer flask for 15 minutes at 37°C under an oxygen atmosphere. The tubule suspension was then centrifuged for 1 minute at 1,000xg in a Sorvall desk top laboratory centrifuge. The pellet was resuspended to a concentration of 1g/10ml in a KRP-buffer containing 1% BSA and 1 mM 1-C^{14}-palmitate (sp. act. 0.4%μCi/ μMole). This mixture was incubated for 30 minutes under oxygen at 37°C. Upon completion of incubation, the mixture was centrifuged (1 min., 1,000 x g) and resuspended in 2-5 ml of .2N H_2SO_4 and recentrifuged

for one minute at 1,000 x g. The resulting pellet was resuspended and washed in ice cold KRP-buffer containing 1% BSA, and then centrifuged 1 minute at 1,000 x g. The tubule pellet was weighed and homogenized in chloroform:methanol (2:1) in a ratio of 1 gm tissue per 20 ml of solvent as described in the Folch procedure (Folch, et al., 1957).

Methanol (0.2 x the volume of chloroform:methanol added) was then added and the mixture centrifuged. The supernate was separated and saved. A volume of 0.05 M KCl 0.2 times the volume of chloroform:methanol was added to induce phase separation. The upper phase was discarded and the lower phase washed 1 time with previously prepared upper phase. The lower phase was saved and an aliquot plated on a 250 μ Silica Gel G thin layer chromatography plate. The thin layer plate was then developed in a solvent system of 80 parts hexane, 20 parts diethyl ether and 1 part glacial acetic acid. The triglyceride and NEFA fractions were identified by comparison to a triglyceride and NEFA standard. The triglyceride, NEFA, and origin (phospholipid fractions were scraped into scintillation vials and counted in an Omniflour-toluene cocktail (New England Nuclear) containing 4% Cab-O-Sil (Eastman Kodak Co.). A second aliquot was taken and added to an Omniflour-toluene cocktail and counted

by liquid scintillation spectrometry. Efficiency was determined by internal standards or by the sample channels ratio.

Glomerular metabolism

Glomeruli were suspended in 5 ml of KRP-buffer containing 1% BSA. The suspension was incubated for 15 minutes at 37°C. The glomeruli were then separated from the medium with a magnet and resuspended in 5 ml of KRP-buffer containing 1% BSA and 1 mM-C^{14}-palmitate (spe. act. 0.4μ Ci/μ Mole). This mixture was incubated for 30 minutes at 37°C. Next, the mixture was centrifuged and the pellet resuspended in 1 ml of .2N H_2SO_4. The mixture was centrifuged and the resulting pellet saved. The lipid was extracted by adding 0.5 ml of 0.5 N KCl followed by 10 ml of 1:2 chloroform: methanol. Phase separation was induced by adding 2 ml of methanol and 2 ml of .05 N KCl. The bottom, organic layer was saved and plated on a thin layer chromatography plate and label determined as described for tubule esterification.

Palmitate extraction from medium

Tubular extraction of NEFA acid was determined by comparing label content of incubation media before and after

incubation. An aliquot of medium containing C^{14} labeled palmitic acid was extracted by the Folch procedure and subjected to thin layer chromatography. The C^{14} content of the NEFA fraction was determined by liquid scintillation spectrophotometry as described for tubular esterification.

Triglyceride and NEFA assay

Animals were killed by a blow to the head and blood removed from the heart and centrifuged immediately. The supernate was separated and allowed to clot. The resulting serum was used for triglyceride and NEFA analysis. Liver tissue was snap frozen in liquid nitrogen. Kidneys were flushed with ice cold KRP-buffer, and the cortex dissected away prior to being snap frozen in liquid nitrogen.

Triglyceride was analyzed as described by Van Handel and Zilversmit (1957) as modified by Butler, et al. (1961). Tristearin (Sigma, St. Louis) was dissolved in chloroform and used as the triglyceride standard. Tissue was homogenized in a 0.33 M pH 7.0 phosphate buffer (1 g / 9 ml). Triglyceride was extracted from 1 ml of tissue homogenate or 1 ml of serum with 20 ml of chloroform. Phospholipids were absorbed out with 4 g of chloroform-moistened,

pulverized zeolite (Doucil). Aliquots of the chloroform extract were then saponified (0.5 ml of 0.2% Ethanolic KOH at 60°C for 30 minutes) or not saponified (0.5 ml of ethanol at 60°C for 30 minutes) and the triglyceride content evaluated by analysis of the difference in glycerol content between saponified and nonsaponified samples. Glycerol was estimated chemically with the metaperiodateoarsenate-chromatropic acid reaction described by Lambert and Neish (1950) and as modified by Butler et al. (1961).

NEFA were extracted from 1 g of tissue by the method of Folch, et al. (1957). The lipid extract was plated on a 250μ or 500μ Silica Gel H thin layer chromatography plate and the plate developed in a solvent system consisting of 70 parts hexane, 30 parts diethyl ether and 1 part glacial acetic acid. Lipid fractions were identified by comparison to appropriate standards after exposure to iodine vapors. Care was taken to avoid exposure of NEFA samples to iodine by covering the spot with a plass plate. The tentatively identified NEFA fraction was then scraped from the plate and the NEFA extracted from the silica gel by the method of Elphick and Lawlor (1976).

Briefly, the NEFA acids were extracted by incubation of the Silica Gel H in 2.62 N KOH (1-3ml). The

resulting slurry was suspended in 2.17 M glycerol (3-9ml) and acidified by adding 2.8 N HCl (1-3ml). The lipid was then extracted with 4 ml of chloroform. The extraction was repeated and the chloroform layers saved and evaporated. The lipid was dissolved in 1 ml of diethyl ether and the fatty acids methylated by exposure to diazo methane as described by Levitt (1973). The fatty acid methyl esters were then injected onto a gas chromatograph using a carrier gas (nitrogen) flow rate of 40 ml, operating at a temperature of 165°C. A six foot column packed with 100 / 120 mesh chromasorb W A W with a liquid phase of 10% SP-2330 (Supelco) was used to separate the various fatty acids. Fatty acids were quantitated by measuring peak height. An internal standard of 10µg heptadecanoic acid (C 17) was added prior to the Folch extraction to correct for loss of sample. The quantities of individual fatty acids were estimated by comparison to known standards.

Diacyl glycerol acyl transferase (DAGAT) Assay

DAGAT is a microsomal enzyme which is dependent upon intracellular proteins for activity (Manley et al., 1974; O'Doherty and Kuksis, 1978). Microsomes and cytosol were

prepared as described by Manley et al. (1974). Tissue was minced in a 0.02 M pH 7.4 phosphate buffer containing 0.1 mM EDTA. The mince was then homogenized in the same medium at a ratio of 1 part tissue per 2 parts medium using a motor driven teflon pestle in glass homogenizer. The homogenate was centrifuged at 1,000 g for 10 minutes and the supernate saved. The supernate was then centrifuged at 20,000 g for 15 minutes. The supernate was saved and centrifuged at 100,000 x g for 60 minutes. The middle layer of the supernate was saved and the microsomal pellet resuspended in the EDTA phosphate buffer. Both fractions were centrifuged again at 100,000 g for 60 minutes. The pellet was resuspended and recentrifuged then resuspended. Cytosol and microsomal fractions were then frozen and stored in a -80°C freezer. Protein content of each fraction was evaluated by the Lowry method (Lowry et al., 1951).

DAGAT was assayed as described by Manly et al. (1974) as modified by O'Doherty and Kuksis (1978). The incubation mixture included 1 mg microsomal protein, 7.5 or 10 mg supernate protein, 5 nM of 1-2 dioleayl - 5 n-glycerol, 1 nM C^{14} palmitoyl - S - Coenzyme A (.2μCi), 4 n Moles $MgCl_2$ and 4 n Moles of ATP in a volume of 1.05 ml.

Diolein was dissolved in 2:1 dioxane:proplylene glycol and added in a 10 ml aliquot. Palmitoyl Coenzyme A was dissolved in 0.1 M pH 6 acetate buffer and added in a 10 ml aliquot. The incubation mixture was incubated for 1 or 2 hours in a gyrorotary incubator at 37°C. The reaction was stopped by the addition of 3.75 ml of chloroform:methanol (1:2). Triglyceride was extracted and phase separation induced by the addition of 1.25 ml of chloroform and 1.25 ml water. The bottom organic layer was saved and plated on a silica gel G thin layer chromatography plate. The plate was developed in a solvent consisting of 80 parts hexane, 20 parts diethyl ether and 1 part glacial acetic acid. The triglyceride fraction was identified by comparison to a triglyceride standard. The triglyceride fraction was then scraped into a scintillation vial and suspended in an omni-flour-toulene 4% cabosil cocktail.

Statistical analysis

Where appropriate, data were analyzed using analysis of variance, completely random design or randomized complete block design, or student's "t" test for paired or group comparisons. Treatment means were compared by Student-Newman

Keul's procedure (Steele and Torrie, 1960). The 0.05 level of probability was used as the criterion of significance.

RESULTS

Effect of palmitate in the medium of PAH accumulation

Tubules from control and penicillin treated 2 week old rabbits were incubated in a KRP-buffer medium containing 1% BSA in the absence of palmitate (-palm) and in the presence of 1 mM palmitic acid (+palm) (Figure 1). Animals were pretreated with 90,000 I.U. of procaine penicillin G 60, 48, 36 and 24 hours before use. Such treatment enhanced the capacity of tubules to accumulate PAH from a T/M of 3.17 ± 0.85 to a T/M of 8.17 ± 1.44 when transport was measured 24 hours after the last treatment dose. Addition of palmitate to the medium consistently decreased transport (Figure 1). The average T/M for all individual determinations of transport in the absence of palmitate was 5.94 ± 1.24. The addition of palmitate decreased the T/M 40% to an average value of 3.57 ± 0.67.

Figure 1. Accumulation of PAH by suspensions of renal cortical tubules in the presence or absence of added palmitate. Tubules were prepared from litters that had been pretreated with 4 doses of saline (open circles) or 90,000 I.U. of procaine penicillin G (closed circles). One aliquot of tubules was incubated in a KRP-buffer-1% BSA medium (-palm); a paired aliquot of tissues was incubated in a KRP-buffer-1% BSA medium containing 1 mM palmitic acid (+palm). Individual data points are displayed. Values determined in the absence of palmitate are connected to corresponding values determined in the presence of palmitate in tubules from the same pool of tubules.

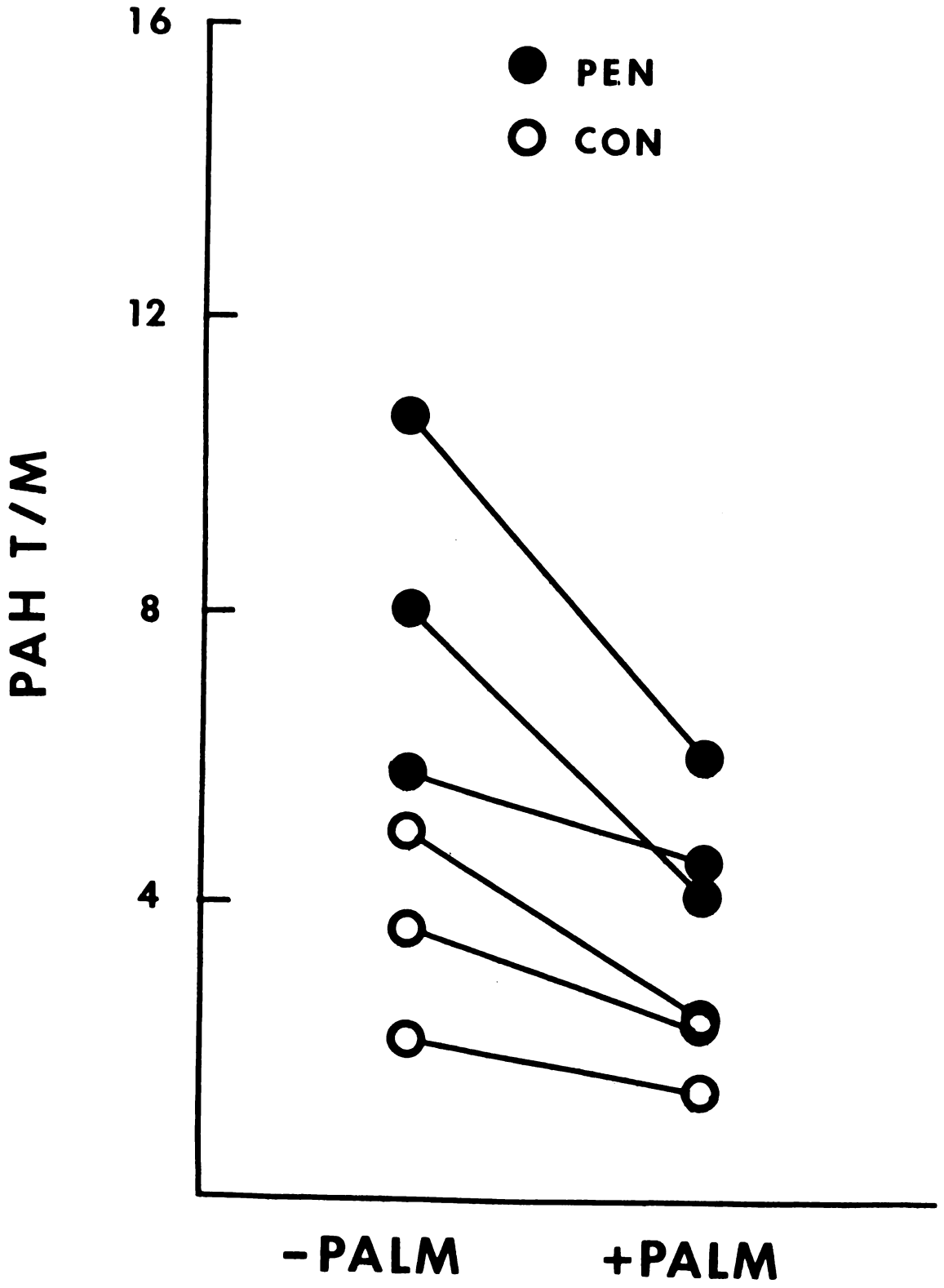

Figure 1

Effect of penicillin treatment on utilization of palmitate

Two week old rabbit litters were treated with saline or 90,000 I.U. of procaine penicillin G at 12 hour intervals for a total of 4 treatments. Twenty-four hours after the last injection transport was determined in a KRP-buffer medium containing 1% BSA and 7.4×10^{-5} M PAH. Penicillin treatment increased transport 100% from a control T/M of 3.04 ± 0.71 to a T/M of 6.22 ± 1.12 (Figure 2). Paired aliquots of tissue were also incubated in a KRP-buffer - 1% BSA medium containing 1 mM palmitic acid. Tubules from control animals extracted 1.08 ± 0.20 micromoles of palmitic acid from the medium during a 30 minute incubation. Penicillin treatment did not significantly increase the extraction of palmitate from the medium (Figure 2).

In a separate series of experiments, using an identical treatment regimen, penicillin treatment increased T/M 138% from 2.97 ± 0.50 to a value of 7.07 ± 0.86 (Table 1). However, penicillin treatment did not increase incorporation of label into tissue total lipid (Table 1).

Figure 2. Effect of penicillin treatment on transport of PAH and on palmitate extraction from the medium. Rabbit litters were treated with saline or procaine penicillin G (90,000 I.U.) at 12 hour intervals to a total of 4 treatments. Twenty-four hours after the last treatment, the capacity of one aliquot to transport PAH was determined while the capacity of a second aliquot, from the same pool of tubules, to extract palmitate from a medium was determined. Values are means ± S.E. *Significantly different from control ($P<0.05$).

