# Renal Tubular Transport of Amino Acids and Phosphate in Normal and Mutant States

by

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## Abstract

The pathophysiology of the major inborn errors of renal transport in man, is reviewed and summarized. The experimental sections describe: 1) the mutant Hyp mouse with X-linked hypophosphatemia and evidence for an intrinsic defect in net tubular reabsorption of phosphate anion; 2) the PRO/Re mouse with autosomal recessive proline oxidase deficiency and evidence that intracellular oxidation of solute comes to influence its transepithelial reabsorption; 3) renal handling of the inert amino acid α-aminoisobutyric acid (AIB) and of phosphate by the rat in vivo. The latter investigations show that AIB is transported by carriers serving the natural L-amino acids. It equilibrates slowly with an intrarenal pool so as to achieve significant cell-to-lumen flux at steady-state. Whereas bovine parathyroid extract enhances fractional excretion of phosphate in the intact and TPTX rat, AIB excretion is not similarly affected in the TPTX rat. On the other hand, calcitonin enhances the fractional excretion of AIB but does not affect phosphate. These findings are significant for the interpretation of the tubulopathy in human vitamin D-deficiency and autosomal recessive vitamin D dependency.

# Transport Tubulaire Rénal des Acides Aminés et des Phosphates dans les Etats normaux et Mutants

Roderick R. McInnes

## Résumé

La physiologie pathologique des principales erreurs innées du transport rénal chez l'homme est examinée et résumée. Les sections expérimentales décrivent: 1) la souris Hyp mutante atteinte d'hypophosphatémie reliée à l'X et les preuves d'un défaut intrinsèque de la réabsorption tubulaire nette de l'anion de phosphate: 2) la souris PRO/Re atteinte d'une carence d'oxydase proline récessive autosomique et les preuves que l'oxydation intracellulaire du soluté en arrivent à influencer sa réabsorption transépithéliale; 3) le traitement rénal de l'acide aminé inerte α-aminoisobutyrique (AIB) et du phosphate par le rat in vivo. Les dernières investigations indiquent que l'AIB est transporté par des porteurs desservant les acides aminés L naturels. Il s'équilibre lentement à l'aide d'une masse commune intrarénal afin d'atteindre un écoulement abondant de cellule à lumière à l'état régulier. Alors que l'extrait de parathyroide bovine augmente l'excrétion fractionnaire de phosphate chez les rats intact et TPTX, l'excrétion d'AIB n'est pas affectée de la même façon chez le rat TPTX. D'autre part, la calcitonine accroit l'excrétion fractionnaire d'AIB mais n'affecte pas les phosphates. Ces constatations jouent un rôle important dans l'interprétation de la tubulopathie dans la carence de vitamine D et la dépendance autosomique à trait récessif de la vitamine D chez les êtres humains.

"My business is to teach my aspirations to conform themselves to fact, not to try to make facts harmonize with my aspirations. Sit down before fact as a little child, be prepared to give up every preconceived notion, follow humbly wherever nature leads you, or you will learn nothing."

- Thomas Huxley

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This thesis has been typed with great care by Huguette Ishmael, Lynne Merid and Janet Winston.

Finally, I am indebted to my family, for permitting it.

# PREFACE

This thesis is submitted according to regulations for thesis style authorized by the Graduate Training Committee of the Biology Department of McGill University. The main body of the thesis is presented in a form suitable for publication. The section entitled "Genetic aspects of renal tubular transport: Diversity and topology of carriers", is a review of the physiological mechanisms of genetic disorders of renal transport, already published in Kidney International. The next two sections describe mutations which alter renal solute transport in mice. The first describes the renal defect in phosphate transport in the Hyp mouse with X-linked hypophosphatemic rickets. This material has been accepted for publication in Kidney International. The second illustrates how deficiency of proline oxidase activity in the PRO/Re mouse affects net reabsorption of L-proline. This work was published in the Proceedings of the National Academy of Sciences (USA).

The next three sections describe renal transport of the non-metabolizable amino acid α-aminoisobutyrate (AIB) in the rat. In the first AIB transport is shown to resemble the transport of natural amino acids. The contribution of cell-to-lumen flux of AIB to fractional excretion (or its reciprocal, net reabsorption) is examined in the second. The last analyzes the relationship between renal transport of AIB and mineral metabolism. The first two reports have been submitted to the American Journal of Physiology, the third to Pediatric Research.

Unless otherwise noted in the introduction to each section, the candidate conceived and carried out the experiments of this thesis alone. The sole exceptions to this statement are that urinary calcium and phosphate measurements were made on a Technicon Autoanalyser by Mr. Peter Lamm, and one-third of the amino acid analyses were done by Mr. Cyril Adams.

# SECTION II

## INTRODUCTION

Knowledge of the normal kidney is essential if one is to comprehend the disruption of structure and function resulting from both acquired and genetic renal disease. Study of the biochemical dysfunction caused by mutant genes, however, has been a powerful investigative tool for students of the kidney. Thus, in a recent review of organic solute transport, Ullrich (45) remarks that "at the moment, the safest indication for the diverse amino acid transport systems (in mammals) seems to be data gained from individuals with genetic defects". Furthermore, in bacteria, multiple transport systems with overlapping specificities have been resolved only through a combination of kinetic and genetic means (33). Since the transport mandate of the kidney is unequivocally greater than that of the microbe (41, section III of thesis) then genetic probes of transport will continue to be essential in the effort to understand the molecular basis of this function.

The transport of hydrophilic solutes through the cell membrane, irrespective of whether or not the latter is ultimately accommodated by the fluid mosaic model (42) or some modification of it (8), must be a mediated process, since passive diffusion through a lipid barrier cannot account for the rates of transport observed (11, chapter 4). The so-called "carriers" mediating solute transport in kidney are probably proteins, but at present the evidence is only indirect (39). When considered together with the certainty that the bacterial amino acid carriers are proteins (33),

that Na+ and Ca++ transport in mammalian tissue is ATP-ase mediated (34), and that soluble protein extracts prepared from rat kidney brush-border membranes contain the D-glucose, L-alanine and phosphate transport systems (26), there is little reason at present to implicate another molecular species. Consequently, it is difficult to argue with the contention that the known inborn errors (18) of renal transport (Section III of thesis) probably result from mutation of the genes that regulate or code for the synthesis of the carrier protein or related proteins.

Regardless of the precise nature of the carriers, a large body of knowledge has been acquired about the characteristics of amino acid (30, 38,39,41,48) and phosphate (28) transport in mammalian kidney. This information has been attained using a combination of in vivo and in vitro methods (25,38). Of the five methods generally used for the study of phosphate and amino acid transport in vivo, (38), only two are used in this thesis: the renal clearance technique and the maximum tubular reabsorption rate (Tm). Renal clearance determination is "The volume of blood which one minute's excretion suffices to clear of solute when the urine volume is large enough to permit maximum solute output" (29). Thus, amino acids whose net reabsorption is poor have high clearance rates, and vice-versa. The Tm measurement is a clearance study in which the amount of solute (i.e. substrate) presented to the nephron is increased in step-wise increments. When this is done, saturation of the reabsorptive or transport capacity of the kidney is observed for many amino acids (41). The relationship between the net reabsorption rate and the filtered solute load is similar to that between the enzyme reaction rate

and substrate concentration described by Michaelis kinetics (37). Demonstration of saturability of the reabsorptive capacity was one of the first indications that the transepithelial movement of amino acids in kidney is carrier-mediated (37). In metabolic or genetic disorders in which excessive solute excretion is found in the presence of a normal substrate (filtered) load, information concerning the nature of the reabsorptive abnormality may be deduced using the Tm technique. Thus, hereditary iminoglycinuria in one pedigree (36) has been found to be due to a decrease in the Tm for proline, with the residual transport system(s) showing a normal affinity for proline. In another unrelated kindred, however, a homozygote had a normal Tm for proline, but an altered affinity for proline by the carrier, as indicated by an abnormal "splay" in the titration curve (22).

The type of information which can be acquired from these and other in vivo methods (38) is limited, however, since it is not at all clear, using these approaches, what intermediate steps occur between the entry of the solute into the tubular lumen, and the formulation of the final urine composition. In this respect, the kidney has been recently referred to as a "black-box" (38,45). Nevertheless, a nascent understanding of the intermediate events has been gained using a variety of histological, biochemical and in vitro physiological methods (38,39,41).

The post-glomerular part of each nephron is termed the proximal tubule. This region constitutes an epithelial sheet (44) in which the individual cells are held together at "tight junctions" (15) which are themselves normally impermeable to solutes. Consequently, solute passage

must occur across the brush-border membrane of the cell, rather than between the cells. Since the great majority of phosphate (21) and amino acids (41) is reabsorbed in this part of the tubule, it is not surprising that membrane preparations of the proximal tubule brush-border demonstrate significant transport activity towards these solutes (25,26,38, and section IV of this thesis). This transport step is active for both amino acids (45) and phosphate (28). This conclusion is partly based on the observation that the intracellular concentration of these solutes, in an osmotically active form, is higher than that of the filtrate or the plasma. Furthermore, this distribution ratio cannot be achieved by electrochemical equilibration according to the Nernst equation (28,38).

Upon entry into the renal tubular cell the solute has three possible fates: (i) it may enter the peritubular capillary by transport across the basolateral membrane. This process is carrier-mediated (39). (ii) it may enter the metabolism of the cell (13), (iii) it may backflux into the tubular lumen to either be reabsorbed again or lost into the urine (4; also, sections V and VIII of this thesis). This description clearly shows how transepithelial membrane transport is a more complex process than the accumulative transport found in non-epithelial cells, or in microorganisms. Unfortunately, none of the in vivo methods currently in use is able to study, as an isolated event, any single one of the transport phenomena constituting this net process. The claim (41) that micropuncture methods examine transport across the luminal membrane as an <u>isolated</u> phenomenon ignores the facts that the cellular concentration of solutes which might counter-exchange with the luminal solute is uncontrolled, and that the metabolism of the renal cell modulates the intracellular concentration of

such"exchangeable" compounds (19, and section V of this thesis). On the other hand, the various micropuncture techniques (where filtrate from individual nephrons is sampled, or a single nephron is infused with a known solution) do give kinetic information on net uptake of solute from lumen (41),and on the parts of the nephron most important in amino acid transport (3,27). They are also capable of directly demonstrating the presence of extracellular fluid to lumen fluxes of solute (4).