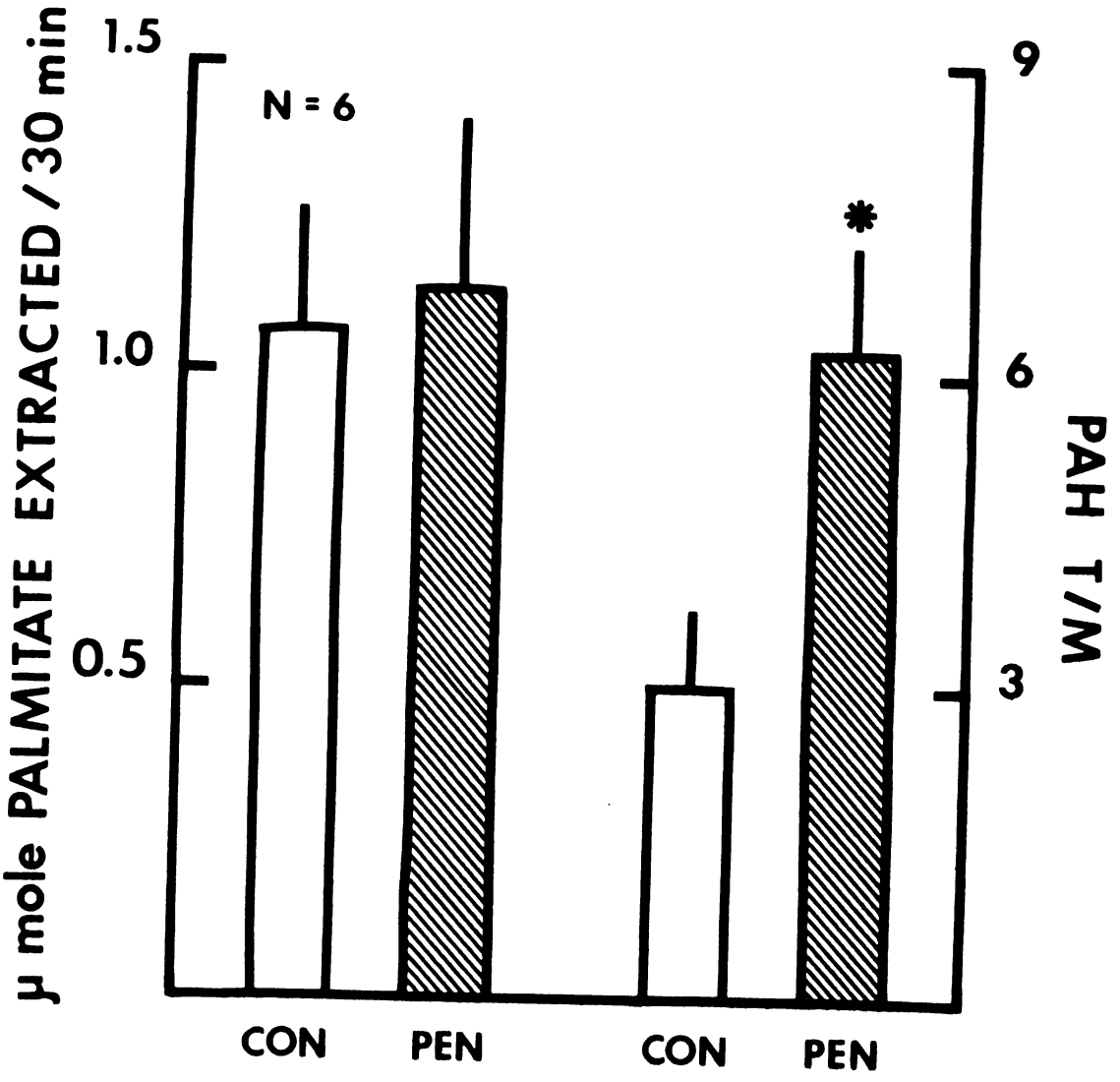
μ mole PALMITATE EXTRACTED / 30 min
1.5
1.0
0.5
N = 6
*
9
6
3
PAH T/M
CON
PEN
CON
PEN

Figure 2

TABLE I. Penicillin Effect on PAH T/M and Palmitate Incorporation into Tissue Total Lipid in 2 Week Old Rabbits

	CON	PEN	% CHANGE
PAH T/M	2.97 ± .50	7.07 ± .86[b]	138
Tissue[c] Lipid	2.31 ± .23	2.44 ± .20	6

[a] N = 9. Values are means ± S.E.

[b] Significant $p<.05$.

[c] μ mol/g/30 min.

Effect of penicillin treatment of endogenous NEFA and triglyceride

Two week old rabbits were treated with 90,000 I.U. procaine penicillin G at 12 hour intervals for a total of 2 doses. Twenty-four hours after the last dose animals were killed and the serum, kidney and liver content of triglyceride determined. Serum and kidney content of several fatty acids was determined by gas-liquid chromatography. The individual fatty acids were summed to estimate total NEFA. Penicillin treatment increased the serum content of several individual NEFA (Table 2) and increased the overall serum content of measured NEFA from 87.7 ± 3.4 to 159 ± 6.8 mg/ml (Figure 3).

Penicillin treatment significantly decreased renal triglyceride from 0.264 ± 0.035 mg/g to 0.177 ± 0.032 mg/g (Figure 4). Serum triglyceride and hepatic triglyceride were not significantly affected by penicillin treatment (Figure 4).

Effect of fasting on endogenous lipids

Adult female rabbits were fasted for 48 hours and endogenous lipid content of serum, liver and kidney

Figure 3. Effect of penicillin treatment on renal and serum concentrations of NEFA. Animals were treated with two doses of 90,000 I.U. of penicillin G with a 12 hour interval, and killed 24 hours after the last dose. Individual fatty acids were quantitated with gas-liquid chromatography and select fatty acids summed to estimate the total NEFA content. Values are means ± S.E.
*Significantly different from control ($p<0.05$)

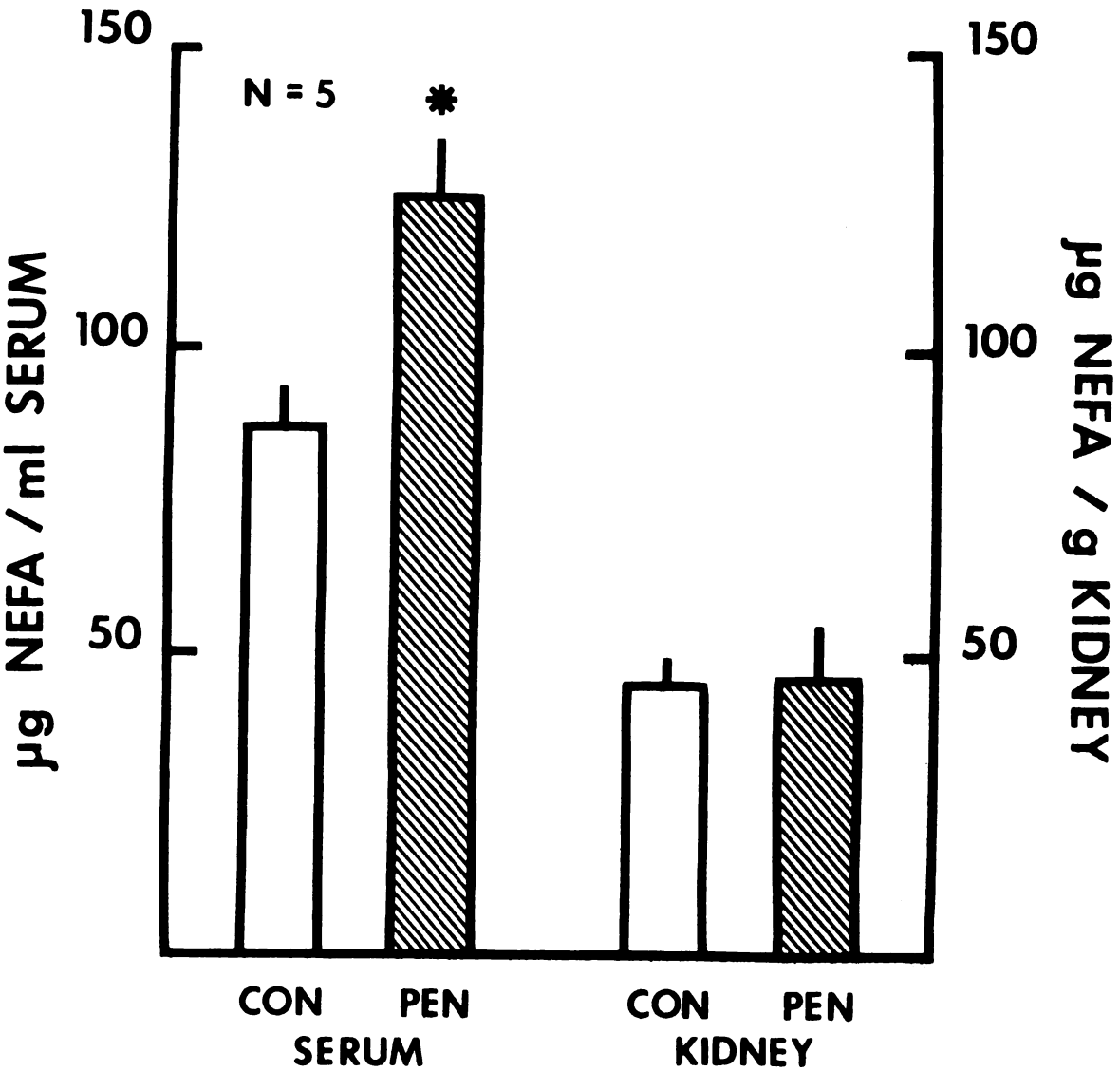
150
100
50
N = 5
*
µg NEFA / ml SERUM
µg NEFA / g KIDNEY
CON
PEN
SERUM
CON
PEN
KIDNEY

Figure 3

TABLE 2.

Effect of Penicillin Treatment on Endogenous Serum and Kidney NEFA Content of 2 Week Old Rabbits[a]

Fatty acids[b]		C 14	C 16	C 16:1	C 18	C 18:1	C 18:2
SERUM	CON	4.84 ± 0.66	60.2 ± 2.42	1.21 ± 0.24	8.34 ± 1.22	8.23 ± 1.03	4.86 ± 1.65
	PEN	8.11 ± 1.65[d]	81.0 ± 7.17[d]	2.20 ± 0.53	11.14 ± 1.96[d]	16.0 ± 1.2[d]	8.99 ± 1.06[d]
KIDNEY	CON	3.29 ± 0.30	27.2 ± 2.49	1.30 ± 0.53	9.18 ± 1.34	3.74 ± 0.80	1.72 ± 0.50
	PEN	3.59 ± 0.76	29.1 ± 4.4	0.90 ± 0.18	7.42 ± 0.23	3.94 ± 0.38	1.14 ± 0.35

a) Animals were treated with saline (con) or 90,000 I.U. of penicillin G (pen) 24 and 36 hours before use.

b) The fatty acids measured were myristic (C 14), palmitic (C 16), palmitoleic (C 16:1) stearic (C 18), oleic (C 18:1) and linoleic acid (C 18:2).

c) Values are means ± S.E.

d) Significantly different from control $p<0.05$.

Figure 4. Effect of penicillin treatment on renal, liver and serum triglyceride. Two week old litters were treated with two doses of 90,000 I.U. penicillin G with a 12 hour interval. Triglyceride was quantified 24 hours after the last dose. Values are means ± S.E. *Significantly different from control ($p < 0.05$)

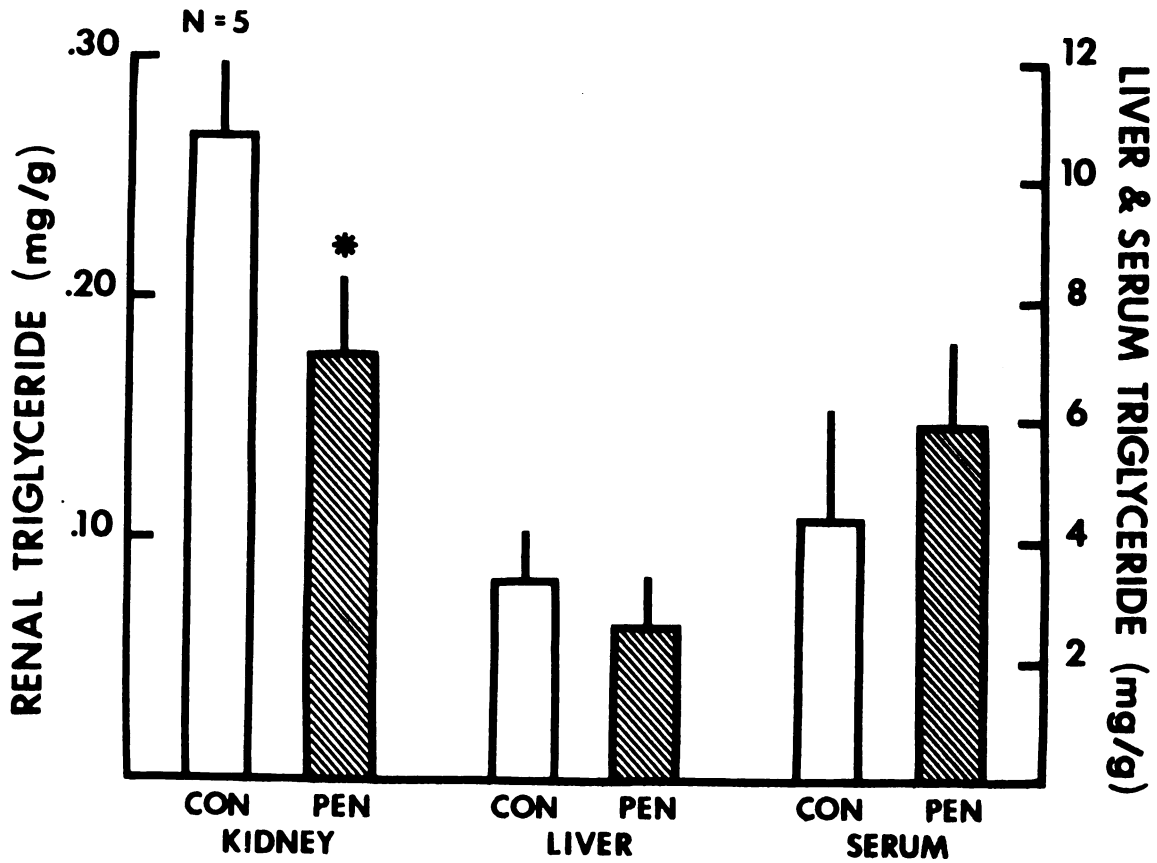
N = 5
.30
.20
.10
RENAL TRIGLYCERIDE (mg/g)
12
10
8
6
4
2
LIVER & SERUM TRIGLYCERIDE (mg/g)
*
CON
PEN
KIDNEY
CON
PEN
LIVER
CON
PEN
SERUM

Figure 4

determined. Individual fatty acids were determined by gas-liquid chromatography. The content was estimated by summing the measured content of myristic, palmitic, palmitoleic, stearic, oleic and linoleic acid. Fasting significantly increased the serum NEFA from 33.9 ± 3.1 to 369 ± 199 mg/ml. Fasting also increased liver NEFA content 160% but renal fatty acid content was not increased from a control value of 65 ± 15 μg/g (Figure 5).

Fasting significantly increased serum triglyceride from 0.208 ± 0.138 mg/ml to 3.59 ± 2.05 mg/g (Figure 6). Renal and hepatic content of triglyceride was not significantly affected but both tended to be increased (Figure 6).

Effect of organic anions on NEFA content of tubules incubated in a medium containing palmitic acid

Tubules from adult rabbits were incubated in a KRP-buffer medium containing 1% BSA and 1 mM palmitate. Tissue content of the various fatty acids were determined after 30 minutes of incubation. The sum of all the fatty acids measured was 665 ± 105 μg/g in control tissue (Figure 7). Of the fatty acids measured 90.5 ± 1.0% was in the form of

Figure 5. Effect of fasting on renal, liver and serum NEFA concentration. Animals were fasted for 48 hours before use. Individual fatty acids were quantitated by gas-liquid chromatography. Values represent the sum of the measured fatty acids. Displayed values are means ± S.E. *Significantly different from control<(p 0.05).

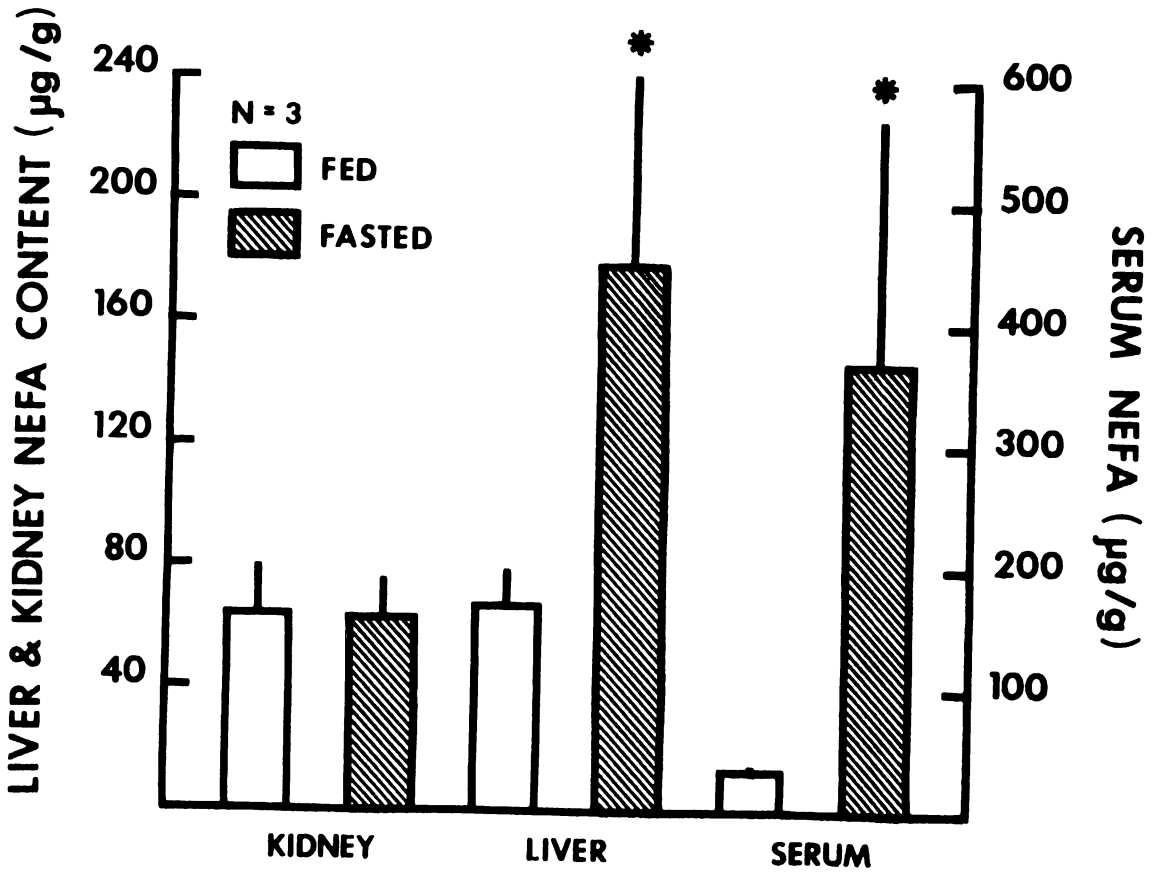
LIVER & KIDNEY NEFA CONTENT (µg/g)
240
200
160
120
80
40
N = 3
FED
FASTED
600
500
400
300
200
100
SERUM NEFA (µg/g)
KIDNEY
LIVER
SERUM

Figure 5

Figure 6. Effect of fasting on renal, liver and serum triglyceride. Animals were fasted for 48 hours prior to use. Values are means ± S.E. *Significantly different from control ($p<0.05$).