Recognition of the importance and precise mechanism of each component of the net reabsorptive process, therefore, has been possible only through a synthesis of knowledge from in vitro and in vivo methods. The in vitro methods (38) include kinetic studies of transport in slices of kidney (cortex, medulla or papilla), isolated glomeruli, isolated tubules, and subcellular preparations of membrane vesicles (25). That studies of renal cortex slices in vitro describe transport events primarily at the peritubular membrane rather than at the brush-border luminal surface (30,38,40 and section III of this thesis) was forcibly proposed by Wedeen & Weiner (46). Using autoradiographic methods, these investigators showed that an extracellular marker,tritium-labelled inulin, does not have access to the lumina of proximal tubules in slices incubated in vitro. On the other hand, efflux of amino acid from cell to lumen appears to occur in the slice (10), as do secretory processes, such as PAH secretion (46). This new understanding of the meaning of transport studies in kidney cortex slices has allowed a more rational interpretation of amino acid transport data acquired from in vivo work (38,40). This fact is demonstrated in Section V of this thesis, and, for example, in studies of

dibasic amino acid and cystine transport by Segal and his colleagues (2, 23). An overly simplistic interpretation of the basic defect in the human disease cystinuria has been avoided because of transport studies in kidney cortex slices from affected patients. Thus, whereas the urinary excretion of cystine, ornithine, arginine and lysine is increased in cystinuria, only the uptake of the dibasic amino acids by the slice is low, cystine transport being normal. The basic defect has not yet been identified, but it will have to account for these divergent findings which describe events at different membranes of the tubular cell (43).

In vitro methods, especially transport studies in the cortical slice, have allowed characterization of the transport step from peritubular lumen to cell. Amino acid uptake at this renal cell surface resembles that found in many other mammalian tissues (11, chapter 5) in being dependent on Na+, pH, and energy, and in demonstrating stereospecificity (L-configuration preferred), saturation kinetics, competitive inhibition by related amino acids, exchange diffusion, and sensitivity to temperature change (38, 39). With the exception of the last variable, analagous studies of luminal surface events in vivo, primarily using micropuncture methods, have shown that the net transport step at the brush-border demonstrates these properties as well. It is evident, however, that these luminal and peritubular characteristics of amino acid influx into the renal cell, though similar, are not identical. The previously quoted problem concerning dibasic amino acid and cystine transport exemplifies these differences. Many other discrepancies are noted in the review of Silbernagl et al (41). A major difference, discussed by Ullrich (45), lies in the fact that amino acid transport at the luminal membrane appears to be entirely Na+ -dependent,

whereas in vitro, this is not necessarily always the case (37). The dependence on Na+ for luminal transport means that "a mutual inhibition of amino acids in their transport does not necessarily indicate... that they share a common transport system" (45). Whether this dependence on Na+ cotransport means, as at the basolateral membrane, that the Na+ participates in a ternary complex (11,36) involving the reactive site of the carrier, the Na+ and the amino acid, or whether Na+ may only participate in the coupling of amino acid transport to cell energy metabolism (i.e. the amino acid transport is secondarily active (45)) is currently unresolved (38). These are not mutually exclusive alternatives.

Despite the appropriateness of the cautionary statement by Ullrich noted above, the competitive interactions observed between amino acids at both the luminal (41) and peritubular surface (37) are often highly selective. For example, L-proline inhibits L-hydroxyproline and glycine reabsorption (35), but has no significant effect on the reabsorption of other amino acids besides L-alanine (41). On the basis of these studies of competitive interactions in vivo, at least five major transport groups are well established: (i) α-amino acids - cationic; includes cystine. (ii) α-amino acids - anionic (iii) iminoglycine group (iv) α-amino acids - the remaining neutral amino acids (v) β-amino acids. The recognition of genetic diseases which selectively affect only one of these groups (section III, this thesis) is, as mentioned previously, the best single proof to date of their existence. In addition to these major divisions, competitive interactions also suggest the presence of smaller

groupings (3,9,41). Just as the designation of several transport systems for amino acids in Ehrlich ascites tumor cells means only that each amino acid uses the different systems to different extents (12), so it is in kidney, that interactions which are not explicable on the basis of the major divisions are also known. For example, the non-metabolizable amino acid α-aminoisobutyrate (AIB), used in the studies of sections VI, VII, and VIII of this thesis, although mainly interacting with the neutral amino acids in Ehrlich cells (11), causes a pronounced increase in dibasic amino acid excretion in man (17). Similar examples are noted in the major review articles (38,39,41).

In contrast to the significant knowledge which has been acquired concerning the influx of amino acids into the cell, across either the peritubular or luminal membrane, relatively little is known about the efflux systems which allow the absorbed solute to return to the extracellular fluid. For some groups of amino acids, the dibasics for example, the specificities for influx and efflux are the same, as shown by exchange diffusion in renal cortical cells (39). In general, however, understanding of the contribution which efflux makes to the net reabsorptive process is poor, a point recently emphasized by Segal (40). The importance of a normal efflux process is indirectly illustrated by the study of prolinuria in hyperprolinemic mice in this thesis (section V). In the renal cortex of these animals, a deficiency of proline oxidase results in an elevation of the proline concentration in the renal cortical cell. This elevated intracellular concentration is interpreted as being responsible for an increased cell to lumen flux of the amino acid, resulting in increased proline excretion. In the same way that this metabolic block

results in decreased intracellular disposition of a solute taken up by the cortical cell, a decrease in cell to capillary flux of amino acid would tend to increase the intracellular concentration of the solute, with a similar consequence - increased net excretion. This latter mechanism has been suggested as one cause of the cystinuria induced by lysine infusion in rats, since lysine infusion increases renal-tubule cell cysteine (23).

The increased cell to lumen flux of amino acid which occurs in either of these situations is an accentuation of a normal process which is usually not of great significance (4,9). In section VII of this thesis, a cell to lumen flux of the non-metabolizable amino acid α-aminoisobutyrate is demonstrated. Our data suggest that approximately half of the urinary excretion of AIB is derived from this cell to lumen flux. This means that a quantity of AIB equivalent to approximately 7% of the filtered load reaches the urine without glomerular passage. Since the fractional reabsorption of the natural amino acids is generally greater than 98%, a cell to lumen flux by them is unlikely to be of the same absolute magnitude as it is for AIB, unless subsequent reabsorption occurs at points further along the nephron.

Increased cell to lumen solute efflux can also result from impaired energy production, as demonstrated by the toxin maleic acid in rats (4), and by bacterial mutants lacking energy coupling, in which the normal energy-dependent elevation of the efflux Km is no longer maintained (47). In pathological situations such as that induced by maleic acid or other

tubular toxins, the increased excretion of solutes is, not surprisingly, generalized. In humans, this generalized abnormality, called the renal Fanconi syndrome (38), has genetic as well as metabolic causes. One of the most studied of the latter group is the abnormality found in patients with hyperparathyroidism accompanying vitamin-D or calcium deficiency (31). Although increased parathyroid hormone (PTH) secretion has been generally considered to be a necessary component of the hyperamino-aciduria (24), section VIII of this thesis shows this interpretation to be an over simplification. We found that PTH does not increase the excretion of α-aminoisobutyrate in thyroparathyroidectomized rats. On the other hand, calcitonin, the other major calciotropic hormone, induced a striking increase in AIB excretion. A major intracellular action of calcitonin appears to be reduction of the cytoplasmic calcium ion concentration (6). This observation suggests that in both vitamin-D and calcium deficiency, low cytoplasmic calcium (7) may reduce membrane permeability (see section VIII) and thus decrease lumen to cell movement of amino acids.

Hyperparathyroidism has not only been considered as a cause of the generalized tubular defect of vitamin-D and calcium deficiency, but also of the hyperphosphaturia found in one of the classical human hereditary disorders of transport, X-linked hypophosphatemia (XLH). This hypothesis was challenged by Arnaud et al (1) and Glorieux and Scriver (20), who found no evidence for hyperparathyroidism, but proposed that the disease resulted

from a defect in one of two phosphate transport sytems (14) present in the normal kidney. The limitations of in vivo studies in humans made the direct evaluation of this hypothesis impossible until the discovery of X-linked hypophosphatemic mice (Hyp/Y) by Eicher et al (16). The similarity of the human and murine phenotypes and the stability of the X-chromosome during evolution (32) suggests that both X-linked mutations involve the homologous gene. Section IV of this thesis describes the in vivo and in vitro investigations of renal phosphate transport in the murine mutant. Serum parathyroid hormone levels are normal in Hyp/Y (16), as is phosphate transport by the peritubular membrane of the cortical cell. The explanation of the increased phosphate clearance observed in vivo was apparent only during the study of phosphate entry into vesicles prepared from renal cortical brush-border membranes of normal and Hyp/Y mice. The mutant mice have a marked decrease in a Na+ -dependent component of phosphate influx across the luminal membrane. Further study should allow definition of this mammalian inborn error of transport at a molecular level.

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SECTION III

## Genetic Aspects of Renal Tubular Transport: Diversity and Topology of Carriers

This review of the inborn errors of renal transport discusses the pathophysiological mechanisms of these disorders in terms of the individual fluxes which constitute net transepithelial transport. Once a solute is transported from the tubular lumen to the cell interior, it has three possible fates: backflux into the lumen, metabolism in the cell, or efflux across the basolateral membrane into the peritubular fluid. Of the thirty inherited disorders of transport discussed in this review, only in X-linked hypophosphatemia (see Section IV of this thesis) has it been possible to identify which of these four steps is altered by the mutation. In many of these disorders, however, the indirect evidence implicating one of the four steps listed above as the precise locus of the transport defect is very compelling. Thirteen of these diseases are analysed in detail, and the apparent target of the mutation is suggested for each one. Four of these conditions are the subject of other sections of this thesis (Sections IV, V, VII and VIII) and the candidate also contributed to the concepts presented on the renal Fanconi syndrome and pseudohypoparathyroidism in this review. The other known mammalian inborn errors of renal transport are tabulated.

Throughout this discussion particular emphasis is given to the role these inborn errors of transport have had in providing insight into the physiology of the normal kidney. The diversity of the transport systems responsible for the uptake of individual solutes is revealed more unambiguously by mutation than by any other probe available.

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MEDICAL REPORT ON

"GENETIC ASPECTS OF RENAL TUBULAR TRANSPORT: DIVERSITY AND TOPOLOGY OF CARRIERS."

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# Genetic aspects of renal tubular transport: Diversity and topology of carriers

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Mutations which cause the inborn errors of membrane transport can provide information about the normal topology[1] of renal transepithelial transport. In recent years various reviews of tubular transport [1–6] have appeared which discussed the interrelation between disease and net tubular reabsorption of organic solutes.[2] Their emphasis was primarily on the functions which served solute transport and less on the diseases associated with the disturbance of transport. In this review we have taken the opportunity to describe, and to speculate on, the probable site in the tubular cell of the defect in transcellular movement of the solute in a number of inborn errors of tubular transport. We hope that the speculations will stimulate debate, formulation of hypotheses and further experimental evaluation to advance our knowledge. The table included in this paper provides a catalogue of the currently accepted inborn errors of tubular transport. It is these clinical "windows" which, through the expression of mutation, have revealed and helped to delineate an impressive array of specific transport functions in tubular membrane.