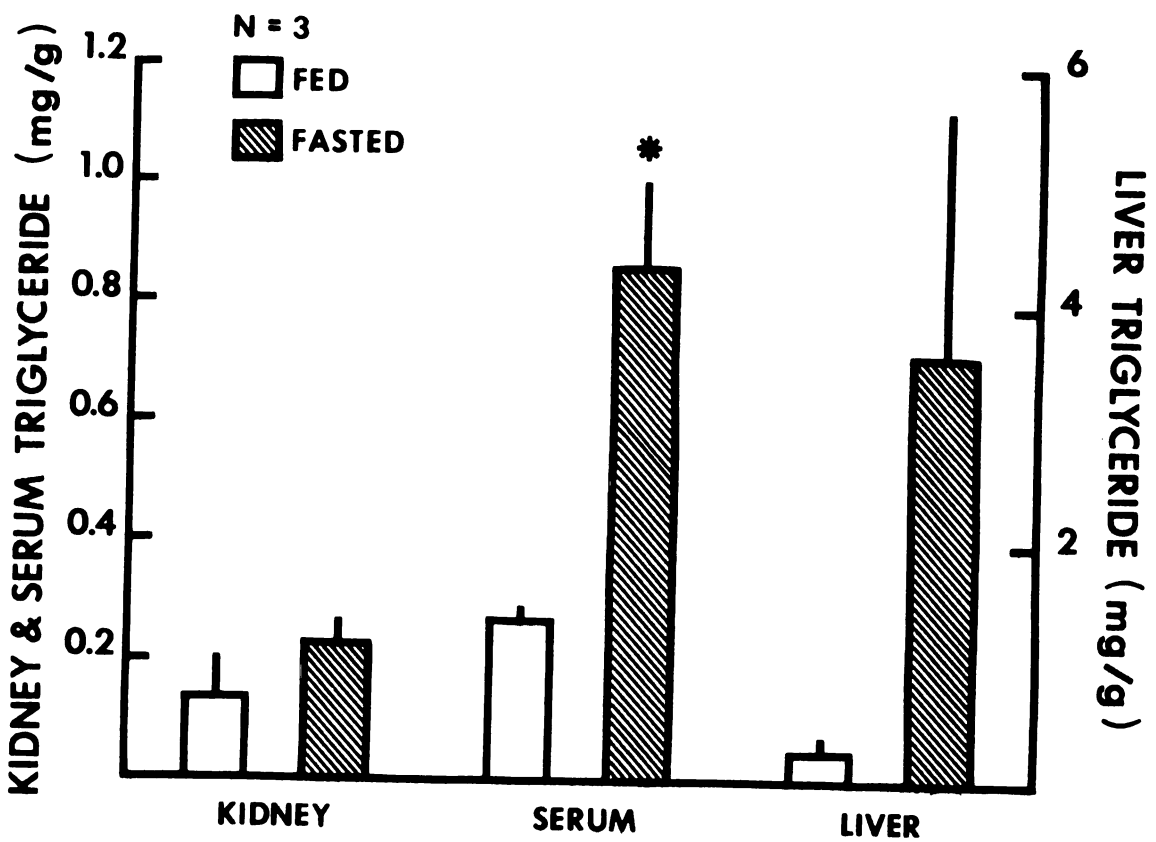
N = 3
FED
FASTED
KIDNEY & SERUM TRIGLYCERIDE (mg/g)
1.2
1.0
0.8
0.6
0.4
0.2
6
4
2
LIVER TRIGLYCERIDE (mg/g)
*
KIDNEY
SERUM
LIVER

Figure 6

Figure 7. Effect of probenecid and PAH on tubule content of NEFA *in vitro*. Tubules were prepared from adult rabbits and incubated for 30 minutes in a KRP-buffer-1% BSA medium containing 1 mM palmitate. Individual fatty acids were determined by gas-liquid chromatography. The individual fatty acids were summed to estimate total NEFA. Values are means ± S.E.

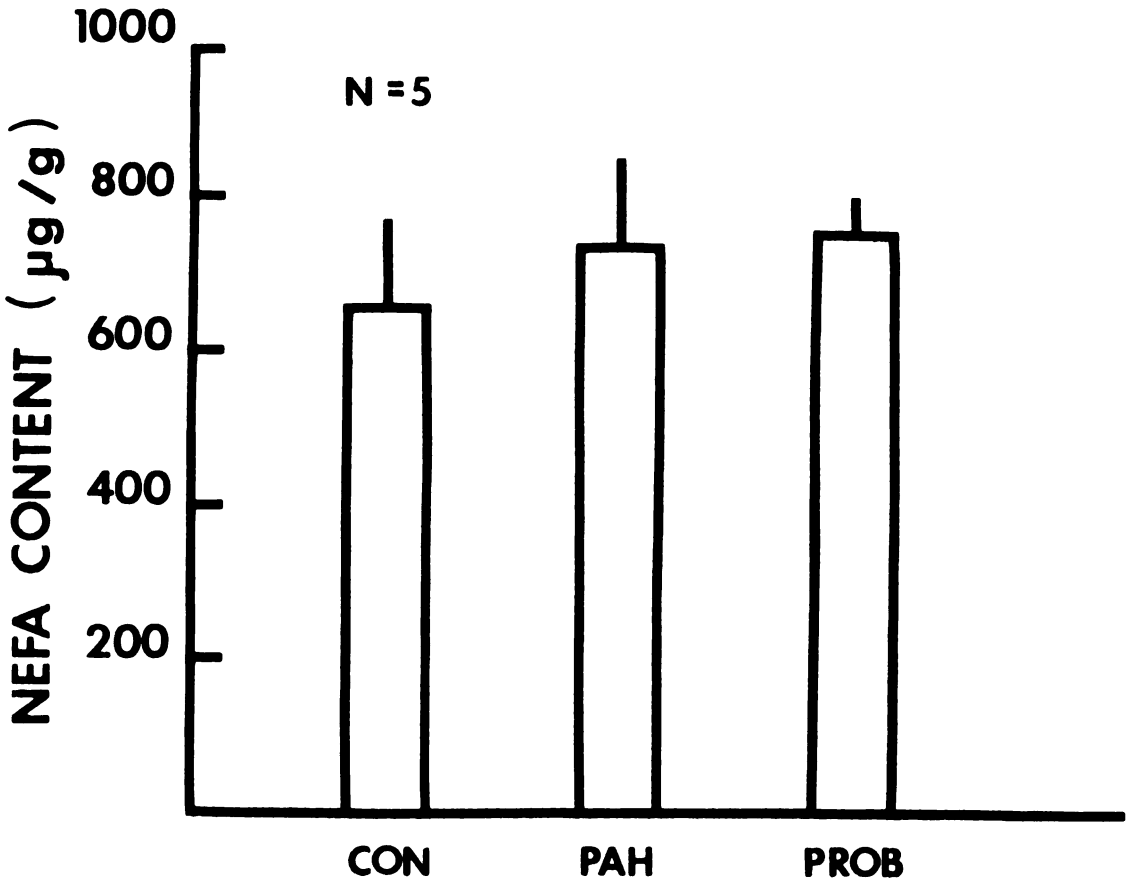

Figure 7

palmitic acid. Probenecid (2mM) and PAH (2mM) did not significantly affect the NEFA content of the incubated tubules.

Effect of fasting on renal transport

Adult rabbits were fasted for 48 hours and transport capacity determined as the ability of tubules to concentrate PAH. Fasting decreased the T/M from a control value of 6.78 ± 1.20 to a fasted value of 3.92 ± 1.04 (Figure 13). Fasting adult rats for 48 hours similarly decreased the transport of PAH from a S/M of 10.3 ± 1.00 to a value of 3.52 ± 0.23 (Figure 8). The addition of bicarbonate to the drinking water (0.28M), increased transport capacity to an S/M of 25.30 ± 0.72. Fasting decreased the capacity of rats drinking 0.28 M bicarbonate to an S/M of 5.10 ± 0.73 (Figure 8). Nicotinic acid (250 mg/kg q.i.d.) failed to enhance transport when transport was measured 6 hours after the last dose of nicotinic acid (Figure 8).

Transport of the organic cation, TEA was not affected by fasting, substituting a bicarbonate solution for drinking water or by treatment with nicotinic acid (Figure 9).

Figure 8. Effect of fasting, nicotinic acid and bicarbonate on the transport of PAH by the rat kidney. Rats were fasted for 48 hours. Nicotinic acid (250 mg/kg) was administered to fasted rats at 6 hour intervals to a total of 4 treatments. Animals were used 6 hours after the last treatment. Bicarbonate was supplied in the drinking water as a 0.28 M solution. Values are means ± S.E. *Significantly different from control ($p<0.05$).

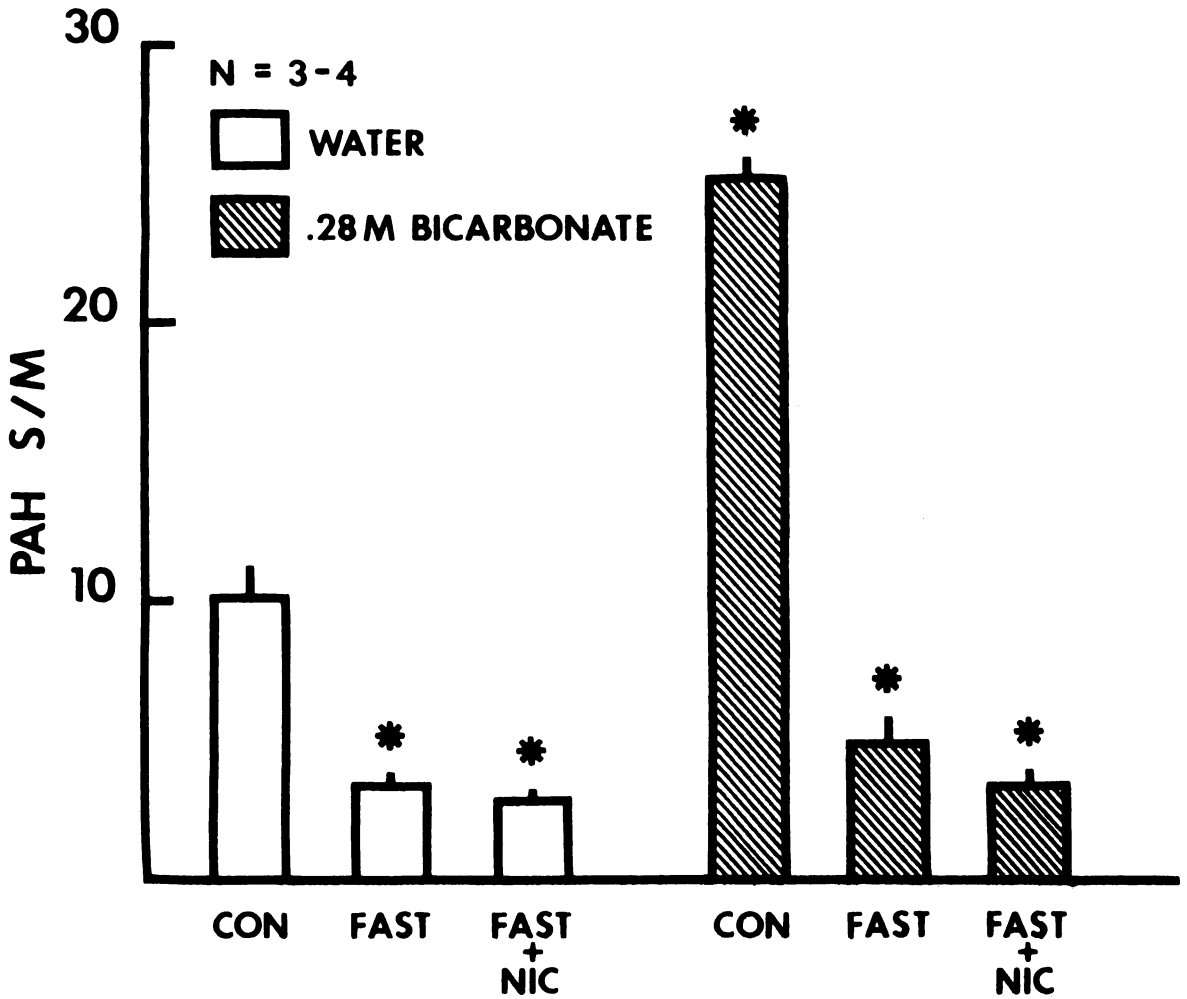
30
20
10
PAH S/M
N = 3-4
WATER
.28 M BICARBONATE
CON
FAST
FAST + NIC
CON
FAST
FAST + NIC

Figure 8

Figure 9. Effect of fasting, nicotinic acid and bicarbonate on the transport of TEA in the rat kidney. Rats were fasted for 48 hours. Nicotinic acid (250 mg/kg) was administered to fasted rats at 6 hour intervals to a total of 4 treatments. Bicarbonate was supplied in the drinking water as a 0.28 M solution. Values are means ± S.E.

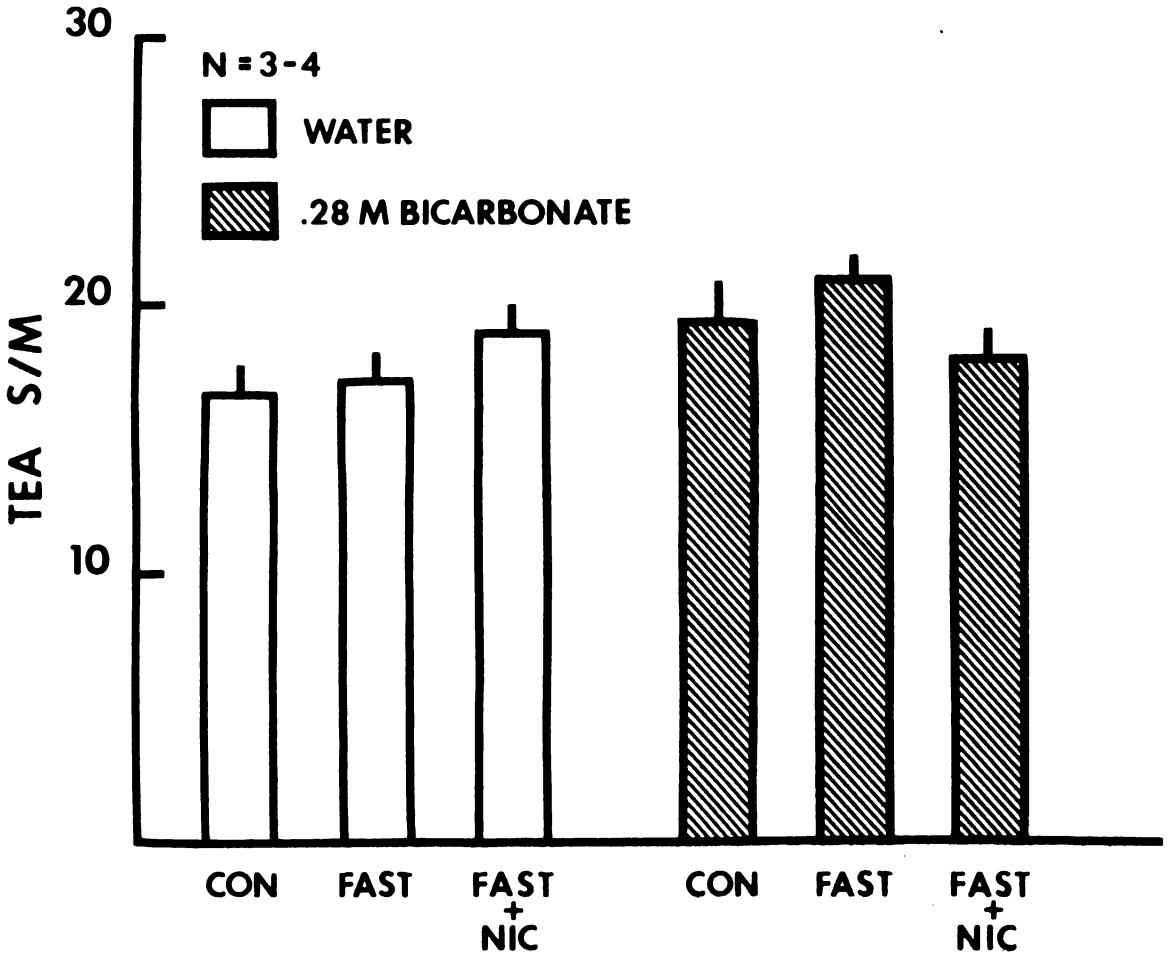
30
20
10
TEA S/M
N = 3-4
WATER
.28 M BICARBONATE
CON
FAST
FAST + NIC
CON
FAST
FAST + NIC

Figure 9

Effect of penicillin treatment on glomerular lipid metabolism

Glomeruli were prepared from two week old rabbit litters pretreated with procaine penicillin G (90,000 I.U.) or saline in a dosage regimen of twice a day for 2 days or twice a day for 1 day. Twenty-four hours after the last dose, the capacity of glomeruli to incorporate label from C^{14} - palmitic acid into triglyceride relative to the incorporation of label into tissue total lipid was determined (Figure 10). Incorporation of label into tissue NEFA relative to the incorporation of label into total lipid was similarly determined (Figure 11). Penicillin treatment did not affect the relative incorporation of label into triglyceride (Figure 10) or into tissue NEFA (Figure 11).