### Cellular uptake and transcellular transport

The epithelial cell of the renal tubule has luminal and basilar poles, with definite orientations to the fluids in contact with them; its luminal pole faces an ultrafiltrate of plasma where solute is topologically *outside* the body while the basilar pole is in contact with peritubular interstitial fluid where solute is *inside* the body. Cells of the proximal tubule, in both the convoluted and pars recta segments where a major fraction of electrolyte and organic solute transport occurs, have a greatly enlarged membranous surface formed of closely packed microvilli: the latter increase the absorptive surface about forty-fold [7, 8]. Microvilli are associated with an unstirred layer at the external liquid-solid interface, which is the major rate-limiting step in trans brush-border permeation at low concentrations of many solutes [9]. The unstirred layer also serves as a locale in which enzymes may act upon substrate, e.g., disaccharides, before transport [10]. Tubular epithelium is also a continuous sheet by virtue of punctuate contacts, or tight junctions, between cells at their luminal poles [11]. These junctions are probably impermeable to organic solutes under normal conditions. Therefore, the solute must enter epithelial cells to reach the peritubular space during reclamative transport and to reach the lumen during secretory transport. To achieve this vectorial process, an asymmetry of net solute flux is required during reclamative and secretory transports. How mutation informs us about the functional organization of two sets of membranes (luminal or brush border, and basolateral) and three pools (luminal, cellular and peritubular) ordered in series, and how they achieve these net transtubular fluxes, is of fundamental interest to us in this brief review.

*Cellular uptake of solute.* The current view of biological membranes emphasizes a fluid-mosaic, structural model, in which the proteins form a mosaic in the bilaminar bed of lipid [12]. In the absence of diffusion channels, permeation of the plasma membrane by hydrophyllic solutes must be facilitated by carriers [13]. Certain types of membrane protein apparently serve as carriers [14], thus providing a pertinent focus for the effect of mutation on the transport process. Various chemical and kinetic probes [13] reveal the specificity of carriers and their ability to recognize solutes. Since the transmembrane move-

[1] Topology—anatomical definition: the structure of a particular region or part of the body (Webster).

[2] The term "solute" in this paper refers to organic solutes except when clearly indicated otherwise: for example, in the section on phosphate reabsorption.

ment of many solutes across the basolateral and brush border membranes of tubular epithelium, both *in vivo* and *in vitro*, can occur against a chemical gradient in all parts of the nephron [5, 6], there must also be an investment of energy in transport in the form of a conjugate-driving force [15].

Assuming that the solute remains osmotically active upon entering the intracellular pool, and there is no evidence yet to the contrary [16], it follows that the plasma membrane plays a critical role in the form of a barrier to exodus after concentrative uptake. A mechanism for attainment of dynamic asymmetry in the carrier as it functions on opposite sides of the membrane is required. A simple kinetic description of this event is possible [6] (Fig. 1). The Michaelis equation can be used to describe carrier-mediated and opposing fluxes[2] of solute across the membrane where entry (or influx) and exodus (or efflux) are described by separate equations:

$$U_{inf} = \frac{V_{max\ inf} \cdot [S]_o}{K_{m\ inf} + [S]_o} \quad (1)$$

and

$$U_{eff} = \frac{V_{max\ eff} \cdot [S]_i}{K_{m\ eff} + [S]_i} \quad (2)$$

$[S]_o$ and $[S]_i$ are the concentrations of solute on the outside and inside of the membrane, respectively. When uptake is concentrative, influx exceeds efflux until the steady state is achieved at which time influx equals efflux. In the latter state, $[S]_i$ will be $>> [S]_o$; it follows from equations 1 and 2 that $K_{m\ eff}$ must then be $>> K_{m\ inf}$. The maintenance of this relationship which is essential to concentrative uptake appears to be a genetically determined property of the carrier.

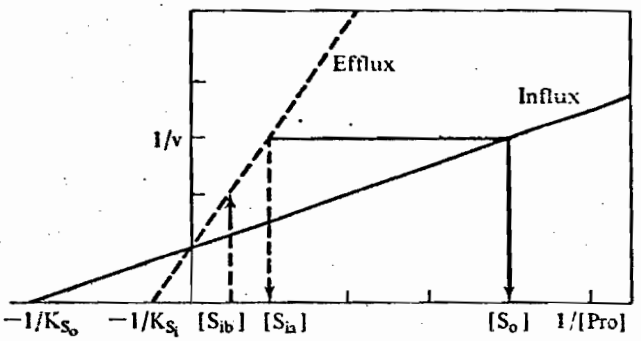



**Fig. 1.** *Diagram showing the apparent kinetics of steady-state entry (influx) and exodus (efflux) under conditions of active transport (accumulation against a chemical gradient) of* L*-proline across the plasma membrane of a cell.* (See text for appropriate Michaelis equations and description of terms.) Where the rate of solute entry is not higher than the rate of its removal by metabolism, the latter comes to influence transport in the transtubular orientation. Diminution of metabolic "runout" may cause the internal solute concentration to rise (i.e., change from $[S]_{ia}$ to $[S]_{ib}$): when this change occurs in relation to the luminal membrane, exodus (backflux) will increase and egressed solute will be removed by the flowing column of urine. These considerations may be relevant to the interpretation of solute loss in some hereditary disorders of tubular transport and metabolism and to the concept of maximal rates of tubular absorption (Tm). (From Scriver, McInnes and Mohyuddin [97].)

Efflux across the luminal membrane occurs during net absorption of amino acids and glucose by the proximal tubule [17–19]; this backflux is quite in keeping with the normal kinetics of efflux depicted in Fig. 1. However, backflux is necessarily small if concentrative uptake is an initial step during net transepithelial reclamation of solute, as is now known to be the case for the transport of some amino acids and for glucose [17–19].

The ability to generate asymmetry in transcellular movement of solute, so that a vectorial flux occurs, has long been a subject of interest [20]. Transport across epithelium has many features which are analogous to transport across the plasma membrane; but transepithelial transport also has unique characteristics which distinguish it from simple uptake into cells. In tubular epithelium the now well-recognized substrate specificity of the transport process, presumably invested in the transport proteins or reactive sites of the plasma membrane [13], and limned by the appropriate kinetic, chemical and genetic probes [1–6], is not necessarily identical at opposite poles of the transporting cell. Uptake into the intracellular pool, as defined by equations 1 and 2, can pertain to both luminal and basilar and lateral membranes. However, if an identical net flux exists at both sets of membranes, net transcellular flux, from urine to peritubular space, or the reverse would not occur. For example, unless the unidirectional flux of solute *outward* at the basilar pole of the cell is greater than the unidirectional flux *outward* at the luminal pole, there is nothing to prevent achievement of a transcellular steady state so that backflux becomes the equal of entry at the luminal site serving uptake from glomerular ultrafiltrate, thus making net absorptive transport impossible. During absorption, a mechanism must be found which maintains the interim instantaneous uptake ratio below the "true" steady-state ratio across the luminal membrane; therefore, an explanation for "run out" from the intracellular space in the direction of the peritubular space is required. Two mechanisms seem plausible. *1) Metabolism.* If there is a large intracellular pool of the solute, membrane transport will not be the rate-limiting step in its transcellular movement. The rate of its intracellular utilization will determine its intracellular concentration and, therefore, the number of molecules of the original solute species available for backflux after uptake. It follows that a disturbance of

intracellular metabolism may perturb tubular absorption through modulation of intracellular pool size. Currently available techniques which measure net reabsorption and transepithelial movement are, in essence, "black box" methods which largely fail to account for intracellular events acting on solute. *2) Dissimilar characteristics of luminal and basilar membranes.* Luminal and basilar membranes are dissimilar both in their morphologic and functional characteristics. Differences at the two poles have been described, for example, in electropotential [21], and in the uptake of amino acids [18, 22–25] sugars [19, 26, 27] and keto acids [28]. This functional asymmetry clearly permits metabolically inert amino acids, which have no "run out" into other metabolic pools, to be reclaimed from urine into blood against a chemical gradient [29]. Accordingly, proximal tubule reclamation may be accomplished by a more permissive efflux at the basilar membrane relative to the luminal pole. Relatively little work has been done to examine the role of solute removal by peritubular blood flow in relation to urine flow, and the relation this may have to transtubular transport kinetics.

We should recognize also that solutes such as, amino acids [6, 30, 31] and glucose [19] which are taken up from peritubular fluid in many portions [16, 26, 27] of the nephron evidently experience only minimal net efflux across the luminal membrane and do not appear in bladder urine in significant amounts. Further evidence for diversification of transport in brush border and basolateral membranes in various parts of the nephron is seen in the distal tubule, which takes up amino acids, not at all from the lumen and only slowly from peritubular fluid [6].

### Functional classification of inborn errors of tubular transport

Two membranes and three solute pools, arranged in series, accommodate transepithelial transport. In principle, the various inborn errors of reclamative transport can then be assigned to specific disorders of membrane function according to Fig. 2. Mutation can effect tubular reabsorption at the following stages: *1A*) uptake activity (entry) at the luminal carrier; *1B*) backflux (luminal exodus) permitted on the carrier; *2*) cellular utilization of absorbed solute (pool size) controlled by its metabolic disposal; *3*) unidirectional flux from cell to peritubular fluid at basilar and lateral membranes (peritubular exodus).

Disorders of secretory transport can also be served by this model with due consideration for the predominant direction of the fluxes.

Hormone-dependent inborn errors of tubular transport require consideration of two additional steps: *1*) binding of hormone to the appropriate plasma membrane and *2*) intracellular translation of the hormonal signal. We have selected examples from Table 1 to describe defective mechanisms at different stages of the transepithelial transport process.