Effect of various penicillin dosing regimens on tubular transport capacity and tubular metabolism of palmitic acid

Litters of two week old rabbits were treated with saline or 90,000 I.U. of procaine penicillin G with a variety of treatment regimens. Tubular capacity to accumulate PAH and tubular metabolism of C^{14} - palmitic acid were determined 12, 24 or 48 hours after the last treatment.

Figure 10. Effect of penicillin treatment on glomerular incorporation of C^{14} palmitate into triglyceride. Litters of two week old rabbits were pretreated with 2 or 4 doses of penicillin G (90,000 I.U./treatment) at 12 hour intervals. Twenty-four hours after the last treatment glomeruli were prepared and their capacity to incorporate labeled palmitate into triglyceride relative to the incorporation of label into total lipid determined. Values are means ± S.E.

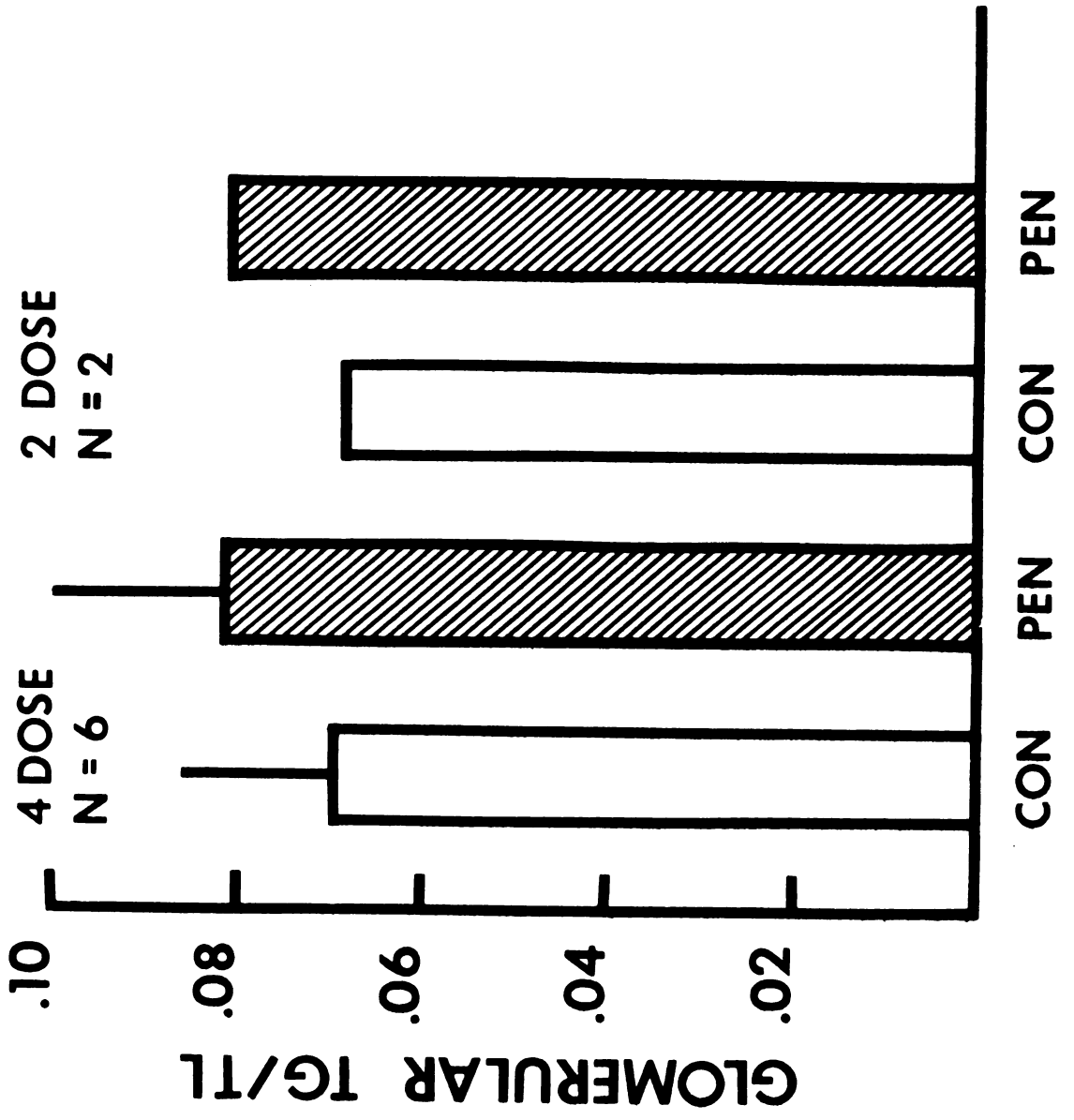
GLOMERULAR TG/TL
.10
.08
.06
.04
.02
4 DOSE
N = 6
2 DOSE
N = 2
CON
PEN
CON
PEN
CON
PEN

Figure 10

Figure 11. Effect of penicillin treatment on glomerular incorporation of C^{14} palmitate into tissue NEFA. Litters of two week old rabbits were pretreated 2 or 4 times with procaine penicillin G (90,000 I.U./treatment) at 12 hour intervals. Twenty-four hours after the last treatment glomeruli were prepared and their capacity to incorporate C^{14} palmitate into tissue NEFA relative to incorporation into total lipid determined. Values are means ± S.E.

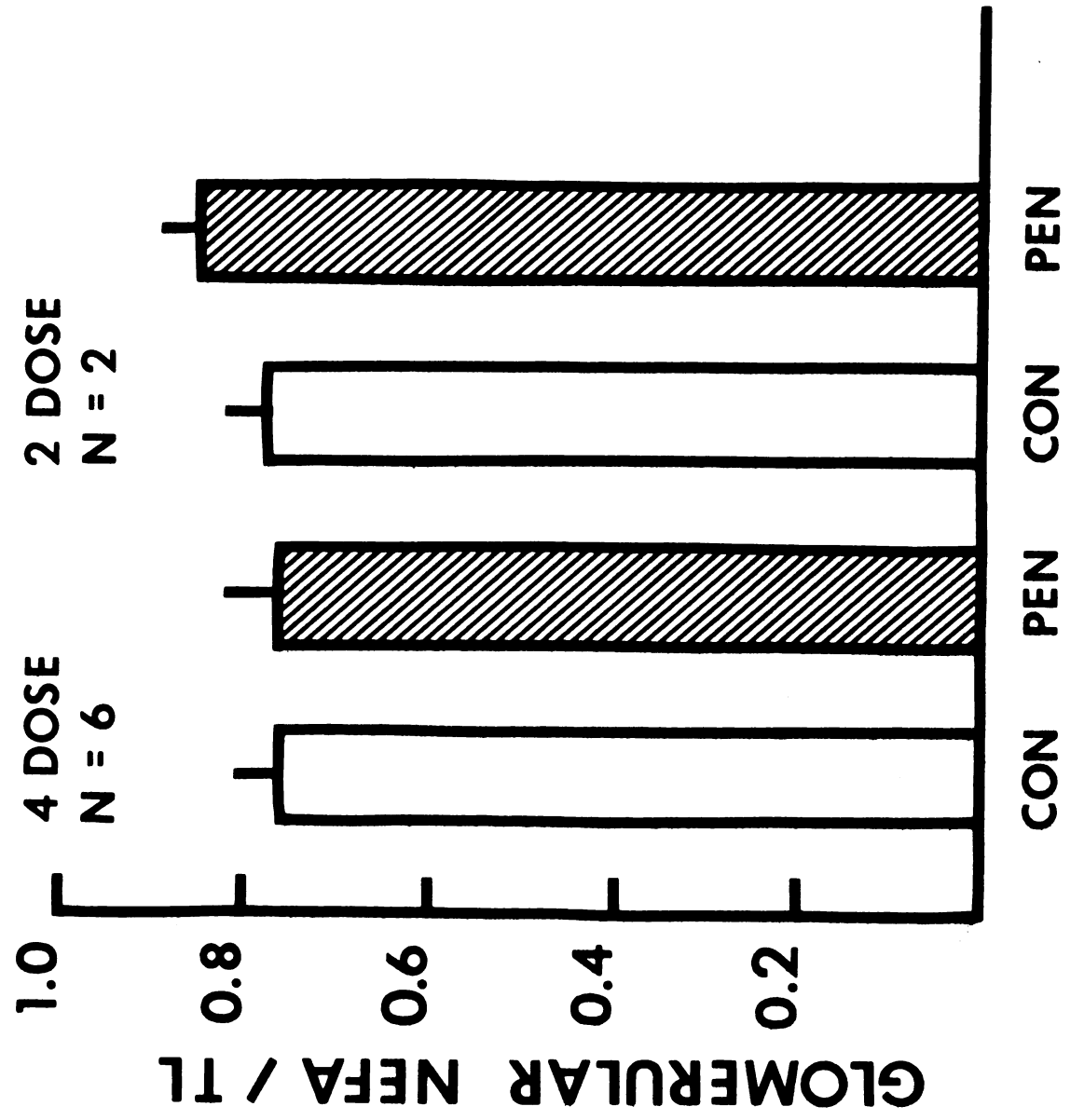
GLOMERULAR NEFA / TL
1.0
0.8
0.6
0.4
0.2
4 DOSE
N = 6
2 DOSE
N = 2
CON
PEN
CON
PEN

Figure 11

Tubules from litters treated with a single dose of penicillin did not exhibit altered metabolism or altered transport when evaluated 12 hours after treatment (Figure 12; Table 3). Treatment with two doses of penicillin at a 12 hour interval enhanced incorporation of label into triglyceride and decreased incorporation of label into NEFA but did not enhance PAH transport capacity when evaluated 12 hours after the last treatment (Figure 12; Table 3).

Treatment with 2 doses of penicillin G significantly enhanced PAH transport 24 hours after treatment (Figure 12; Table 3). Incorporation of labeled palmitate into tissue NEFA was slightly, but significantly decreased (Figure 12; Table 3).

Treatment with four doses of penicillin G was associated with increased transport 24 and 48 hours after the last treatment dose. (Figure 12; Table 3). The four dose regimen was also associated with a significant increase in incorporation of label into triglyceride 24 hours after the last dose (Figure 12; Table 3). Incorporation of labeled palmitate into tissue NEFA was decreased 24 hours after treatment but the effect appeared to have decreased markedly 48 hours after the last dose (Figure 12; Table 3).

Figure 12. Effect of various penicillin treatment regimens on tubular transport capacity and tubular metabolism of palmitate. Litters were treated with 1, 2 or 4 doses of penicillin (90,000 I.U./dose), at 12 hour intervals and the effect of treatment determined 12, 24 or 48 hours after the last treatment. Values are mean differences (treated-control). Values represent 3 or 9 individual determinations.

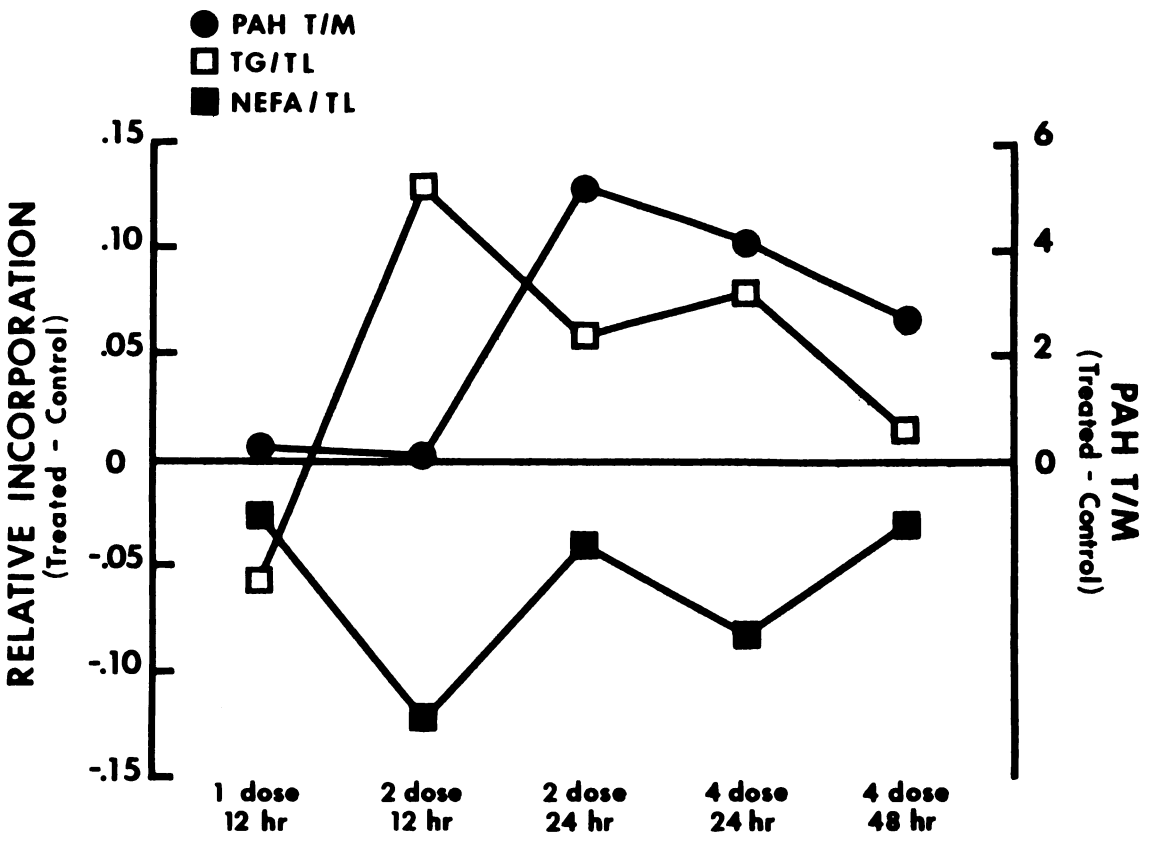
PAH T/M
TG/TL
NEFA/TL
RELATIVE INCORPORATION
(Treated - Control)
.15
.10
.05
0
-.05
-.10
-.15
6
4
2
0
PAH T/M
(Treated - Control)
1 dose
12 hr
2 dose
12 hr
2 dose
24 hr
4 dose
24 hr
4 dose
48 hr

Figure 12

TABLE 3

Effect of Various Penicillin Treatment Regimens on NEFA Metabolism and Organic Anion Transport in the 2 Week Old Rabbit[a]

# of DOSES[b]	1	2	2	4	4
Time after last treatment[c]	12	12	24	24	48
TG/TL[d]	0.053 ± .085	0.087 ± .020[e]	0.056 ± .005[e]	0.079 ± .011	0.013 ± .007
NEFA/TL[d]	0.023 ± .094	0.121 ± .013[e]	0.038 ± .028[e]	0.080 ± .013[e]	0.030 ± .016[e]
PL/TL[d]	0.005 ± .022	0.032 ± .040	0.024 ± .012	0.011 ± .025	-.007 ± .032
TL[d]	0.35 ± .35	-.066 ± .031	-.076 ± .072	0.134 ± .121	0.263 ± .093
PAH T/M	0.27 ±0.89	0.15 ± .50	5.21 ±1.83[e]	4.13 ±0.67[e]	2.76 ±0.37[e]

a) Values are mean differences (treated-control) ± S.E. of 3 or 9 determinations.

b) Neonates were treated at 12 hour intervals with 90,000 I.U. penicillin G to the total indicated.

c) Animals were killed 12, 24 or 48 hours after the last treatment.

d) Incorporation into total lipid (TL) was expressed as micromoles/g/30 min. Incorporation into triglyceride (TG/TL), phospholipid (PL/TL) and NEFA (NEFA/TL) was expressed relative to incorporation into total lipid.

e) Significantly different from control. $p < 0.05$.

Effect of fasting on transport and palmitate metabolism

Adult rabbits were fasted for 48 hours or allowed free access to food. Tubule suspensions were then prepared and the effect of fasting on transport or upon the metabolism of C^{14} - palmitic acid determined. Fasting significantly decreased the accumulation of PAH into tubules (Figure 13). Fasting did not affect the incorporation of palmitate into tissue total lipid (Figure 13). While fasting did not significantly alter incorporation of palmitate into tissue triglyceride, tissue NEFA, or tissue phospholipid, incorporation of label into triglyceride and phospholipid tended to be decreased, and the incorporation of label tended to be increased in tissue NEFA (Figure 14).

Effect of nicotinic acid on accumulation of PAH and lipid metabolism in the fasted rabbit

Adult rabbits were fasted for 48 hours before use. Twenty-four hours before use 250 mg/kg nicotinic acid or saline was injected intraperitoneally. The ability of tubule suspensions to accumulate PAH and to metabolize palmitate was then determined. Nicotinic acid did not

Figure 13. Effect of fasting on accumulation of PAH into tubules and the incorporation of labeled palmitate into tissue total lipid. Adult rabbits were fasted for 48 hours and the ability of tubules to metabolize palmitate and accumulate PAH were determined. Values are means ± S.E. *Significantly different from control ($p<0.05$).