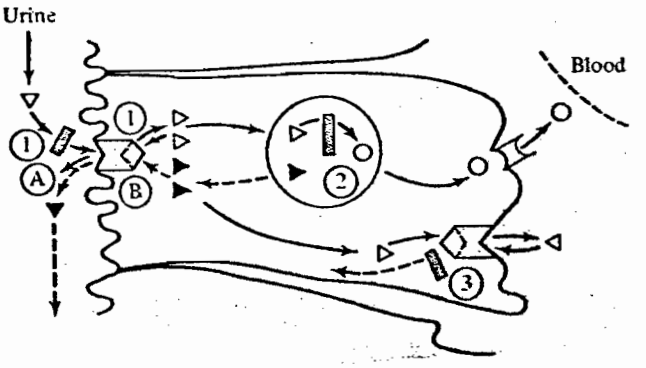



**Fig. 2.** *A model delineating possible sites for expression of mutant alleles causing hereditary disorders of tubular reabsorption.* Appropriate recognition of the vectorial flux in secretory transport allows the model to be adapted also to disorders of secretion. Defect 1A, mutant site (carrier) blocks entry; defect 1B, mutant carrier permits excessive exodus (backflux) from internal pool (△); defect 2, blocked catabolic mutant state prevents metabolic "runout" to alternate form (○) leading to accumulation of original solute in the internal pool (▲) and exaggerated exodus (backflux) on normal carrier; defect 3, defect in exodus at basilar (or lateral) membrane leads to intracellular accumulation as in defect 2. All lead to reduction of net transepithelial flux. Not shown are hormone-modulated defects involving hormone binding at specific sites on the basilar membrane, and transduction of signal by internal messenger to influence the primary transport process (e.g., phosphate transport modulation by parathyroid hormone or calcitonin, and water transport modulation by vasopressin).

*1A. Disorders of solute uptake of the luminal membrane.* The casual student of biological transport is likely to assume that the hereditary disorders of tubular transport express themselves at the luminal membrane. We know from studies of electrolyte transport that other peritubular membranes play vital roles in solute migration across the tubule; no direct evidence for a luminal membrane defect has been obtained in any disease listed in Table 1; only indirect evidence, and only in certain disorders of net reclamation, is at hand. The examples we have chosen will highlight a number of themes including the diversity of membrane sites used by even a single solute during tubular transport, and the great specificity among sites exposed to a wide mixture of solutes. Consequently, mutation is likely to ablate only a fraction of any given transport function. Diversity has adaptive advantage.

*The Hartnup trait.* Typical homozygotes have a selective impairment in the intestinal and tubular absorption of a particular group of neutral $\alpha$-amino

Table 1. Heritable disorders of renal tubular transport in man[1]

| Trait (McKusick cat. No.) | Substance affected | Presumed (or possible) mutant gene product | Other tissues affected | Apparent inheritance pattern | Comment[2] |
|---|---|---|---|---|---|
| Hyperdibasic aminoaciduria (12600) (22270)[3] | Lysine, ornithine arginine, ("dibasic" group) | Shared "dibasic" amino acid transport system (uptake) | Intestine liver? brain? | AD/AR | Two alleles (different loci?) Type I (22270) protein intolerance; failure to thrive, hyperammonemia (mitochondrial defect?) hets silent. Type II (12600) assoc. with mental retardation in recently discovered homoz. Hets have modest dibasic aminoaciduria. |
| Cystinuria (22010)[3] | Lysine, ornithine arginine and cyst(e)ine | Shared membrane efflux system? | Intestine brain? (skin fibroblasts are normal | AR | "Negative" reabsorption of affected amino acid can occur. Three alleles (same locus?) each causing different phenotypes: in type-I hets, no excess amino acids in urine ("silent"); in type-III homoz, intestinal transport intact. |
| Hypercystinuria (23820) | Cystine | Specific system for cyst(e)ine (uptake) | ? | AR? | One pedigree only |
| Iminoglycinuria (24260)[3] | Proline, hydroxy-proline, glycine | Shared system for iminoacids, glycine (& sarcosine) (uptake) | Intestine | AR | Four alleles (same locus?) I, II, silent hets III, IV hyperglycinuric hets I, with intestinal defect IV, Km mutant. |
| Hartnup (23450)[3] | Neutral amino acids (excluding iminoacids and glycine) | Shared system for large neutral amino acid group (uptake) | Intestine (skin fibroblasts are normal) | AR | Two alleles (same locus?) I, intestine affected; II, intestine normal. Hets "silent" in both. |
| Dicarboxylic aminoaciduria (glutamate-aspartate transport defect) (23165) | Glutamic aspartic | Shared dicarboxylic acid transport system (?) | Intestine | AR | |
| $\beta$-aminoaciduria (hypertaurinuria) See text | Taurine, $\beta$-alanine $\beta$-AIB | No primary transport defect in man; taurinuria in mouse | | | System defined by competitive inhibition in hyper-$\beta$-alaninemia in man (McKusick [23740]) (& taurinuria phenotype in mouse). |
| Renal glucosuria (glycosuria) (03260)[3] | Glucose | Glucose carrier (uptake) | Intestine normal | AR | Two forms; Km variant & low-Tm variant (Reubi type A). |
| Glucose-galactose malabsorption (23160)[3] | Glucose galactose | Shared glucose-galactose carrier (uptake) | Intestine | AR | Minimal renal glucosuria and no galactosuria under usual conditions. Diarrhea principal symptom; mimics disaccaridase deficiency |
| Bartter syndrome (hypokalemic alkalosis) (24120)[3] | $Na^+$ ($K^+$ secondarily?) | A $Na^+$ carrier? | Erythrocyte ($Na^+$ content increased) | AR | Secondary juxtaglomerular cell hyperplasia, normal blood pressure, secondary hyperaldosteronism & hypokalemic alkalosis |
| Albright's hereditary osteodystrophy (pseudohypopara-thyroidism, etc.) (30080)[3] | Calcium | Regulation of parathyroid hormone receptor mechanism (or messenger system) | Bone | XL (dominant) (or sex-influenced AD) | More then one allele (at different gene loci?) Type I, no increase in urinary cAMP after PTH challenge; cortical adenyl cyclase present; circulating PTH increased. Type II, urinary cAMP response intact but not effective. |

Table 1. *(Continued).*

| Trait (McKusick cat. No.) | Substance affected | Presumed (or possible) mutant gene product | Other tissues affected | Apparent inheritance pattern | Comment[a] |
|---|---|---|---|---|---|
| Familial hypophosphatemic rickets (30780)[a] | Phosphate | Phosphate carrier (luminal membrane?) (retaining cellular pool) | Intestine? Bone? | XL (dominant) | Low $Tm_{Pi}$; "negative" reabsorption of phosphate can also occur, suggesting excessive backflux from cell to urine. Trait responds to phosphate replacement. |
| Renal tubular acidosis (type II) (31240) | Bicarbonate | Proximal tubular mechanism for reclamation | Intestine (probable) | XL (?) (recessive) (or AR?) | Prevalent in males. Low capacity (Tm) for reabsorption of $^-HCO_3$. Daily requirement of $^-HCO_3$ is 10 to 15 mEq/kg. |
| Renal tubular acidosis (type III) (26720) | Bicarbonate | Late proximal or early distal tubular mechanism for reclamation | | AR (?) | "Dislocation" (Km) type assoc. with hypokalemia & osteomalacia/rickets. |
| Renal tubular acidosis (type I) (17980)[a] | $H^+$ | Distal tubular collecting duct hydrogen ion secretion mechanism | | AD | Bicarbonate reclamation normal. Ability to secrete $H^+$ in distal system vs. gradient is defective. Daily requirement of $HCO_3$ is 1 to 2 mEq/kg. |
| Distal renal tubular acidosis with nerve deafness (26730)[a] | $H^+$ | Carbonic anhydrase B | Erythrocyte | AR | Progressive nerve deafness is marker finding. RTA responds to $HCO_3^-$, 1 to 2 mEq/kg. |
| Diabetes insipidus (vasopressin resistant) (30480)[a] | $H_2O$ | Antidiuretic hormone receptor mechanism (or messenger system) | | XL (recessive) ? | Two alleles (different loci?). Type I, no urinary cAMP response to ADH. Type II, female proband urinary cAMP response intact but not effective. |
| Idiopathic Fanconi syndrome (22770)[a] (22780)[a] | Generalized effect on all solutes & water | Coupling of energy (?) Tight junction integrity (?) | Secondary to renal phenotype | AR (& AD?) | Adult-onset (22780) and infantile childhood forms (24270) are differentiated. Basic defect unknown; probably several alleles. |
| Symptomatic forms of Fanconi syndromes: | | | | | |
| a) Cystinosis Type I (21980)[a] Type II (21990)[a] Type III (22000)[a] | Same (secondary response) | Cystine storage (lysosomal defect) with secondary damage to tubule & glomerulus (later) | Same | AR (for each type) | Several alleles. Infantile (type I) & adolescent (type II) forms have differing rates for onset of nephropathy. "Adult" form (type III) has no nephropathy. |
| b) Hereditary fructose intolerance (22960)[a] | Same (+ fructose) | Fructose-1-phosphate aldolase (with secondary effects on cellular ATP) | Same (hepatic cirrhosis) | AR | Nephropathy dependent on phosphate depletion in kidney. Responds to fructose withdrawal. |
| c) Galactosemia (23040)[a] | Same (+ galactose) | Galactose-1-phosphate uridyltransferase (with secondary effects on cellular ATP) | Same (cataracts, CNS) | AR | "Galactosemia" due to galactokinase deficiency does *not* have Fanconi syndrome. Fanconi syndrome responds to galactose withdrawal. |
| d) Hereditary tyrosinemia (27670) | Same (+ tyrosine metabolites) | Unknown (with secondary effects on cellular ATP) | Same (hepatic cirrhosis) | AR | Fanconi syndrome responds to tyrosine restriction. |

Table 1. (*Continued*).

| Trait (McKusick cat. No.) | Substance affected | Presumed (or possible) mutant gene product | Other tissues affected | Apparent inheritance pattern | Comment[2] |
|---|---|---|---|---|---|
| c) Wilson's disease (27790) | Same (with proximal and distal RTA) | Unknown (seondary effects on cytochrome oxidase system?) | Hepatolenticular degeneration | AR | Fanconi syndrome responds to depletion of copper storage. |
| f) Lowe's oculocerebro renal syndrome (30900)[3] | Generalized disfunction with defective urinary ammonia production | Unknown | An oculocerebro-intestinal-renal syndrome (involving tissues with high γ-glutamyl cycle activity?) | XL (recessive) | Basic defect still unknown. Treatment for tubular reclamation defects does not improve mental retardation or the cataracts & hydrophthalmia. |
| Vitamin D dependency (pseudodeficiency rickets) (26470)[3] | Generalized defect. (Secondary response) | 25-hydroxyvitamin D-1α-hydroxylase | (Vitamin D hormone synthesis occurs in kidney mitochondria; deficiency affects intestinal absorbtion of calcium & initiates PTH response.) | AR | Nephropathy dependent on PTH excess & hypocalcemia (phenocopy occurs in vitamin D deficiency.) |
| Miscellaneous | | | | | |
| a) Glucoglycinuria (13810) | Glucose & glycine | Unknown (the two solutes do *not* share a common carrier. | | AD | Asymptomatic. Normal-Tm (type-B) glucosuria. Possibility that this is a heterozygous manifestation of a Fanconi-like tubulopathy merits consideration. |
| b) Luder-Sheldon syndrome (15250) | Generalized amino acids glucose & phosphate | Unknown | | AD | Same as for previous entry. Symptoms of Fanconi syndrome have occurred in probands. |
| c) Rowley-Rosenberg syndrome (26850) | Generalized aminoaciduria | Unknown | | AR | Associated components of syndrome: growth, retardation, muscular hypoplasia, pulmonary involvement & right ventricular hypertrophy. |