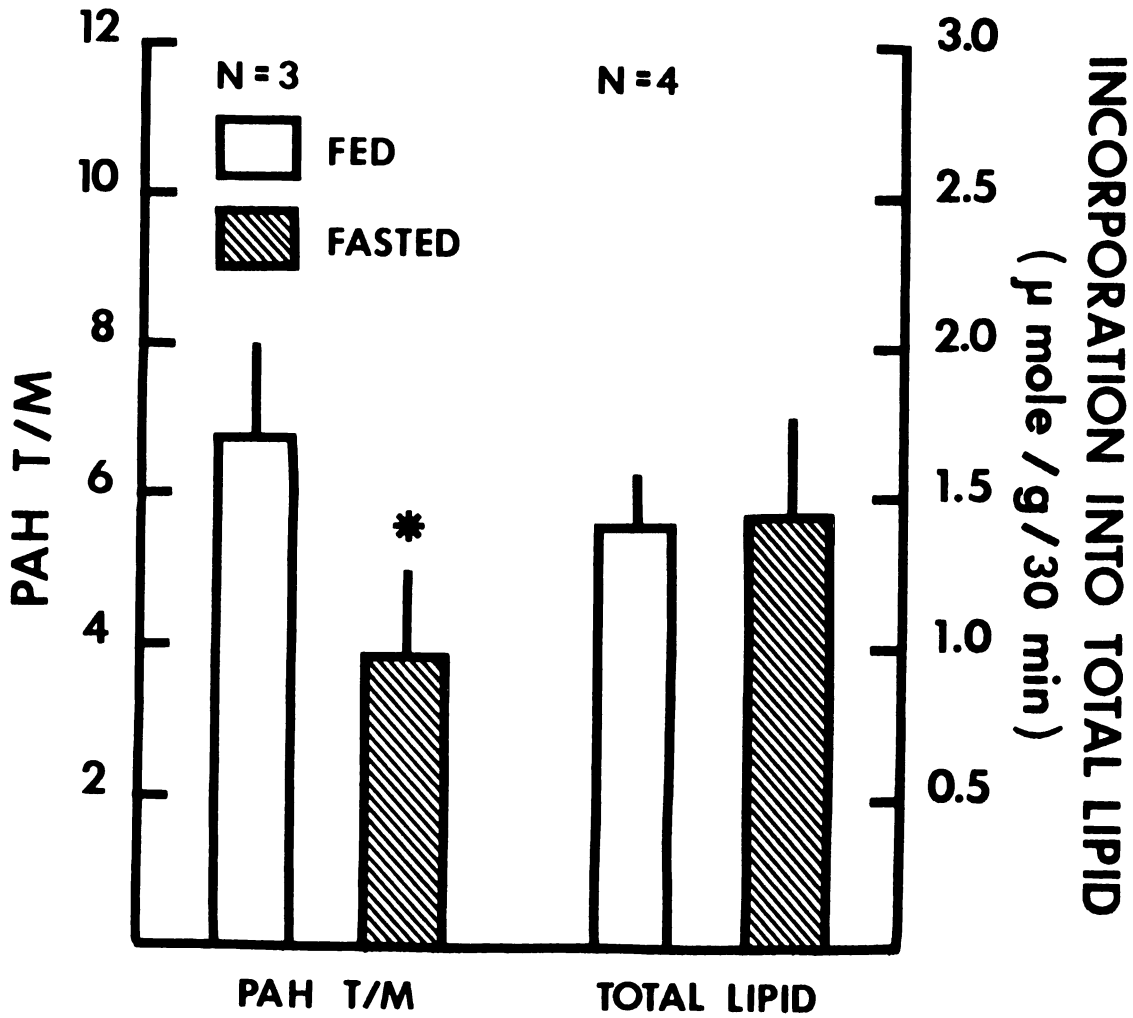
12
10
8
6
4
2
PAH T/M
N = 3
FED
FASTED
N = 4
*
3.0
2.5
2.0
1.5
1.0
0.5
INCORPORATION INTO TOTAL LIPID
(μ mole / g / 30 min)
PAH T/M
TOTAL LIPID

Figure 13

Figure 14. Effect of fasting on the distribution of labeled palmitate into tissue lipids. Adult rabbits were fasted 48 hours and tubule suspensions prepared. Tubules were incubated in a medium containing C^{14} palmitate and the distribution of label in various lipid classes determined relative to incorporation of label into tissue lipid. Values are means ± S.E.

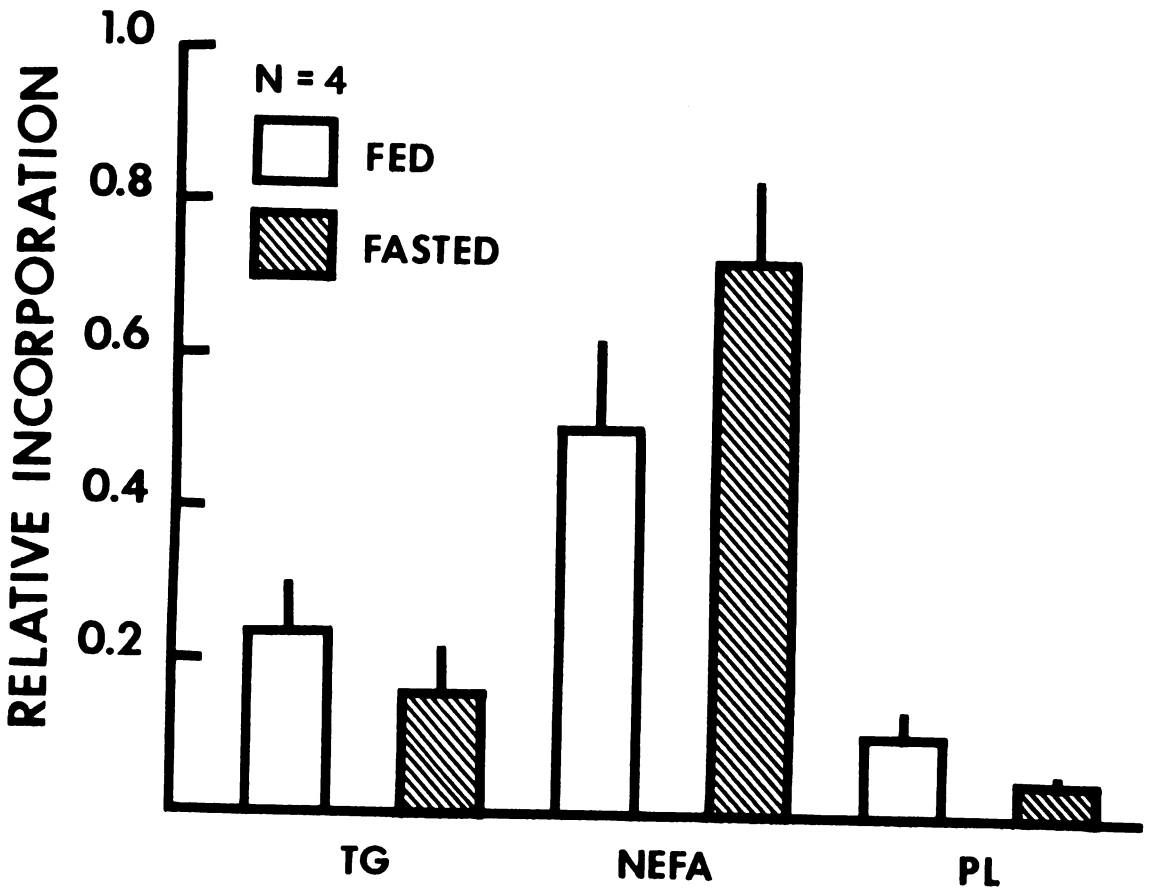
RELATIVE INCORPORATION
1.0
0.8
0.6
0.4
0.2
N = 4
FED
FASTED
TG
NEFA
PL

Figure 14

affect PAH transport capacity nor the incorporation of label into tissue total lipid (Figure 15). Nicotinic acid did, however, significantly alter the renal utilization of C^{14} - palmitate. Nicotinic acid treatment increased incorporation of labeled palmitate into triglyceride about 60% compared to control from a relative incorporation of 0.144 ± 0.034 to a relative incorporation of 0.241 ± 0.040 (Figure 16). Nicotinic acid also decreased incorporation into tissue NEFA about 20% from a relative incorporation of 0.752 ± 0.026 to a relative incorporation of 0.599 ± 0.066 (Figure 16).

Effect of probenecid, PAH and nicotinic acid in vitro on the metabolism of palmitic acid

Incubation of tubules in the presence of 2 mM probenecid, 2 mM PAH or 2 mM nicotinic acid did not affect incorporation of label into tissue total lipid (Figure 17; Figure 20). In fed rabbits, probenecid and PAH significantly decreased the relative incorporation of label into tissue triglyceride (25 and 20% respectively compared to control) (Figure 18).

In fasted rabbits, PAH and nicotinic acid decreased the relative incorporation of labeled palmitic acid into

Figure 15. Effect of nicotinic acid on renal accumulation of PAH and incorporation of label into tissue total lipid in the fasted rabbit. Rabbits were fasted 48 hours before use. Rabbits were treated with nicotinic acid (250 mg/kg) or saline 24 hours before use. Values are means ± S.E.

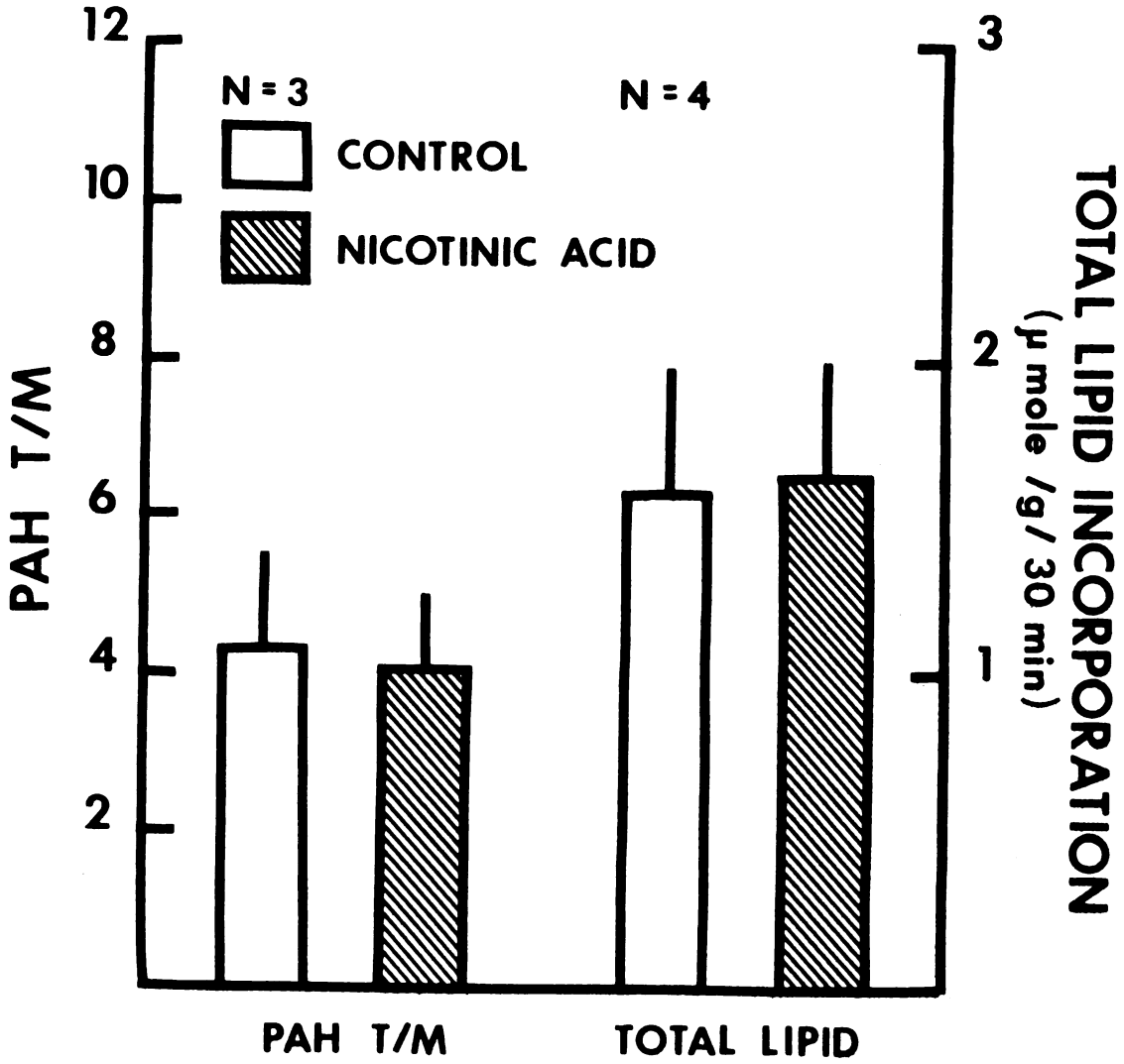
12
10
8
6
4
2
PAH T/M
N = 3
N = 4
CONTROL
NICOTINIC ACID
3
2
1
TOTAL LIPID INCORPORATION
(μ mole /g/ 30 min)
PAH T/M
TOTAL LIPID

Figure 15

Figure 16. Effect of nicotinic acid on the distribution of labeled palmitate into tissue lipid. Adult rabbits were fasted 48 hours before use. Nicotinic acid (250 mg/kg) or saline was injected intraperitoneally 24 hours before use. Incorporation of label into lipid classes by tubule suspensions was determined relative to the incorporation of label into total lipid. Values are means ± S.E. *Significantly different from control ($p<0.05$).

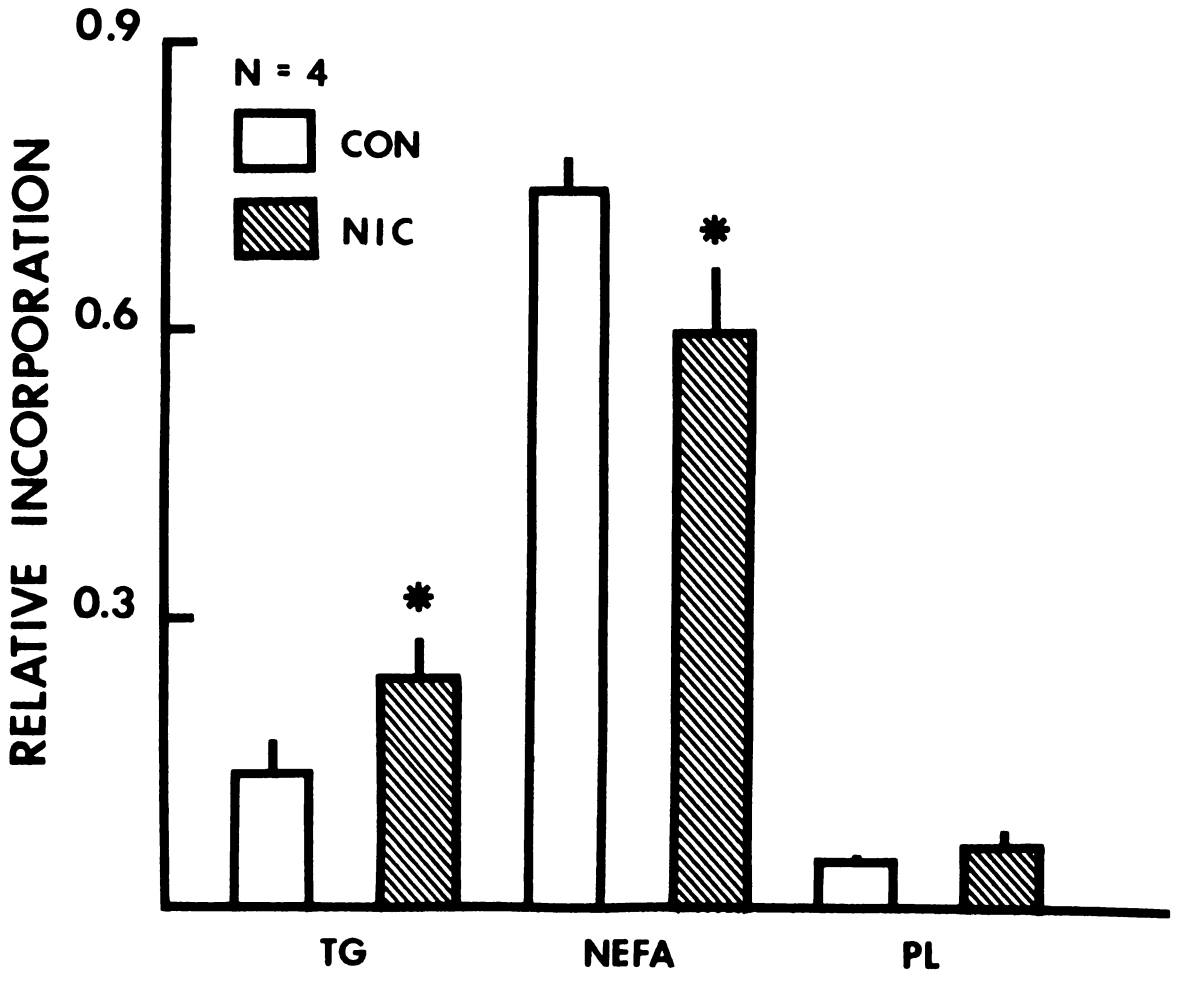

Figure 16

Figure 17. *In vitro* effect of probenecid or PAH on incorporation of label into tissue total lipid. Tubules were prepared from fed rabbits and incubated in a medium containing 1 mM C^{14} - palmitate and either in the presence of, or the absence of 2 mM probenecid or 2 mM PAH. Values are means ± S.E. of 7 or 9 determinations.

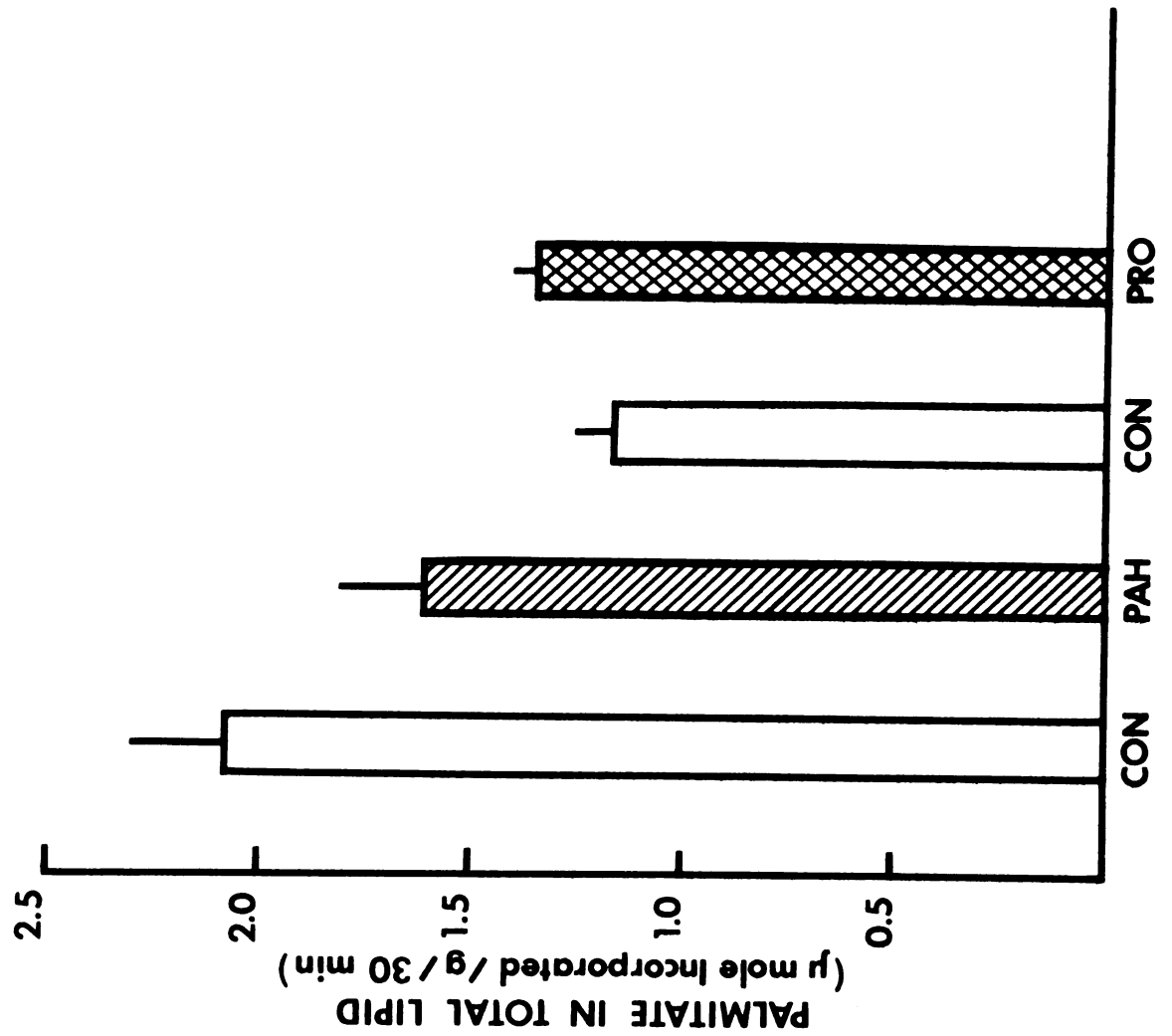
PALMITATE IN TOTAL LIPID
(μ mole incorporated /g / 30 min)
2.5
2.0
1.5
1.0
0.5
CON
PAH
CON
PRO

Figure 17

Figure 18. *In vitro* effect of probenecid or PAH on incorporation of label into tissue triglyceride. Tubule suspensions were prepared from fed rabbits and incubated in a medium containing 1 mM C^{14} - palmitic acid and in the presence or absence of added organic anion. The organic anions probenecid or PAH were added to a concentration of 2 mM. Values are means ± S.E. of 7 or 9 determinations. Values are expressed as incorporation of label into triglyceride relative to incorporation of label into total lipid. *Significantly different from control ($p<0.05$).

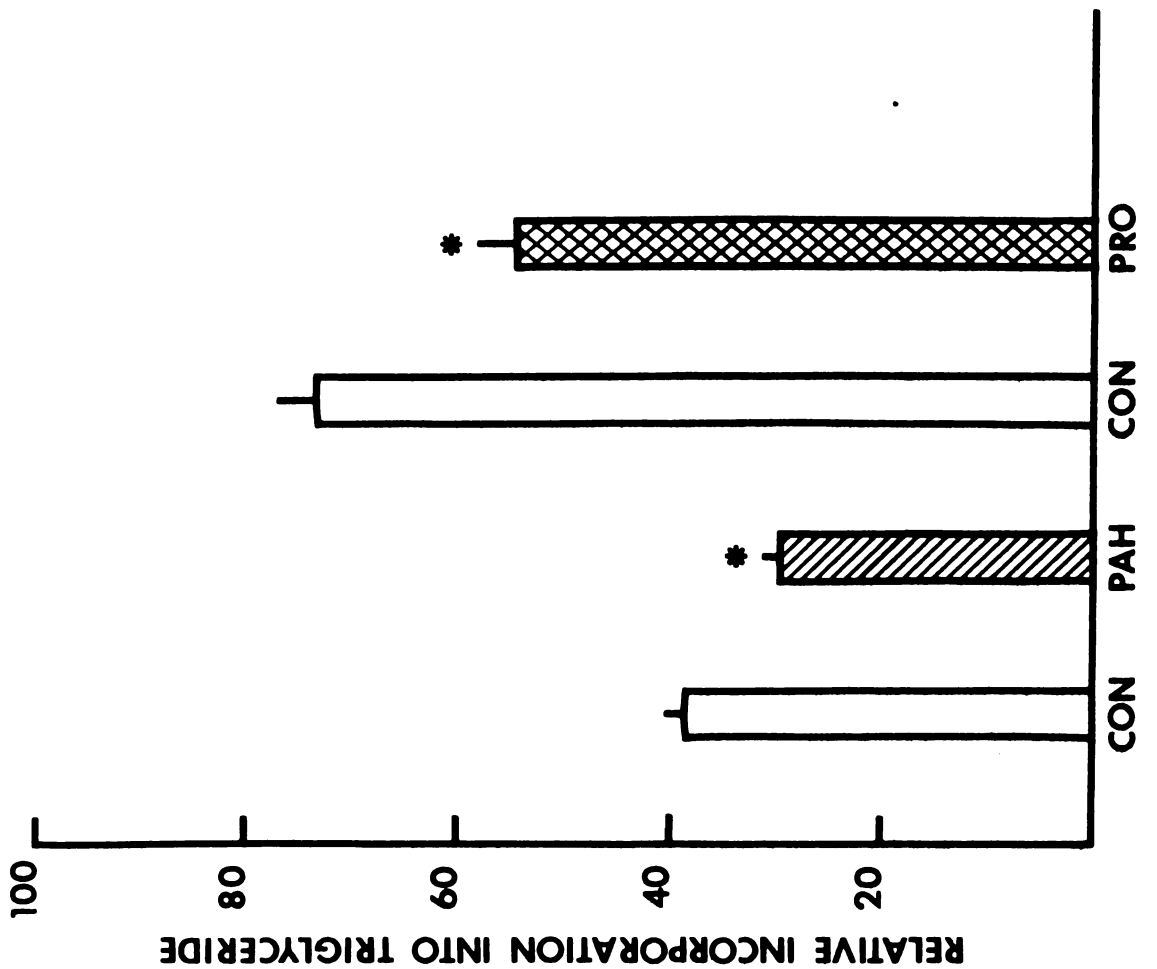
RELATIVE INCORPORATION INTO TRIGLYCERIDE
100
80
60
40
20
CON
PAH
CON
PRO
*
*

Figure 18

triglyceride, (27 and 39% respectively compared to control) (Figure 21).

PAH and probenecid tended to increase incorporation of label into tissue NEFA in fed rabbits (Figure 19). Similarly, PAH and nicotinic acid slightly, but significantly, enhanced incorporation of label into tissue NEFA (Figure 21).

Effect of penicillin treatment and organic anions on diacyl glycerol acyl transferase (DAGAT)

Two week old litters were pretreated with 90,000 I.U. of procaine penicillin G 24 and 36 hours prior to preparation of hepatic and renal microsomes and a 100,000 x g supernate. Supernates prepared from control and penicillin treated rabbit kidneys were added to a standard amount of hepatic microsomes and the synthesis of triglyceride from diolein and palmitoyl coenzyme A determined. Supernate dependent synthesis of triglyceride from penicillin treated rabbits was not significantly different from synthesis supported by supernate from control rabbits (Figure 22). Similarly, microsomes from control rabbit kidneys were not different from microsomes from penicillin treated rabbits in their capacity to support triglyceride formation (Figure 22).

Finally, the addition of 2 mM PAH or 2 mM nicotinic acid did not affect the synthesis of triglyceride by DAGAT (Figure 22).

Figure 19. *In vitro* effect of probenecid or PAH on incorporation of label into tissue NEFA. Tubule suspensions were prepared from fed rabbits and incubated in a medium containing 1 mM C^{14} - palmitic acid and in the presence or absence of added organic anion. The organic anions probenecid or PAH when added were added to yield a 2 mM concentration. Values are means ± S.E. of 7 or 9 determinations. Values are expressed as incorporation of label into NEFA relative to incorporation of label into tissue total lipid.

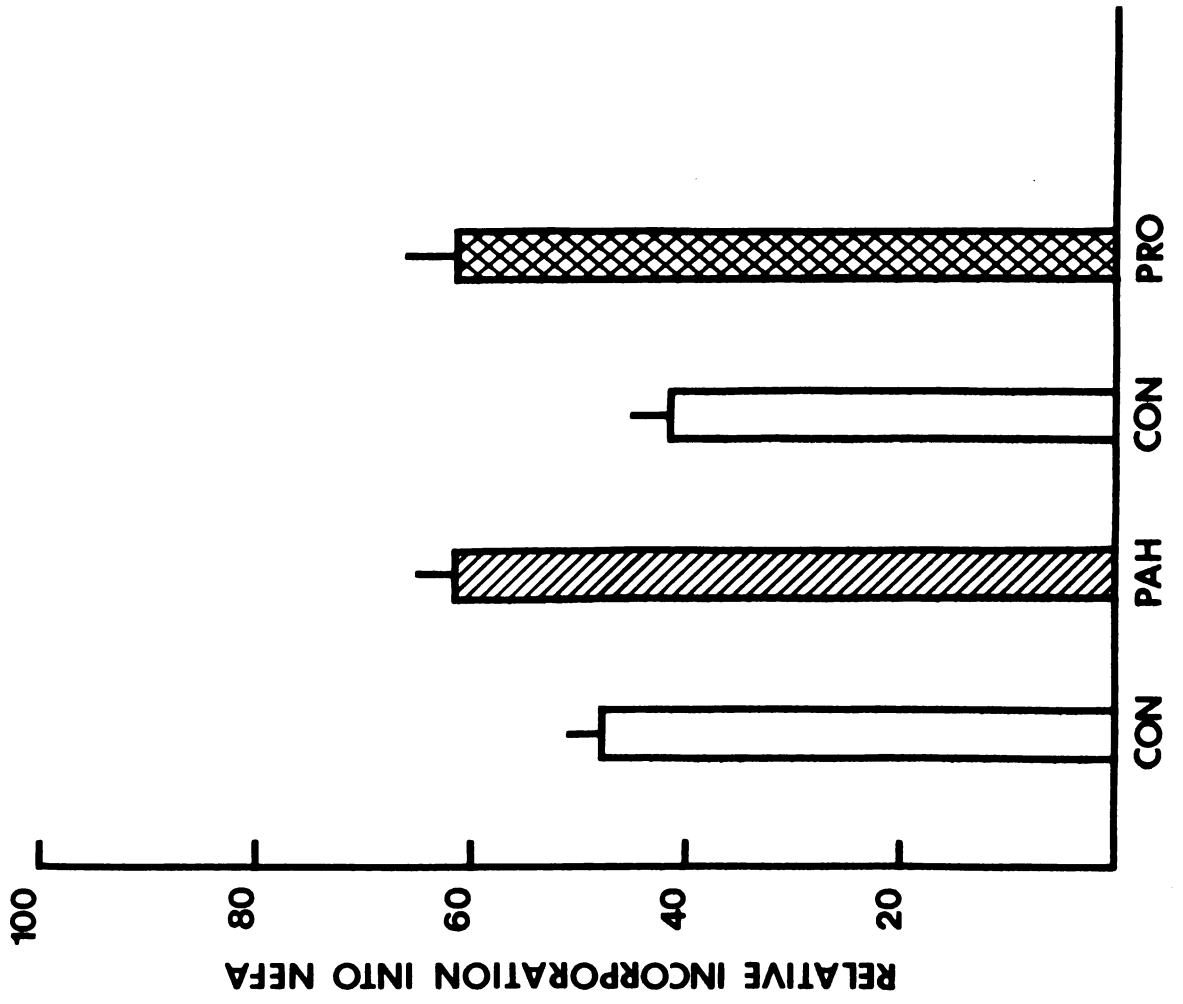
RELATIVE INCORPORATION INTO NEFA
100
80
60
40
20
CON
PAH
CON
PRO

Figure 19

Figure 20. In vitro effect of 2 mM nicotinic acid or 2 mM PAH on incorporation of label into tissue total lipid. Tubule suspensions were prepared from fasted rabbits (48 hour fast) and incubated in a medium containing 1 mM C^{14} - palmitic acid and in the presence or absence of added organic anion. Values are means ± S.E.

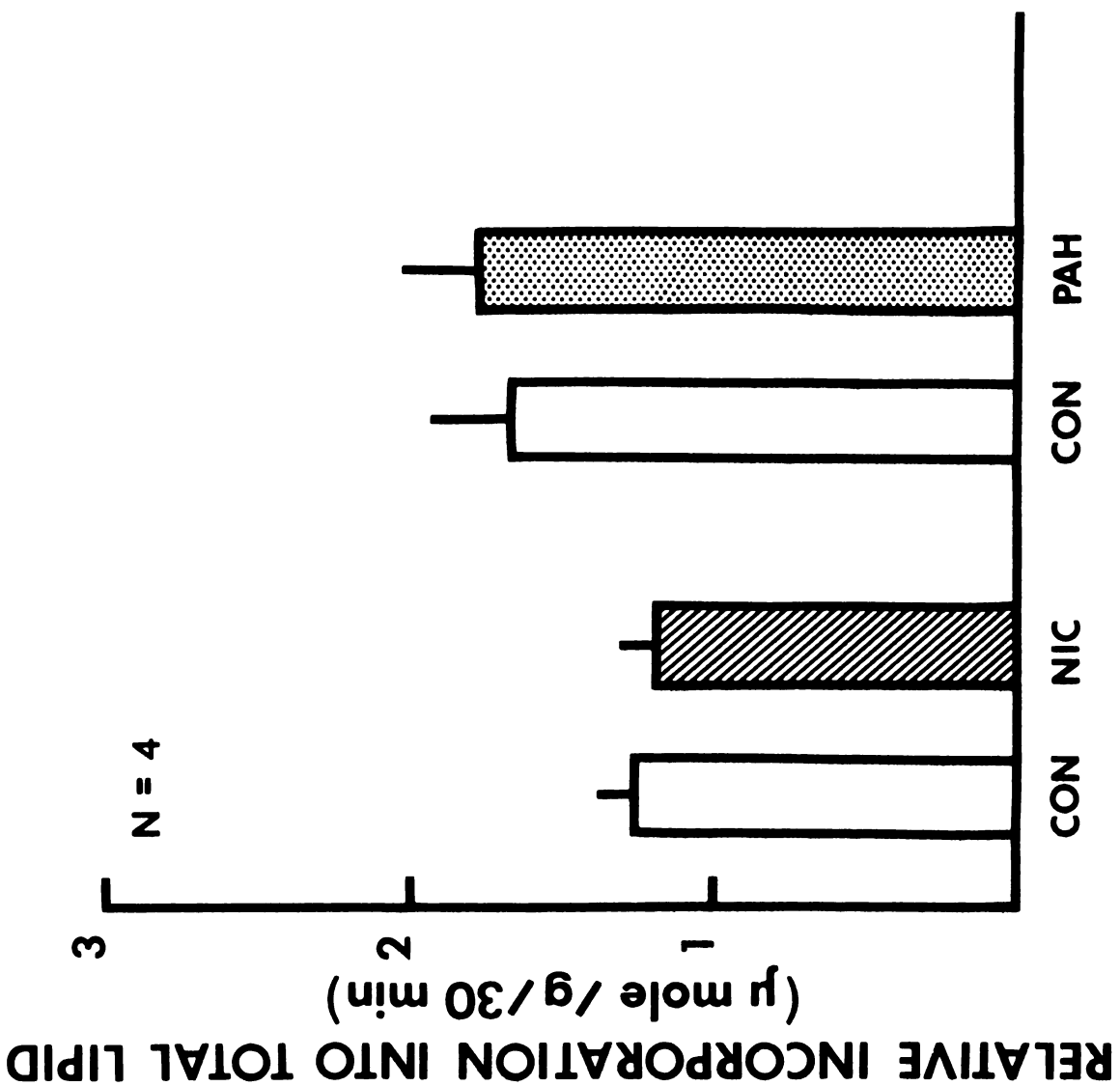
RELATIVE INCORPORATION INTO TOTAL LIPID
(μ mole /g/30 min)
3
2
1
N = 4
CON
NIC
CON
PAH

Figure 20

Figure 21. In vitro effect of 2 mM nicotinic acid or 2 mM PAH on incorporation of label into various lipid classes. Tubule suspensions were prepared from fasted (48 hour fast) rabbits and were incubated in a medium containing 1 mM C^{14} palmitic acid. Tubules were incubated in the presence or absence of the organic anions probenecid or PAH. Values are expressed as a % of control and are means ± S.E. *Significantly different from control ($p<0.05$).