[1] A catalogue of 28 inherited disorders of tubular transport is provided herein. Each disease included in the table has a proven (see footnote 3) or suspected pattern of inheritance and is to be found under its own five-digit catalogue number in the appropriate section (autosomal dominant, 10,000 series; autosomal recessive, 20,000 series; and X-linked, 30,000 series) of McKusick's *Catalogue of Mendelian Inheritance in Man* [161]; selected literature citations are given with each entry. Vignettes covering the major clinical features and the genetic aspects of many of these traits will also be found in the *Compendium of Birth Defects*, published by the National Foundation-March of Dimes [162]. Numerous probable inborn errors of tubular transport are not included in Table 1 because their tubular manifestations have yet to be clearly understood. These conditions (and their McKusick catalogue number, if available) include the following: *Pyroglutamic aciduria* (26613) due to a defect in glutathione synthesis and secondary overproduction of the pyroglutamic acid (5-oxo-proline) intermediate of the γ-glutamyl cycle; idiopathic *hypercalcuria* (hyperexcretory form) (23810); some patients with *Leigh's necrotizing encephalopathy* (25600); *Familial nephrosis* (25630) with a generalized tubulopathy; and *Immerslund's* syndrome (26110) with unexplained tubular proteinuria.
[2] hets = heterozygote, homoz = homozygote.
[3] Proven pattern of inheritance.

acids [6, 32]; nonepithelial cells, such as cultured skin fibroblasts, do not have the defect [33]. Intestinal uptake of various dipeptides containing amino acids affected by the Hartnup trait is not impaired [34–36], because dipeptides are transported in the gut and kidney, at membrane sites which are independent of those used by their constituent free amino acids [37, 38]. Following uptake, dipeptides are cleaved by intracellular peptidases; the free amino acids then enter metabolic pools or leave the cell. The normal plasma response curve in the Hartnup trait, following dipeptide feeding, indicates that cleavage and absorption of dipeptide-derived amino acids are normal. Therefore, efflux of the released free amino acids across the basilar plasma membrane must be intact, and the defect in transepithelial absorption must be confined

to a specific uptake carrier serving the large "Hartnup" group of amino acids on the *luminal* membrane (viz. Fig. 26–21 in [6]). In the absence of any comparable studies of dipeptide reabsorption by kidney, we reason by analogy that a similar location of the defect in proximal tubule epithelium accounts for the specific Hartnup hyperaminoaciduria.

*Renal glucosuria and glucose-galactose malabsorption.* Two autosomal recessive disorders of hexose transport [39, 40] reveal the likelihood that renal tubular epithelium possesses two (or more) genetically distinct mechanisms for glucose transport: this may not be the case in the intestine. The transport defect in each trait almost certainly involves an uptake system on the luminal membrane.

The characteristics of D-hexose transport in kidney are complex. Hexose transport mechanisms provide substrates for the metabolic systems yielding energy for basal renal work, and also for a component of renal transport work itself [41]. Hexoses enter proximal tubular cells, *in vivo*, from luminal and basilar poles [26, 42]. However, luminal and basilar membranes clearly possess differing characteristics for hexose transport. By means of the sudden-injection, multiple-indicator dilution method [26], it has been shown that there are D-glucose-preferring (G) sites (shared with D-galactose) and D-mannose-preferring (M) sites in the luminal membrane [42]. Confirmation of these data, and evidence for $Na^+$-dependent D-glucose transport at the luminal membrane, has been obtained by a stop-flow microperfusion method [43] and by kinetic analysis of isolated preparations of brush border membranes [44]. It is likely also that G sites of luminal and basilar membranes are not identical [26, 42]. Furthermore, this delineation of hexose transport in the luminal membrane of kidney leads one to believe that its properties are qualitatively different from those previously defined for the luminal membrane of intestinal epithelium [45].

By means of a technique using isolated, perfused proximal tubule segments, the characterisitcs for true transcellular transport of D-glucose have been revealed. D-glucose can be transported against a chemical gradient out of the tubule lumen. Active transport is therefore a property of the luminal membrane. Glucose reclamation occurs predominantly in the convoluted portion of the proximal tubule but it also takes place in the pars recta. The unidirectional fluxes of D-glucose, from cell to lumen, and from cell to peritubular fluid, were dissected from the net transepithelial fluxes; outward flux at the basilar pole exceeds exodus at the luminal border by a four-fold margin. Basilar permeability to D-glucose is apparently carried-mediated. When the maximum rate of reabsorption ($Tm_G$) is reached, the limiting component is, accordingly, uptake at the luminal membrane (or intracellular metabolism), not permeability at the basilar membrane.

Tubular reabsorption of D-glucose has long been known to observe a Tm in mammalian kidney *in vivo* [46, 47] (Fig. 3). The observed "splay" in the titration curve relating filtered load to the threshold for glucosuria ($Fmin_G$), and the reabsorption rate, has evinced much argument. Some consider the observation to be compatible with ordinary Michaelis-Menten kinetics for uptake by a single saturable system; others take it as evidence for anatomical heterogeneity among the nephrons performing the functions of filtration and reabsorption [1]. We believe a resolution of this classical argument among renal physiologists is to be found in the hereditary disorders of glucose transport.

Reubi [48] observed two variations upon the normal titration curve in *familial renal glucosuria.* The *type-A variation* is characterized by a low $Fmin_G$ and low $Tm_G$; *type-B glucosuria* has a low $Fmin_G$ but a normal $Tm_G$ (Fig. 3). A kinetic interpretation [3, 6, 49] of glucose reabsorption ascribes the type-A variant to a reduced number of transport sites, while the type-B variant reflects reduced affinity of the hexose carrier for glucose. With this in mind, should a hereditary disorder of glucose transport involve the intestine as well as the kidney, or should the type-A and type-B phenotypes be found in the same pedigree, there would then be little room for the nephron heterogeneity hypothesis as an explanation for splay in solute absorption curves.

When *in vivo* data for hexose transport are compared with data obtained by the kidney cortex slice method, additional points of interest are found. The slice method, which exposes only the basilar membranes of proximal tubular epithelium to the incubation medium (see following), reveals active transport (uptake) of hexoses across these membranes [50, 51]; this finding is analogous to the evidence for active transport *in vivo* [19]. The slice data also reveal more than one type of D-hexose uptake [48]; this finding is also in keeping with the *in vivo* observations [26, 42]. Kleinzeller [49] has delineated a number of homologies between D-hexose uptake *in vitro* by kidney slices and during absorption *in vivo*. However, Silverman, Aganon and Chinard [26, 42] have shown with different techniques that basilar membranes and luminal membranes are not identical in their hexose carrier properties. Therefore, the genetic probes of glucose transport in man assume great importance since they may inform us

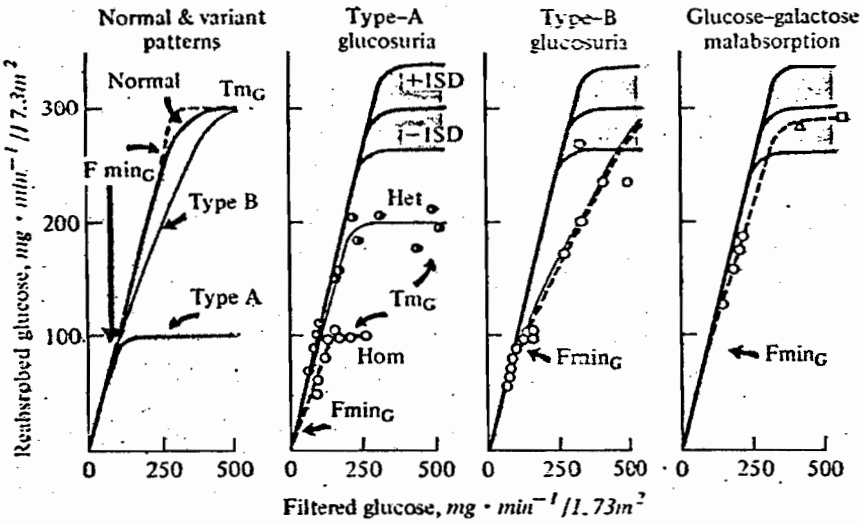



**Fig. 3.** *Glucose titration curves showing maximum transport (reabsorption) rate ($Tm_G$), and venous plasma threshold for glucosuria ($Fmin_G$), in normal subjects and in the hereditary glucosurias.* Theoretical type-A and type-B glucosuria titration curves according to Reubi (1954) are shown in *left panel*; note that the hypothetical curves do not conform to the actual observations *in vivo* shown in the three adjacent panels. Actual type-A glucosuria data (*2nd panel from left*) were redrawn from Elsas, Busse and Rosenberg [39]; type-B glucosuria data (*3rd panel from left*) were redrawn from Elsas and Rosenberg [49]. The data for renal handling of D-glucose in glucose-galactose malabsorption (*right panel*) are taken from Elsas et al (○) [40], Beauvais et al (□) [54] and Abraham et al (△) [55].

about the disposition of hexose carriers in the renal tubule in a manner not revealed by any previous study.

*Glucose-galactose malabsorption* is characterized by severe impairment of hexose transport in the gut and minimal deficit in the renal tubule; *familial renal glucosuria* is a disorder of renal tubular reclamation of D-glucose without an intestinal defect.

In glucose-galactose malabsorption, an uptake system, which resembles the G-system in kidney brush border [26, 42], is deleted in the luminal membrane of intestinal epithelium of homozygotes [40, 52–54]. On the other hand, renal titration studies reveal little [40] or no [54, 55] deviation of $Tm_G$ from normal in homozygotes (Fig. 3). Endogenous glucose metabolism is normal in the disease [55, 56] and the hexose transport defect is not expressed in the erythrocytes, as a representative of nonepithelial tissues [57].

In contrast to these findings in glucose-galactose malabsorption, there is no aberration of glucose transport in the intestine of homozygotes with familial renal glucosuria [58]. However, the renal titration data reveal a very complex picture compared to the renal findings in glucose-galactose malabsorption. (Fig. 3). Studies in three unrelated pedigrees [39, 58] have revealed a "mild" form of homozygous type-A glucosuria inherited from "silent" heterozygotes (COV pedigree, [58]); "severe" type-A glucosuria inherited from "mild" type-A glucosuric heterozygotes (Hold pedigree, [39]) and type-B and "severe" type-A glucosuria in sibs of a pedigree in which there are relatives with "mild" type-A glucosuria (Hol pedigree, [58]. A compelling argument for at least three mutant alleles, at a gene locus specifying a renal glucose transport system, is offered by these observations. Of particular interest is the evidence that even "severe" type-A homozygotes retain about one-third the normal $Tm_G$ (Fig. 3); and that type-B probands (subject II-4, Hol, [58]) have a normal threshold for glucosuria ($Fmin_G$) which is about one-third the normal $Tm_G$.