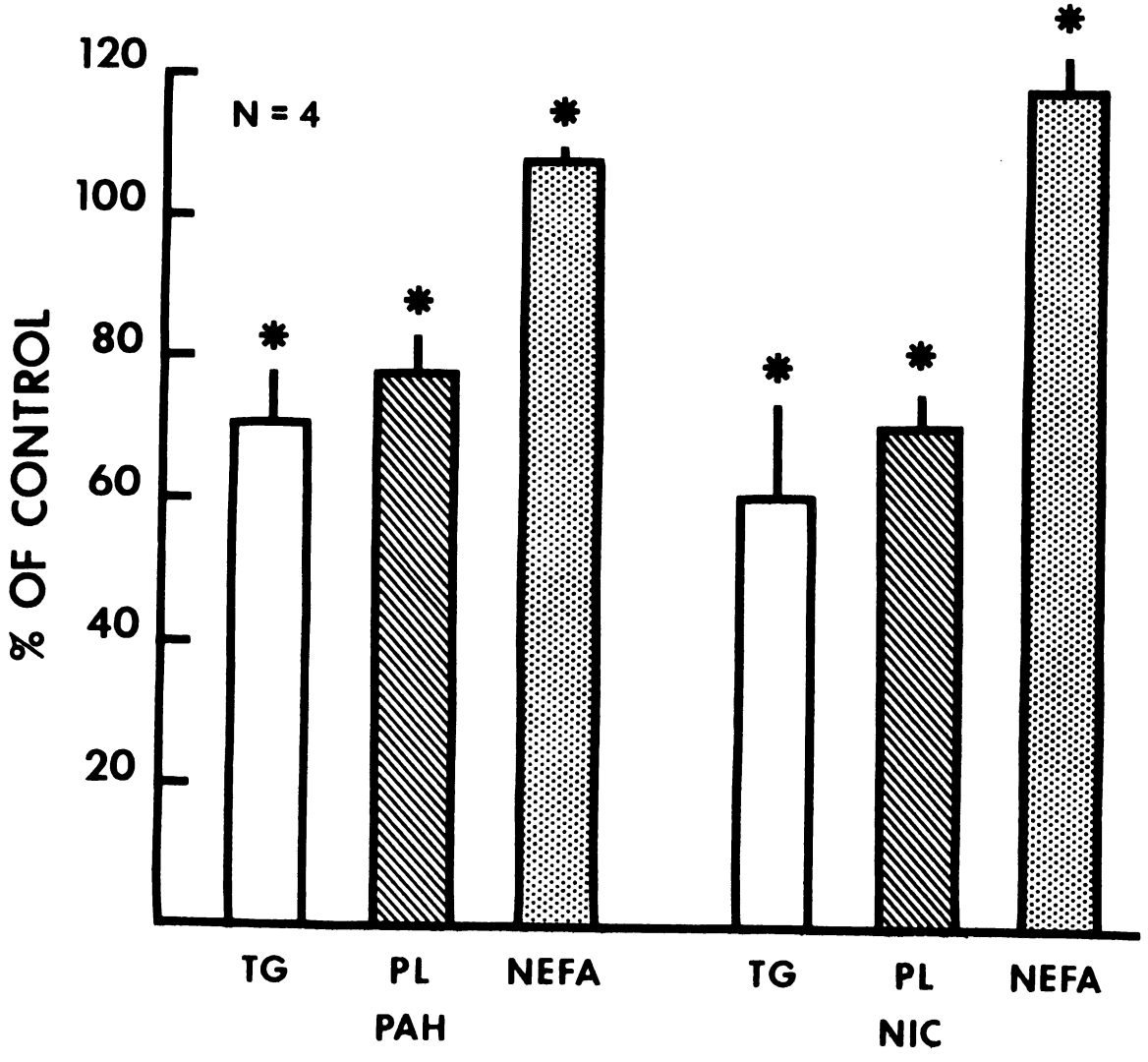

Figure 21

Figure 22, Effect of penicillin treatment and added organic anions on the activity of DAGAT. Microsomes (pell) and 100,000 x g supernate (super) were prepared from kidney and liver tissue of penicillin treated rabbit neonates (2 weeks old). Penicillin (90,000I.U.) was administered 24 and 36 hours prior to preparation of the microsomes and 100,000 g supernate. Nicotinic acid (2 mM) or PAH (2 mM) was added to a complete reaction mixture (supernate + microsomes) and compared to values for the corresponding mixture lacking organic anion. Supernate from kidneys was incubated with a standard hepatic microsome preparation. Similarly, microsomes from rabbit kidneys was incubated with a standard hepatic supernate preparation. Values are means ± S.E. and are expressed as a % of control.

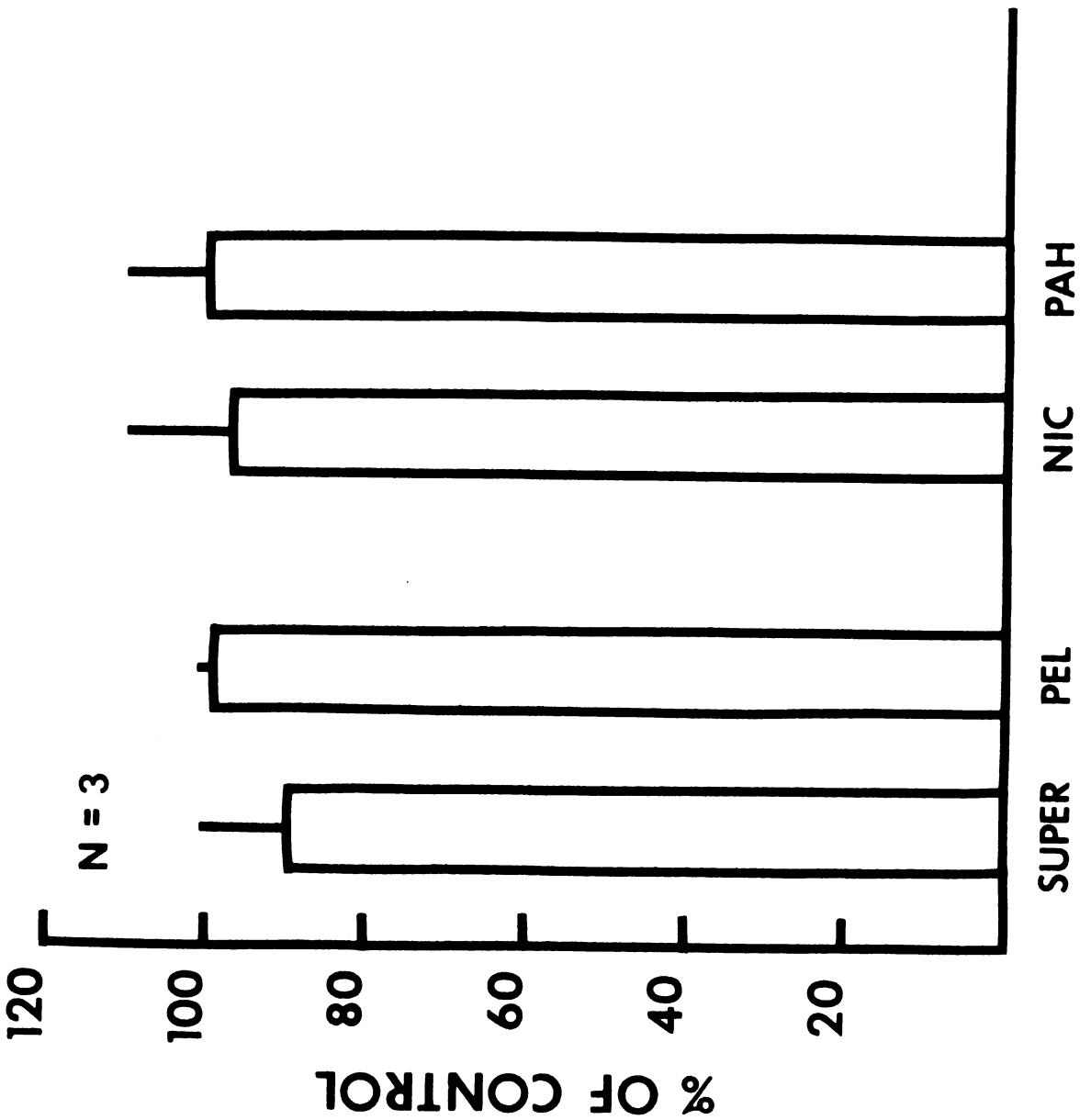

Figure 22

DISCUSSION

The hypothesis that penicillin stimulation of organic anion transport in the immature rabbit is due to disinhibition of the transport system by removal of NEFA is based on the hypothesis that NEFA interact competitively with the organic anion transport system (Hewitt and Hook, 1978). Analysis of the relationship between the renal organic anion transport system and NEFA requires the resolution of three separate, but related questions: First, are NEFA transported by the renal organic anion transport system? Second, can NEFA act as endogenous inhibitors of the renal organic anion transport system? Third, is the altered renal metabolism of palmitate produced by penicillin treatment of the neonate a specific manifestation of altered transport? The results of this study support the conclusion that NEFA are not transported by the organic anion transport system and do not, in physiological concentrations, act as inhibitors of the transport system. The data demonstrate that altered metabolism of palmitate is not specifically related to transport. Consequently, penicillin apparently

does not act to increase transport in the neonate by decreasing NEFA-mediated inhibition.

Renal fatty acid utilization and organic anion transport

The kidney readily utilizes NEFA to support renal work *in vivo* and *in vitro* (Cohen and Barac-Nieto, 1973). Since NEFA are extensively bound to plasma proteins *in vivo* (Goodman, 1958; Goodman, 1961), Cohen and Kamm (1976) concluded that the principle pathway for the uptake of fatty acids into renal cells is across the peritubular membrane. However, the mechanism by which NEFA enter the renal cell remains obscure.

The uptake of fatty acids into a variety of tissues has been characterized as a two step process, since there appear to be two cellular pools of NEFA (Spector et al., 1965). A pool which is readily accessible to extracellular fluid is indicated since tubules which are washed with an albumin containing medium readily releases 45 to 75% of the cellular NEFA (Spector, 1968; Spector et al., 1965). Uptake into and release from this pool occurs at low temperature and in the presence of metabolic inhibitors (Spector, 1971), indicating that flux into this pool is essentially passive.

Human erythrocytes appear to contain a lipoprotein binding site for NEFA (Spector, 1971; Goodman, 1958). Binding is readily reversible and not dependent on cellular metabolism, which suggests that the readily reversible pool corresponds to those fatty acids bound to membrane sites (Spector et al., 1965; Weismann and Korn, 1966).

A second tissue pool of NEFA is indicated since 25 to 55% of tissue NEFA is not readily removed by washing (Spector, 1968; Spector et al., 1965). Entry into this pool can occur passively (Spector, et al., 1965). However, it appears that entry into the second cellular pool is also in part enzymatically mediated and energy dependent (Shohet et al., 1968).

Spector (1971) reviewed three possible enzymatically mediated mechanisms for the penetration of fatty acids into the cell. Each of the mechanisms shared an initial activation step in which the fatty acid is esterified with coenzyme A (CO A) by an ATP dependent thiokinase. In the first mechanism the fatty acyl CO A diffuses into the cellular water after being formed. Fatty acyl CO A, however, cannot penetrate mitochondrial membranes. Penetration into the mitochondrial matrix is mediated via the synthesis of a fatty acyl carnitine, from fatty acyl CO A, which can penetrate

the mitochondrial membrane (Fritz and Yue, 1968). The addition of carnitine thus increases mitochondrial utilization of NEFA.

The third mechanism has been postulated to involve the cyclic acylation of 1-acyl glycerol-3-phorphoryl choline to form a phosphatidyl choline. Intracellular NEFA can then be formed by deacylation of the phosphatidyl choline to regenerate 1-acyl glycerol-3-phosphoryl choline.

The demonstration that probenecid, a classic inhibitor of renal organic anion transport, significantly decreased renal utilization of NEFA suggests that in the kidney, fatty acids may penetrate into the renal cell via the renal organic anion transport system (Barac-Nieto and Cohen, 1968). Several observations support this suggestion. First, palmitate can inhibit renal organic anion transport in an apparently competitive manner (Barac-Nieto, 1971). Second, the dog kidney preferentially extracts palmitic acid from the blood (Geyer et al., 1949) and third, NEFA exists primarily as organic anions at physiological pH (Goodman, 1958b). In the context of the previous discussion of the mechanism of NEFA penetration into the cell, it is interesting to note that carnitine not only increases mitochondrial use of NEFA, it also stimulates transport of PAH into renal

cortical slices (Barac-Nieto, 1971). Furthermore, Holohan et al. (1976) have reported a dependency of the renal organic anion transport system for phosphatidyl choline.

Hewitt and Hook (1978) attempted to clarify the relationship between renal organic anion transport and the renal uptake of NEFA by using the phenomenon of enhanced PAH transport produced by penicillin pretreatment of the immature rabbit. Penicillin treatment of the two week old rabbit enhances the renal capacity for the transport of the organic anions PAH, penicillin, phenosulfonphthalein and cephaloridine (Hewitt et al., 1977; Wold et al., 1977). These results suggest that if NEFA are transported into the cell by the same mechanism which transports PAH, then penicillin treatment should enhance NEFA utilization. Penicillin treatment, however, failed to significantly increase incorporation of palmitate into tissue total lipid or to increase renal oxidation of palmitate. These results suggest that palmitate is not taken up into renal tissue via the renal organic anion transport system.

However, careful examination of their (Hewitt and Hook, 1978) data indicates that while neither oxidation nor incorporation into total lipid was significantly increased, both tended to increase in conjunction with

penicillin, enhanced transport of PAH. Since metabolism and not actual transport of palmitate was evaluated, the possibility should be considered that the failure to observe increased oxidation or incorporation into tissue lipid may be due to factors apart from the primary interaction with the transport system. Three possibilities exist. First, the measurement of the several fates of the labeled palmitate introduces a very significant source of variance. This suggests that if total utilization were determined by summing the various fates, overall NEFA utilization may have been enhanced without a significant increase in the various individual metabolic fates. Second, since oxidation of fatty acids is a saturable process (Spector and Steinberg, 1965; Spector and Steinberg, 1967; Barac-Nieto, 1976) the failure to observe increased utilization of NEFA may be due to saturation of the metabolic pathways by excess substrate in the incubation medium. Third, the failure of several investigators (Maxild, 1971; Kippen and Klinenberg, 1971) to observe palmitate inhibition of organic anion transport, an observation which is critical to the concept of an endogenous inhibition of organic anion transport, suggests the inhibitory effects of palmitate on the organic anion transport system should be confirmed in this model.

Incubation of tubule suspensions in the presence of 1mM palmitate significantly decreased the capacity of the tissue to accumulate PAH (Figure 1). This result confirms the inhibitory character of palmitate and demonstrates that incubation conditions were appropriate for demonstrating accumulation of NEFA by the organic anion transport system.

The failure to observe increased utilization does not however, appear to be due to the multiple metabolic fates of NEFA. Consistent with the results reported by Hewitt and Hook (1978), penicillin treatment failed to enhance the incorporation of labeled palmitate into tissue total lipid despite a significant increase in the capacity to accumulate PAH (Table 1; Table 3). Furthermore, in a separate series of experiments, penicillin treatment enhanced the accumulation of PAH but did not affect the extraction of labeled palmitate from the medium (Figure 2).

Finally, the failure to observe increased utilization of NEFA related to the increased transport does not appear to be due to saturation of the metabolic pathways. If at the palmitate concentration used in the incubation medium, the metabolic pathways had been saturated, then increased uptake of NEFA should be observed as an increase

in tissue NEFA. Penicillin treatment did not, however, appear to increase renal NEFA content.

Incubation of tissue in the presence of labeled fatty acids leads to the rapid accumulation of fatty acid and achievement of maximum values within a matter of minutes (Spector, 1971). Content of label present as NEFA remains constant during prolonged incubation (Spector, 1971). This result suggests that an evaluation of the uptake of fatty acids into the cell can be estimated from the tissue content of labeled NEFA. If palmitate were being accumulated by the transport system then penicillin enhanced transport should increase the tissue content of labeled NEFA. However, penicillin treatment decreased incorporation of label into tissue NEFA (Figure 12; Table 3; Hewitt and Hook, 1978). Conversely, inhibition of transport with competitive inhibitors would be expected to decrease label incorporation into tissue NEFA if fatty acids were being transported into the cell by the anion transport system. However, the organic anions probenecid, PAH and nicotinic acid tend to increase label incorporation into tissue NEFA (Figure 19; Figure 21).

The failure to correlate tissue NEFA with altered transport has been confirmed by direct measurement of fatty acids in renal tissue. Incubation of tubules in the presence

of palmitate increased tissue content compared to endogenous levels (Figure 7; Figure 3; Figure 5). However, probenecid 2 mM or PAH 2 mM not only failed to decrease tissue NEFA but, instead, tended to increase the tissue NEFA (Figure 7). Furthermore, penicillin treatment failed to increase endogenous tissue NEFA at a time when transport was enhanced in vitro (Figure 3).

While the possibility exists that an organic anion transport system other than the one responsible for the secretion of PAH may be responsible for uptake of NEFA (Hewitt et al., 1978) the failure of probenecid to decrease NEFA content suggests that the alternate transport systems are not involved, since 2 mM probenecid would be expected to effectively inhibit all organic anion transport.

Thus, these results are inconsistent with the concept that the renal organic anion transport system functions in the renal uptake of NEFA. These results are thus consistent with the speculations of Pakarinen (1970) and Cohen and Kamm (1976) that any interactions which occur between NEFA and the organic anion transport system must occur at some point subsequent to the penetration of fatty acids into the cell.

NEFA as endogenous inhibitors of the renal organic anion transport system

While it is apparent that the renal organic anion transport system is not responsible for the penetration of NEFA into the cell, the possibility remains that endogenous NEFA could be acting as endogenous modulators of the renal organic anion transport system.

Extensive study of a variety of organic anions has demonstrated, in general, a positive correlation between the potency of a compound as an inhibitor of transport with its molecular weight, lipid solubility, accumulation in the renal cell and binding to plasma proteins (Weiner, 1973). Significantly, there is a negative correlation between potency as an inhibitor and rapidity of net secretion (Weiner, 1973). It is thus of interest that NEFA are extensively bound to plasma proteins (Goodman, 1958; Goodman, 1961). Similarly, fatty acids are bound to intracellular proteins (fatty acid binding protein; Z protein) (Kamisaka et al., 1975a, b; Listowsky et al., 1976). Furthermore, fatty acids appear to be more tightly bound to these proteins than do organic anions such as PAH (Kamisaka et al., 1975a; Listowsky et al., 1976). NEFA reach maximal concentrations in tissue very rapidly (Spector, 1971) and are soluble in

nonpolar organic solvents which suggests they are readily soluble in membranes. Furthermore, NEFA exist at cellular pH primarily as organic anions (Goodman, 1958b) and, as discussed in the previous section, are not extensively transported by the renal organic anion transport system even though they can inhibit the transport of PAH (Figure 1; Barac-Nieto, 1971). These observations suggest that NEFA could act as effective inhibitors of renal organic anion transport.

Several observations suggest physiological relevance of the concept that fatty acids act as inhibitors of renal organic anion transport. Fasting markedly increases plasma NEFA (Cahill et al., 1966; Goldrick and Hirsch 1964; Dahlquist et al., 1972) and significantly decreases the clearance of uric acid (Fox et al., 1976). Feeding rats a high fat diet resulted in rapid and specific inhibition of PAH transport (Johnson et al., 1973a, b). Finally, the neonatal animal is relatively hyperlipemic in comparison to the adult (Hardmann and Hull, 1969; Persson et al., 1966; Dahlquist et al., 1972), and also exhibits decreased capacity to transport organic anions (Rennick et al., 1961; Horster and Lewy, 1970; Lewy and Grosser, 1974).

Investigations of the renal metabolism of NEFA support the concept that NEFA act as endogenous inhibitors of transport. Hewitt and Hook (1978) observed that a penicillin treatment regimen which enhanced organic anion transport decreased the incorporation of labeled palmitate into tissue NEFA. As discussed above the decreased incorporation of label into NEFA presumably indicates a decrease in the "steady state" level of tissue NEFA since labeled palmitate achieved maximum, stable levels of radioactive NEFA within minutes (Spector, 1971). Hewitt and Hook (1978) also observed increased triglyceride synthesis from labeled palmitate which suggested to them that the increased triglyceride synthesis was decreasing cellular NEFA content and thus disinhibiting transport. Consistent with this speculation, Barac-Nieto (1971) observed that the addition of carnitine disinhibited the decrease in transport produced by added palmitate.

These results support the suggestion that penicillin-enhanced transport of PAH in the neonate, the development of transport with maturation and decreased transport of PAH with fasting are all due to alterations in NEFA-produced inhibition of transport. This hypothesis suggests that increased transport should be correlated with decreased tissue or serum

NEFA and decreased transport should be correlated with increased serum and tissue NEFA concentrations.

Consistent with this speculation and consistent with the results of others, the decreased transport of fasting was associated with elevated serum concentrations of NEFA (Figure 5; Figure 13). Furthermore, consistent with previous observations the increase in PAH transport capacity in adults (compared to newborns) was associated with decreased serum NEFA concentrations (Figure 3; Figure 5). However, penicillin-enhanced PAH transport in the neonate was associated not with a decrease in serum NEFA; but, rather, penicillin treatment increased serum NEFA (Figure 3). This result suggests that altered transport is not the result of alterations in serum NEFA.

Analysis of renal NEFA content further supports the suggestion that NEFA are not responsible for altered transport. While fasting decreased transport (Figure 13) renal NEFA content did not decrease (Figure 5). Furthermore, penicillin enhanced transport (Table 3) was not associated with decreased renal NEFA content (Figure 3). Increased transport capacity in the adult was associated with, if anything, higher NEFA concentrations (Figure 5) than those observed in the neonate (Figure 3). These results clearly

suggest that alterations in transport are not directly related to alterations in tissue NEFA.

The failure of the hypolipidemic agent, nicotinic acid, to alter fasting-induced decreased PAH transport further supports the contention that altered NEFA is not responsible for altered transport. Nicotinic acid reduced tissue and plasma concentrations of NEFA and triglyceride (Carlson 1967; Trout et al., 1967; Carlson et al., 1966). However, treatment with nicotinic acid according to a treatment regimen that could be expected to decrease tissue and serum NEFA failed to enhance renal capacity to accumulate PAH (Figure 8).

Finally, in the experiment reported here (Figure 1) and by Barac-Nieto (1971) in which palmitate decreased PAH transport, the tissue NEFA measured concommitantly was 10 times that found in the fasted adult (Figure 7; Figure 5). This result coupled with the failure of other investigators to observe an effect of palmitate on PAH transport at physiological palmitate concentrations (Maxild, 1971, Kippen and Klinenberg, 1978) suggests that inhibition of transport by palmitate occurs only at pharmacological concentrations of NEFA and not at levels expected under physiological conditions.

These results support the conclusion that NEFA do not act as physiologically effective endogenous inhibitors of organic anion transport.

Altered renal metabolism of NEFA and altered transport

The demonstration in the previous section that generalized alterations in NEFA content are not responsible for alterations in organic anion transport in the kidney leaves unresolved the relationship between altered transport in the neonate and altered metabolism. Conceivably, renal organic anion transport could be linked to lipid metabolism by a mechanism other than by a generalized reduction in NEFA. If, for example, renal metabolism was associated with a change in a small specific pool of tissue NEFA, then measurements of overall tissue NEFA might not detect a change in the NEFA content of the effective pool. However, the observation that the kidney readily oxidizes and esterifies fatty acids (Cohen and Barac-Nieto, 1973) and the observation that incubation of tissue in the presence of fatty acids yields stabile maximal levels in minutes (Spector, 1971) suggests that fatty acids rapidly equilibrate between pools. Thus, if metabolism were selectively disinhibiting the transport

system by removal of inhibiting NEFA then one would expect that altered transport and altered metabolism would be intimately and specifically integrated. Alternatively, if the enzymes of NEFA metabolism were actual components of the transport system, then altered metabolism and altered transport would also be expected to be tightly integrated.

Several observations suggest that fatty acid metabolism and organic anion transport in the kidney are integrated. Treatment of neonates with iodipamide or treatment of adults with penicillin failed to alter metabolism and failed to alter transport (Hewitt and Hook, 1978). However, the possibility must be considered that the failure to observe altered metabolism could have been due to inadequate dosage (see Introduction).

A relationship of altered metabolism with the organic anion transport system is confirmed by the failure to observe altered glomerular metabolism at a time when tubular metabolism and transport was altered (Figure 10; Figure 11; Figure 12; Table 3). The localization of the organic anion transport system to the proximal tubule is well known (Weiner, 1973); consequently the failure to observe altered glomerular metabolism supports the suggestion that altered metabolism is related to the transport system. However, the

possibility should be considered that tubules were affected and glomeruli were not, simply because the organic anion transport system concentrated penicillin in the tubular cells.

Finally, fasting did decrease organic anion transport and did tend to indicate that the increased transport capacity of the fed animal was associated with an increased triglyceride synthesis and decreased incorporation of label into tissue NEFA. This observation is consistent with the suggestion that in the penicillin treated neonate, increased transport capacity was associated with increased triglycerice synthesis and decreased incorporation into NEFA.

However, other observations suggest that altered metabolism and altered transport are not tightly integrated. In the penicillin treated neonate, lipid metabolism was affected before any change in transport was observed (Figure 12; Table 3). Similarly, nicotinic acid treatment of the fasted adult did not affect transport (Figure 15) but did enhance incorporation of labeled palmitate into triglyceride and decreased incorporation of label into tissue NEFA (Figure 16). Clearly, the failure to observe altered transport at a time when metabolism was altered demonstrates that altered metabolism and altered transport were not tightly

integrated. This observation suggests that altered metabolism does not cause altered transport.

The lack of correlation between transport and metabolism suggests that penicillin treatment of neonates alters lipid metabolism by a mechanism unrelated to its effects on organic anion transport.

Penicillin - hypolipidemic drug?

Penicillin treatment of neonates clearly affects lipid metabolism *in vivo* since treatment significantly decreased renal triglyceride (Figure 4) and produced a significant increase in serum NEFA (Figure 3). A number of organic anions are effective hypolipidemic agents. Analysis of a number of nicotinic acid derivatives indicates that the effective chemical is nicotinic acid (Bailey et al., 1972). Similarly, clofibrate is rapidly metabolized to an organic anion which may be the effective agent (Thorp and Waring, 1962). These observations suggest that penicillin may act in a manner similar to that of other hypolipidemic agents to produce altered lipid metabolism.

Several observations support the suggestion that penicillin and hypolipidemic agent, nicotinic acid, share a

similar mechanism of action on renal lipid metabolism. Nicotinic acid treatment decreases the triglyceride content of several tissues including kidney (Carlson et al., 1966; Trout et al., 1967). Furthermore, subsequent to nicotinic acid treatment plasma NEFA are first decreased. Following excretion of the drug, plasma NEFA increased (Pereira, 1967). Similarly, penicillin treatment decreases renal triglyceride (Figure 4) and 24 hours after the last dose increased serum NEFA (Figure 3). Nicotinic acid treatment decreased triglyceride synthesis in the liver (Solyom and Puglisi, 1967) and similarly decreased triglyceride synthesis in the kidney (Figure 21) an action which it shared with probenecid and PAH (Figure 18). Furthermore, probenecid, PAH and nicotinic acid all tend to increase the incorporation of labeled palmitate into tissue NEFA (Figure 19; Figure 21). These results suggest a common mechanism of action.

The mechanism by which nicotinic acid alters lipid metabolism remains obscure (Howe, 1973; Kudchodkar et al., 1978; Vek-Mo and Mjøs, 1978). Several possibilities exist: lipid metabolism might be altered by an interaction with the triglyceride synthesizing enzymes. The <u>in vitro</u> effects of probenecid, PAH and nicotinic acid (Figure 18; Figure 19; Figure 21) are similar to those observed with

α-bromopalmitate in the small intestine (Ockner and Manning, 1976) and in the kidney (Trimble et al., 1977). Ockner and Manning (1976) suggested that the effects of α-bromopalmitate to decrease triglyceride synthesis were mediated by its binding to Z protein. Triglyceride synthesis via diacyl glycerol acyl transferase is dependent on Z protein (O'Doherty and Kuksis, 1978); consequently, the effect of penicillin pretreatment on this reaction and the in vitro effect of nicotinic acid and PAH were determined. Penicillin pretreatment failed to alter either DAGAT activity or the stimulatory effectiveness of 100,000 x g supernate, indicating that neither Z protein nor enzyme activity were altered (Figure 22). Nicotinic acid and PAH similarly failed to alter triglyceride synthesis (Figure 22) indicating that this metabolic step was not the site of action. Consistent with this observation, Brindly (1977) demonstrated that altered DAGAT activity correlates poorly with altered triglyceride synthesis in the liver. The failure to observe an effect directly on DAGAT does not, however, rule out an action on other enzymes responsible for lipid synthesis. These results thus leave unresolved the mechanism by which penicillin alters lipid metabolism, but the results do suggest an action which is similar to that of nicotinic acid.

Conclusions

The data support four conclusions. First, NEFA do not penetrate into the renal proximal tubule cell via the renal organic anion transport system. Second, NEFA do not act as endogenous inhibitors of the transport of PAH at physiological levels. Third, altered lipid metabolism observed after penicillin treatment of neonatal animals is not specifically linked to altered transport, and may be acting in a manner similar to the hypolipidemic agent, nicotinic acid. Fourth, alterations in lipid metabolism do not appear to be due to an action on DAGAT.

These conclusions are not consistent with the hypothesis that penicillin enhanced transport in the neonate is the result of disinhibition of transport by the metabolism of inhibiting NEFA.

SPECULATION

The mechanism by which penicillin treatment enhances transport of PAH in the immature rabbit kidney remains obscure. While it is clear that NEFA do not act as endogenous inhibitors of transport, the possibility remains that other endogenous organic anions may be acting as inhibitors. Thus, penicillin enhanced transport may be, in fact, a process of disinhibition of latent "transporters". Alternatively, the involvement of (protein) synthesis of new transporters cannot be ruled out. It is not surprising that increased labeling of a single protein can not be observed against the background of synthesis of the myriad of other cellular proteins. The failure of approaches taken to elucidate the mechanism of penicillin enhanced transport suggests that a fresh approach will be required to elucidate these effects.

The recent demonstration of increased transport capacity in isolated hepalocytes produced by a number of enzyme inducers, (Eaton and Klaasen, 1978) suggests that such an approach might be useful with kidney tissue. The

capacity to induce transport *in vitro* would allow for a more precise analysis of the role of protein synthesis in the mechanism of penicillin induced transport.

Alternatively the demonstration of Hong et al. (1978) of an interaction of SITS with the anion transport system suggests that the membrane transporter may be identifiable as was the anion transporter in the red blood cell membrane (Rothstein et al., 1976). Thus, while the mechanism remains obscure, fresh approaches promise to broaden our understanding of the biochemical mechanisms of organic anion transport.

SUMMARY

The purpose of this investigation was to evaluate the hypothesis that altered transport of organic anions in the neonatal kidney observed after penicillin treatment, was related to altered renal metabolism of NEFA. Hewitt and Hook (1978) observed that penicillin treatment decreased incorporation of labelled palmitate into tissue NEFA *in vitro*. They suggested that NEFA acted as endogenous inhibitors of transport of organic anions and that altered transport in the neonate was due to a disinhibition of transport by the removal of inhibitory NEFA. The evaluation of this hypothesis requires the resolution of three basic questions: First, are NEFA transported into the renal cell via the renal organic anion transport system? Second, do NEFA act as endogenous inhibitors of the organic anion transport system? Third, is the altered renal metabolism of NEFA observed after penicillin treatment integrally related to altered transport?

Penicillin treatment failed to increase the incorporation of labelled palmitate into tissue total lipid *in*

vitro, failed to increase the extraction of labelled palmitate from the medium *in vitro* and also failed to increase the endogenous renal content of NEFA. Furthermore, inhibitors of the organic anion transport system failed to decrease the renal content of NEFA *in vitro*. These results are not consistent with the suggestion that NEFA enter the renal cell via the organic anion transport system.

Penicillin treatment of the neonatal rabbit did not decrease renal NEFA content and in fact increased serum levels of NEFA. This result suggests that altered serum levels of NEFA are not responsible for altered transport of organic anions *in vitro* and that the increased transport of organic anions observed in the kidney is not related to a decrease in inhibition produced by tissue NEFA. This suggests that NEFA do not act as endogenous inhibitors of the organic anion transport system. Several observations support this suggestion. Fasting decreased transport *in vitro* and increased serum NEFA but did not increase tissue NEFA content. Furthermore, while transport increased with age and serum levels of NEFA tended to decrease, increased transport with aging was associated with, if anything, an increase in renal NEFA. Finally, NEFA decreased transport

in vitro was associated with tissue levels of NEFA 10 fold greater than the endogenous content of NEFA observed in the adult or neonate. These results are consistent with the conclusions that NEFA do not act as endogenous inhibitors of the transport system.

Penicillin treatment altered tubular transport of organic anions and altered tubular metabolism of NEFA but did not affect metabolism of NEFA in glomeruli. Since glomeruli do not transport organic anions, this observation indicates that the expression of altered metabolism was dependent on the organic anion transport system. However, altered metabolism preceded altered transport in the penicillin treated neonate. Furthermore, an identical pattern of altered lipid metabolism was observed in the adult rabbit after treatment with nicotinic acid, but transport of organic anions by the kidney was not altered. These results indicate that altered lipid metabolism is not integrally linked to altered transport.

These results are consistent with the conclusion that NEFA do not enter the renal cell via the renal organic anion transport system, that NEFA are not effective at physiological concentrations as inhibitors of the renal organic anion transport system and that altered NEFA

metabolism is not integrally linked to altered transport. These conclusions are inconsistent with the hypothesis of Hewitt and Hook (1978), therefore, this hypothesis can be rejected.

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