The combined evidence both from physiologic observations in several mammalian species [19, 26, 42, 47], and from the genetic studies in man suggests the following synthesis and hypothesis. Efficient reclamation of hexose by the proximal tubule is controlled by the luminal membrane. Three types of luminal hexose carriers operate in parallel in this capacity. One is the M carrier [26, 42], a diffusional system of no further interest to us here. The second, which we will call the $G_1$ system, corresponds to the G-system of Silverman et al [26, 42]; this carrier interacts with glucose *and* galactose and is under control of a gene we will call the "glucose-galatose" carrier ($G_1$) locus. We propose that the integrity of the $G_1$ carrier is unmasked in one condition—severe type-A glucosuria which causes deletion of a second ($G_2$) glucose carrier. Evidence for the third carrier, which we call $G_2$, is revealed in homozygous glucose-galactose malabsorption which causes deletion of the $G_1$ carrier and "unmasks" the activity of the $G_2$ carrier.

We estimate that the maximal capacity of the $G_1$ carrier is about one-third the total capacity for D-glucose transport in the normal nephron (Fig. 3). From observations in type-A glucosuria, we deduce that the affinity of the $G_1$ system for D-glucose is less than that of the principle carrier ($G_2$); this fact is revealed by the displacement of the titration curve for the residual glucose transport in homozygous type-A glucosuria (Fig. 3). The $G_2$ carrier appears to be very specific for D-glucose and it has a high affinity and high capacity for D-glucose transport (Fig. 3).

Mutation at the $G_1$ locus causes glucose-galactose malabsorption; mutation at the $G_2$ locus causes familial renal glucosuria. The $G_2$ locus may not be expressed in the intestine according to physiologic and genetic evidence; or if there is an intestinal $G_2$ carrier, it has not been affected by the mutations which permit homozygotes with renal glucosuria to survive and to be recognized [58]. More than one mutant allele has already been described for the proposed $G_2$ locus. One type impairs the capacity of the $G_2$ carrier for D-glucose and causes "mild" or "severe" Reubi type-A glucosuria; genetic and phenotypic evidence [40, 58] implies that there exist two different type-A alleles in this respect. The other allele alters the affinity of the $G_2$ carrier for substrate and causes type-B glucosuria.

*Hereditary taurinuria.* We divert briefly to discuss the first of three animal models of hereditary loss of organic solute. Such examples are rare [3] and, when they occur, the opportunity they provide for *in vitro* investigation is valuable.

We have used the kidney cortex slice method and *in vivo* clearance studies, in parallel, to delineate the location of an inherited impairment of taurine reclamation [59, 60] which has been observed in the mouse. Taurine is the prevalent $\beta$-amino acid in mammalian body fluids and it serves as a marker for a well-documented, $\beta$-amino-acid-preferring transport system in the kidney [38, 60–63].

Wedeen and Weiner have shown that kidney slices do not necessarily expose proximal tubule luminal membranes to uptake from the incubation medium [64-66]. In response, some investigators have been quick to reject the slice method as a useful technique to study solute transport in kidney. We have less reactionary views; we believe the slice method provides an opportunity to study pools-in-series (Fig. 4) which can highlight the topology of transepithelial transport. Quick-freeze, soluble-label autoradiography has revealed the distribution of tritium-labelled, inert solutes after their uptake by cortex slices [64] (Fig. 4, top half). Inulin is confined to an extracellular space in contact with basilar and lateral membranes of proximal tubular epithelium; only in the distal tubule does inulin penetrate the lumen. $\alpha$-Aminoisobutyric acid (AIB) is concentrated within cells of the convoluted and straight portions of proximal tubule, little being found in the lumen; if AIB fluxes into the luminal pool, it is avidly reclaimed *in vitro*. *p*-Aminohippuric acid (PAH) is accumulated maximally in the lumen of straight segment but also in the convoluted portion of proximal tubules. Integrity of the punctate contacts between epithelial cells at their luminal pole is essential for these solute distributions to occur in kidney cortex slices. Specific, net flux orientations across the isolated luminal membrane of the proximal tubule are also necessary to achieve the independent spatial distribution of AIB and PAH. The findings indicate that the peritubular, cytoplasmic and luminal pools of proximal tubules in slices exist in series, and that net reabsorptive and secretory fluxes across the luminal membrane remain intact in slices.

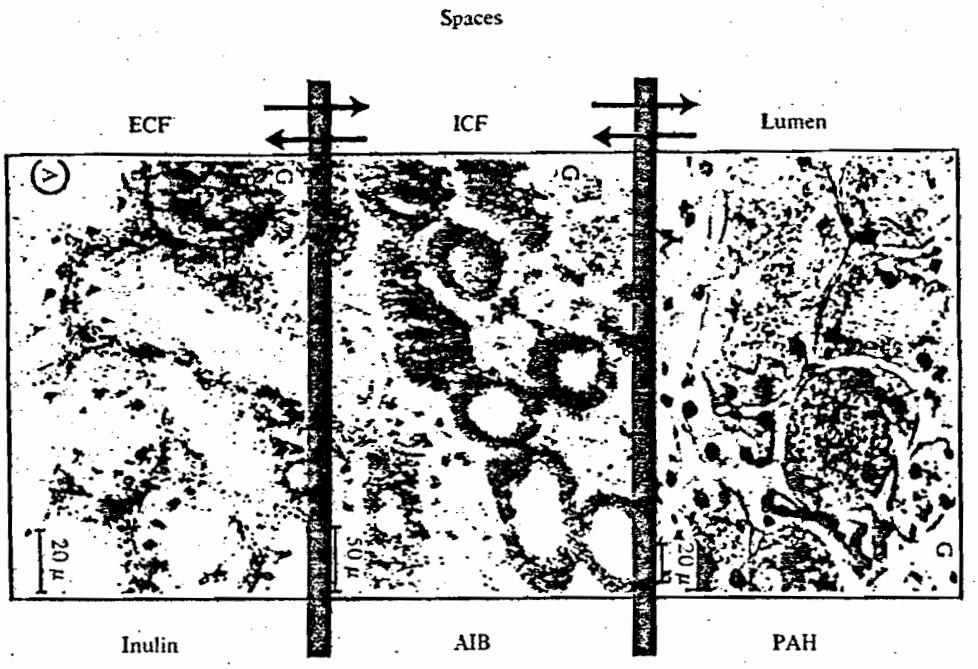
The unique topology of slices was put to use in our study of hereditary taurinuria in the mouse. Taurine is an inert metabolite in mouse kidney and is therefore a useful probe of its transport functions. We investigated three inbred strains of mice available from the Jackson Laboratory, Bar Harbor, Maine: A/J is a normal taurine excretor ($tau^{t+}$) and C57BL/6J and PRO/Re are two homozygous hypertaurinuric strains ($tau^{t-}$). Urine taurine is ten-fold greater in $tau^{t-}$ animals, while plasma taurine is comparable in the three strains. Net tubular reabsorption of taurine is 96.7 ± 1.3% (mean ±SD) of the filtered load in A/J, and 83.9 ± 0.8% and 78.7 ± 5.0% in C57BL/6J and PRO/Re, respectively. Intracellular taurine concentration in outer cortex *in vivo* is similar in the three strains. This important finding, when interpreted according to the kinetics described in Fig. 1, indicates that backflux from an expanded intracellular pool of taurine is not the cause of hypertaurinuria.

Other *in vivo* findings were of interest. $\beta$-Alanine is a competitive inhibitor of taurine transport in kidney [38, 60, 63]. $\beta$-Alanine inhibits taurine reabsorption *in vivo*, in both $tau^{t+}$ and $tau^{t-}$ strains, indicating the retention of a residual taurine transport activity in the nephrons of the latter.

Steady-state uptake of taurine at physiologic concentrations (about 0.5 mM) by thin, outer-cortex slices is clearly greater in $tau^{t-}$ than in $tau^{t+}$ tissue. The higher uptake ratio by $tau^{t-}$ slices is not the result of altered efflux at the basilar membrane slice; efflux from slices is the same in $tau^{t+}$ and $tau^{t-}$ strains.

$\beta$-Alanine, which shares the taurine transport system *in vitro* [38] and *in vivo* [60–63], is also taken up more avidly by $tau^{t-}$ slices. $\beta$-Alanine which is vigorously oxidized by $tau^{t+}$ kidney cortex slices [38] is oxidized less by $tau^{t-}$ slices but normally by slice homogenates with disrupted architecture.

These data suggest a block in concentrative uptake of taurine at the luminal membrane of proximal tubule (Fig. 4). $\beta$-Taurine is not reclaimed efficiently from the luminal "lacuna" of slices (the innermost of the three pools in series) once it has fluxed from cell into lumen. Retention of solute in this pool leads to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Lumen
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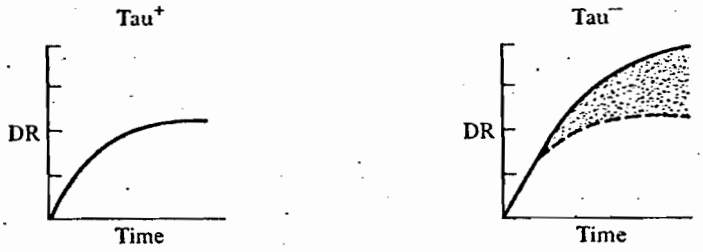
Inulin
AIB
PAH


Tau+
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Tau−
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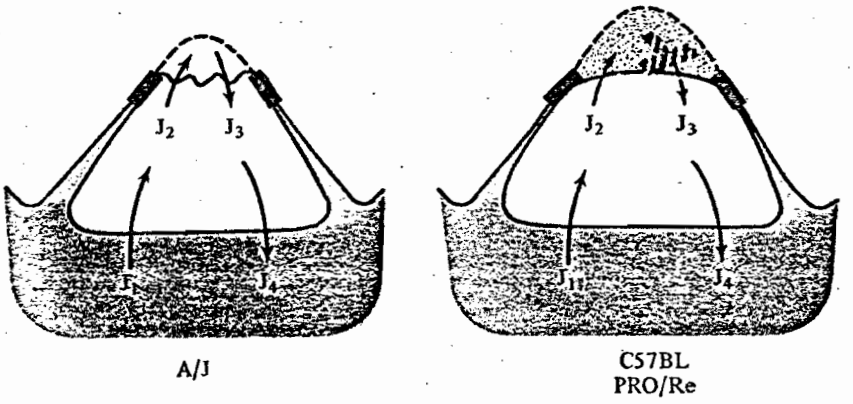
Time


J2
J3
J1
J4
A/J
J2
J3
J1
J4
C57BL
PRO/Re

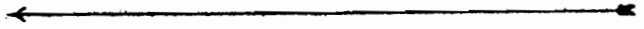


Fig. 4. *An interpretation of enhanced taurine uptake as observed in kidney cortex slices obtained from hypertaurinuric mice (C57BL/6J and PRO/Re strains designated tau$^{t-}$ in text), when compared with normal mice (A/J, designated tau$^{t+}$). Upper half:* Figure shows distribution of $^3$H-inulin, $^3$H- (or $^{14}$C-) α-amino-isobutyrate (AIB) and $^3$H-*p*-aminohippuric acid (PAH) after incubation of rat cortex slices. Inulin does not penetrate the lumen of the proximal tubule, and is confined to extracellular peritubular and glomerular spaces; AIB is concentrated by proximal tubule cells. PAH is maximally concentrated in the lumen of proximal tubule. A concept of "pools-in-series" is implied by these findings (see text). (Rephotographed from Wedeen and Weiner [63].) *Lower half:* Figure shows the enhanced distribution ratio (DR) during time course for taurine uptake by slices. The interpretation for this anomaly is shown in the two sketches at the bottom; it utilizes the "pools-in-series" hypothesis. The *shaded area* indicates the inulin space; the adjacent *clear area* is the cytoplasmic pool; the luminal pool is drawn at the apex bounded by luminal membrane and tight junctions (shown by *solid bars*). The luminal pool can be entered from the extracellular space only through the cytoplasm. Taurine becomes trapped in the luminal pool (*stippled area*) of tau$^{t-}$ slices. Four relevant permeation fluxes are shown: influx across basilar membrane ($J_1$); efflux into luminal pool ($J_2$); reclamation flux from luminal pool ($J_3$); and efflux across basilar membrane ($J_4$). $J_3$ exceeds $J_2$ under normal conditions so that little amino acid is retained in the luminal pool. Assuming $J_3$ = [Tau$_l$] × permeability$_{lc}$, where lc is the lumen-to-cell movement, accumulation of taurine in luminal pool will occur if luminal membrane permeability$_{lc}$ is decreased, all other events being unchanged. The change in $J_3$ in tau$^{t-}$ kidney is presumed to be the result of an hereditary impairment of transport at the urinary surface of the taurine carrier in the luminal membrane (from Chesney RW, Scriver CR, Mohyuddin F, *J. Crin Invest*, vol. 57, 1976, in press).

the higher uptake ratio observed *in vitro*. The *in vivo* data indicate "sequestration" of taurine in the urine pool, a finding also compatible with a luminal membrane transport defect. *In vitro* and *in vivo* data are thus concordant. We believe that *in vivo* and *in vitro* data have been used in parallel, in this case, for topological assignment of a hereditary transport defect to a specific membrane surface in the mammalian nephron. Appropriate studies—both with quick-freeze, soluble-label autoradiography to discern whether labelled taurine accumulates in excess in the lumen of tau$^{t-}$ slices, and with isolated brush border membranes to study taurine binding—will either affirm or dispute these conclusions.

*1B. Defects in the integrity of the (luminal) plasma membrane to efflux.* No hereditary disorder of tubular transport has been proven in this class. However, there is precedent for genetic control of exodus in prokaryocytes [67, 68]; and two disorders in man are reasonable candidates for this type of defect.

Wong, Kashket and Wilson [67] describe a genetic defect of thiogalactoside transport in *Escherichia coli*, and Hectman and Scriver [68] found a mutant strain of *Pseudomonas fluorescens* defective in β-alanine accumulation. Both mutants are unable to concentrate the relevant free solute against a gradient in the intracellular pool, yet both retain the pertinent carrier in their plasma membranes. It was surmized in both that the mutant carrier was unable to prevent solute efflux following uptake into the intracellular pool. With these precedents in mind, it has been proposed [69, 70] that classical cystinuria and X-linked hypophosphaturia may be disorders in which the relevant, specific carriers in the luminal membrane are mutant so as to allow abnormal backflux, while still retaining their carrier functions for facilitated entry.

*Cystinuria* is an autosomal recessive disorder characterized by defective transport of the diamino dicarboxylic amino acid cystine, and the diamino monocarboxylic amino acids lysine, ornithine and arginine [71]. Two or more mutant alleles exist at the gene locus controlling the transport function involved in cystinuria [72]. Net tubular reabsorption of the four amino acids is greatly impaired in mutant homozygotes and in "genetic compounds". It is partially impaired in heterozygotes for two of the three proposed alleles [72].

While $Fmin_{Lys}$ and $Tm_{Lys}$ may both be zero in homozygous cystinuria, Lester and Cusworth [73] have shown that lysine infusion will still provoke enhanced excretion of ornithine, arginine and cystine. They, and others [74, 75], have also shown that the endogenous renal clearance of cystine and the other affected amino acids can exceed the clearance of in-

ulin, so that the "negative reabsorption"[3] of amino acids occurs in cystinuria. On the other hand, cyst(e)ine uptake is only slightly depressed, if at all, in slices prepared from human cystinuric kidney [72, 76, 77, 78]; the representative dibasic amino acid lysine observes a reduced rate of uptake without change in its apparent $K_m$ for uptake [72, 76, 78]. The carrier retains its ability to interact with the dibasic amino acids. By contrast with these findings in kidney, the uptake of both cystine and the dibasic amino acids is clearly impaired in the intestine in homozygous cystinuria [72].

The original hypothesis of Dent and Rose [79], and of Robson and Rose [80], stated that cystinuria is a defect in a selective transport (uptake) system of the tubule shared by cystine and the dibasic amino acids. However, subsequent work *in vitro* has shown that cystine and the dibasic amino acids do *not* share a common system for uptake across the basilar plasma membrane of the tubule as it is exposed in slices of mammalian kidney [60, 81, 82]. On the other hand, cystine, cysteine and the three dibasic amino acids all interact with each other at the luminal membrane, in the normal proximal tubule, presumably on a shared site [83]. The discordance between the properties of uptake sites on luminal and anti-luminal membranes is further ramified when efflux is considered. Schwartzman, Blair and Segal [84, 85] showed that cysteine and the dibasic amino acids shared a membrane efflux site in kidney cortex slices. The properties of the efflux system appear not to be duplicated completely at the corresponding influx site; nor does the efflux carrier experience the customary properties of counterflow. These properties might be accomodated by the behavior of carriers on the outer surfaces of luminal and basilar membranes when exposed in the slice model (see above, hereditary taurinuria discussion) except for the following facts. We know also that a mixed disulfide (cysteine-homocysteine) is prominent in cystinuric urine [86]; that the renal arterial:venous extraction ratio is normal for cysteine, and for cystine, in cystinuria [87]; and that the intracellular cysteine:cystine ratio in normal human and cystinuric renal cortex is normally about 10:1, regardless of the form in which cyst(e)ine enters the cell [77, 88]. Each of these latter observations suggests that intracellular cysteine is the source of excess urinary disulfide in cystinuria.

These disparate observations at first defy coherent interpretation. Moreover, the genetic evidence (Table 1) tells us that mutation, in the form of isolated *hypercystinuria*, and as *hyperdibasicaminoaciduria*, can impair tubular reabsorption of cystine and the dibasic amino acid quite independently, a finding which is explicable only if we assume that the luminal membrane contains reactive sites which are cyst(e)ine-specific and dibasic amino acid-specific, each under the control of separate genes. However, to reiterate, the physiological evidence [83] tells us that a third species of uptake site in the luminal membrane of the mammalian nephron is shared by the five naturally occurring amino acids, but that this site is apparently not present in the basilar membrane [61, 81, 82]. Loss of only the luminal membrane system [83] (shared by cysteine, cystine, lysine, ornithine and arginine) would indeed account for the cystinuria trait as we know it. But this simple interpretation would not explain the stimultaneous observation of zero $Fmin_{Lys}$, zero $Tm_{Lys}$, negative reabsorption of amino acids and competitive interaction between the four amino acids during reabsorption. An alternative and seemingly unifying interpretation is to propose that cystinuria is a defect in a shared efflux system of the plasma membrane so that the relevant intracellular amino acids (cysteine, lysine, ornithine and arginine) experience exaggerated exodus. This interpretation explains most of the *in vitro* data and would account for backflux into urine from the intracellular pool to yield negative reabsorption under certain conditions. The relevant amino acids still interact on a shared luminal membrane uptake system which permits competitive inhibition to occur under certain conditions.

The second candidate for defective luminal efflux is *X-linked hypophosphatemia* (familial hypophosphatemic rickets). In this "phosphopenic" form of rickets [70] tubular reabsorption of phosphate is selectively affected [89]; a corresponding defect in intestinal absorption may also exist [90]. The venerable hypothesis of Albright ascribed the defect in tubular reabsorption of phosphate in familial hypophosphatemia as a consequence of secondary hyperparathyroidism initiated by a primary disorder of vitamin D-dependent calcium absorption in the intestine [91]. However, this is implausible as there are no elevated serum concentrations of C-terminal immunoreactive PTH in mutant hemizygotes unless calcium homeostasis has been altered [70]. Moreover, the anticipated generalized defect in tubular reab-

[3] The term "negative reabsorption" is sometimes used when it is found that the excretion rate (UV) of a solute exceeds its load in glomerular filtrate (F), whereas the customary relationship is F > uv. The term "net secretion" could also be used in this instance provided it was not implied that UV > F was the result of a specific energy-dependent secretion process. When used in this paper, negative reabsorption implies an abnormal finding; secretion is reserved for normal functions in which UV > F is the customary relationship.

sorption of solute which accompanies "calciopenic" secondary hyperparathyroidism [70] is not observed in the X-linked trait. Furthermore, normal urinary cyclic-AMP excretion under basal conditions [89, 92] is partial evidence against the defect being a selective hyperresponsiveness of the tubule to normal levels of circulating PTH [93]. Mutant hemizygotes also manifest a low $Tm_{Pi}$ and negative reabsorption of orthophosphate [89, 92, 94]; these two findings focus interest on a primary transport defect involving orthophosphate.

The fortunate discovery by Eicher and Southard, at the Jackson Laboratory, of an X-linked mutation which causes hypophosphatemic rickets in the mouse has allowed us to investigate, in preliminary fashion, the mechanism of hyperphosphaturia in this presumed model of the human disease. Renal tubular reclamation of orthophosphate is equally diminished in the mutant mouse and in man. Concentrative uptake of phosphate by cortical and medullary slices appears to be normal in the mutant hemizygous mouse; and the intracellular concentration of total and inorganic phosphate also appears to be normal [95].

A hypothesis is proposed to accomodate the findings in X-linked hypophosphatemia. We suggest that the mutation permits excessive luminal efflux (backflux) of cytoplasmic phosphate ion to account for negative reabsorption in the trait. We also suggest that the equilibrium of phosphate between four pools in series may determine its net transepithelial flux; the luminal, cytoplasmic, mitochondrial and peritubular spaces comprise the four phosphate pools. Partition of phosphate in the mitochondrial pool may be found to be important in the transepithelial movement of phosphorus, modulating it in a manner analogous to that proposed for transepithelial movement of calcium [96]. The hypothesis requires us to know whether concentration of phosphate in the cytoplasm available to backflux is dependent to any extent on the amount in the mitochondrial pool. It may be of significance that mitochondria, particularly of proximal tubule cells, are palisaded at the basilar membrane [7], forming an interface between peritubular and cytoplasmic pools. Events which diminish or increase phosphate activity in mitochondria may come to influence phosphate activity in cytoplasm in a series model of transport and may help to explain the various effects of calcium and PTH infusions reported in X-linked hypophosphatemia [89, 92, 93].

It is implied that a disorder of luminal membrane backflux could be expressed in any portion of nephron and could come to influence net reabsorption in both the proximal and distal tubules. Any test of the luminal membrane efflux hypothesis, in cystinuria and X-linked hypophosphatemia for example, should consider this possibility.

*2. Disorders of intracellular pool size.* Interference with metabolic disposal ("run out") of solute could lead to expansion of the intracellular pool in the presence of continuing uptake (Fig. 2). Consequently, the effective concentration of solute which interacts with the luminal carrier at the intracellular interface could increase; under this condition, backflux into the lumen and the moving column of urine must increase, all other events being equal, as the efflux component at the luminal membrane responds to the elevated, internal solute concentration (Fig. 1). In this respect, intracellular metabolism of solute comes to influence its transtubular migration. One cannot avoid thinking of the possibility, that under this circumstance the $T_m$ of a solute may be influenced by its renal metabolism [97, 98].

Renal tubular reabsorption of amino acids has been carefully measured in *phenylketonuria* and *sarcosinemia*, two blocked-catabolic mutant states in man, to determine whether impaired catabolism of the solute impedes its reabsorption [99–102]. In phenylketonuria the hepatic conversion of phenylalanine to tyrosine is almost completely blocked [103]. Phenylalanine hydroxylase activity, with about one-fifth the specific activity of the hepatic enzyme, is found in human renal cortex [104]. However, it is not known whether the renal enzyme is an isozyme or whether it is deficient in phenylketonuria. It is known from measurement of phenylalanine reclamation that there is no abnormality of tubular reabsorption of this amino acid in phenylketonuria [99, 100, 101].

A similar observation has been made for sarcosine reabsorption in sarcosinemia [102]. It is likely that less than 10% of total body sarcosine oxidation takes place in mammalian kidney [102, 105]; a severe loss of sarcosine oxidation in one of our patients was not accompanied by impaired renal reabsorption of this amino acid [102], indicating that renal oxidation of this amino acid is unimportant in its reabsorption. Accumulation of sarcosine in the mutant state actually enhances glycine reabsorption [102] even though sarcosine and glycine interact competitively on their shared uptake sites [6, 102]. Counterflow between the raised intracellular sarcosine and urinary glycine on the luminal carrier appears to be a satisfactory explanation for the behavior of glycine reabsorption in sarcosinemia. The latter finding also suggests a normal backflux exchange activity at the luminal membrane in sarcosinuric kidney and no loss of carrier integrity. It follows from the examples of phenylketonuria and sarcosinemia that an initial increase in

the amount of an amino acid in its peritubular and luminal pools does not come to influence the capacity for its net tubular reabsorption, beyond the normal kinetics of concentration-dependent uptake [13] at the plasma membrane.

Another animal model (the third and last to be mentioned in this review) has provided a valuable opportunity to examine directly the effect on net tubular absorption of an *initial* increase in intracellular pool size [97]. The mutant homozygous PRO/Re mouse has *hyperprolinemia* and less than 1% of the normal proline oxidase activity in kidney [106, 107]. Under normal conditions, proline is reclaimed avidly from urine by the mammalian nephron [61, 108, 109]; the kidney also takes up proline from peritubular plasma *in vivo* [110]. In the PRO/Re mouse, the endogenous proline concentration is eight times normal in plasma and four times normal in kidney cortex; by contrast it is fifty times normal in the urine [97]. The *in vivo*, steady-state uptake of proline across the basilar plasma membrane is not impaired and efflux at this surface is normal; the integrity of the well-documented proline transport systems in these membranes [111, 112] is also retained in PRO/Re kidney. Appropriate studies also reveal the luminal membrane uptake of proline to be intact in the PRO/Re mouse [97].

Proline oxidation in normal mouse kidney is of such large capacity that the intracellular proline pool is kept at a low level [97]; consequently, the normal metabolic outflow comes to influence proline uptake rather dramatically. Normal slices do not observe any expansion of the soluble proline pool until the external substrate concentration is greatly elevated; and saturation of tubular reabsorption *in vivo* ($Tm_{Pro}$) is not observed in the normal mouse until the filtered proline load is augmented far beyond the limit which is required to delineate the $Tm_{Pro}$ in man.

The extraordinary hyperprolinuria which characterizes the PRO/Re mouse can be understood only by taking into account the elevated intracellular concentration of free proline [97]. When we apply Michaelis kinetics (Fig. 1), we see excessive prolinuria in the PRO/Re phenotype as a simple consequence of the primary elevation in the concentration of intracellular proline *in vivo* (depicted as $S_{1b}$ in Fig. 1). Efflux of proline is thus enhanced on the same normal carrier that moves it normally into the cell either from urine, or from peritubular fluid. However, when exodus occurs across the luminal membrane, proline will emerge into a moving column of fluid; since its distal tubular reclamation is insignificant [6], the "lost" proline will appear in bladder urine. Fractional excretion of proline is accordingly elevated in the PRO/Re mutant. The *in vivo* topology of transepithelial transport permits this phenomenon to be observed in PRO/Re kidney whereas the *in vitro* topology does not.

With the hindsight afforded by the PRO/Re model, it is of interest that the venous plasma threshold ($Fmin_{PRO}$) for prolinuria in some human homozygotes with autosomal recessive hyperprolinemia appears to be slightly lower than the threshold observed in normal persons [108]. That finding suggests that renal proline oxidase activity may subtly influence proline reabsorption even in man. It is also apparent from these observations that the classical Tm concept will require reevaluation since renal metabolism of a solute does indeed influence its rate of tubular absorption.

*3. Disorders of exodus at the antiluminal pole.* This type of disorder would impede net transtubular migration of solute (Fig. 3). The result would be enhanced intracellular accumulation of the substrate leading to exaggerated backflux at the luminal surface. Thus far, only experimental models of this mechanism have been reported.

The rapid-injection, multiple-indicator dilution technique has shown [10, 42, 113] that most solutes which interact competitively to augment their fractional excretion do so at the luminal membrane. However, an equivalent competition between solutes during unidirectional exodus at the basilar plasma membrane [84, 85] could also impede net reabsorption. The effect of artificially blocked exodus on fractional excretion has been studied in the ligated ureter preparation *in vivo* [31, 114]. L-Arginine enhances renal clearance of L-lysine in the ligated dog [31] in part by causing the intracellular lysine concentration to increase through competitive interaction between lysine and arginine at the efflux site in the basilar membranes. Corresponding experiments in the rat [114] showed that L-lysine provokes cellular accumulation of $^{35}$S-labelled products derived from extracellular L-$^{35}$S cystine, and increases the renal clearance of L-cystine. These findings illustrate the general theme of this review, that renal uptake and transtubular migration are independent phenomena [115]; they also support the likelihood that blocked efflux of solute from the basilar pole of the epithelial cell can impede net reabsorption. It remains for investigators to find a disease of tubular function in man which fits this interpretation.

### Generalized disorders of tubular transport

The *renal Fanconi syndrome* [116] of various etiologies, and the X-linked, *oculo-cerebro-renal syn-*

*drome* of Lowe, Terry and MacLachlan [117] are examples of generalized disruptions of tubular transport activities.

The Fanconi syndrome can be defined as the integrated clinical manifestations, of whatever cause, resulting from excessive urinary loss of three or more classes of solutes including the amino acids, monosaccharides, electrolytes (phosphate, calcium, bicarbonate, potassium, sodium), uric acid and protein (particularly $\beta$-globulins); water loss also occurs. Substances not normally observed in any quantity in urine may also appear in excess, including lithium, magnesium, insulin and vitamin D and even lysozyme [118]. The increase in fractional excretion of these solutes is largely due to reduced net tubular reclamation. However, impairment of tubular secretion is also observed in the syndrome; for example, $Tm_{PAH}$ is depressed [119].

Morphologic abnormalities may accompany the Fanconi syndrome. The swan-neck lesion, which involves the initial portion of the proximal tubule, is a hallmark [120]. In all likelihood, this lesion is secondary to the underlying cause of the syndrome. The atrophy of the absorptive surface in the anatomial lesion further reduces the membrane activity available for transport. Many species of transport sites are nonetheless still active, in the atrophied epithelium, since the normal competitive interactions between molecules, which share reactive sites, are retained in the Fanconi syndrome [79, 121] (Fig. 5).

A reduced Tm value characterizes the tubular transport of various solutes in the Fanconi syndrome (Fig. 5). This finding alone could be attributed to a simple reduction in the activity or the number of carriers in the membrane (equation1). However, negative reabsorption has also been observed at high plasma solute concentrations in some patients with the syndrome (Fig. 5) and this finding implies more than the loss of carrier activity. The work of Bergeron, Vadeboncoeur and Laporte with the maleic acid model of the Fanconi syndrome [25, 122, 123] is of particular interest in the latter context. Maleic acid is a noncompetitive inhibitor of solute uptake by kidney *in vitro* [125], and it causes the Fanconi syndrome *in vivo* [124]. Within a few hours of exposure to sodium maleate (400 mg/kg i.p.), there is a profound deterioration in the network of perimitochondrial membranes [123]; this anatomical abnormality and the Fanconi syndrome appear and then abate in parallel after the maleate injection. Following peritubular capillary injection of leucine in the maleate-treated rat, the amino acid appears in tubular urine at a rate indicating direct transtubular flux [25]. There is also a reduction in the cellular concentration of the amino acid while its fractional excretion is elevated [122]. Exaggeration of these findings to the point of "negative reabsorption" can be imagined.

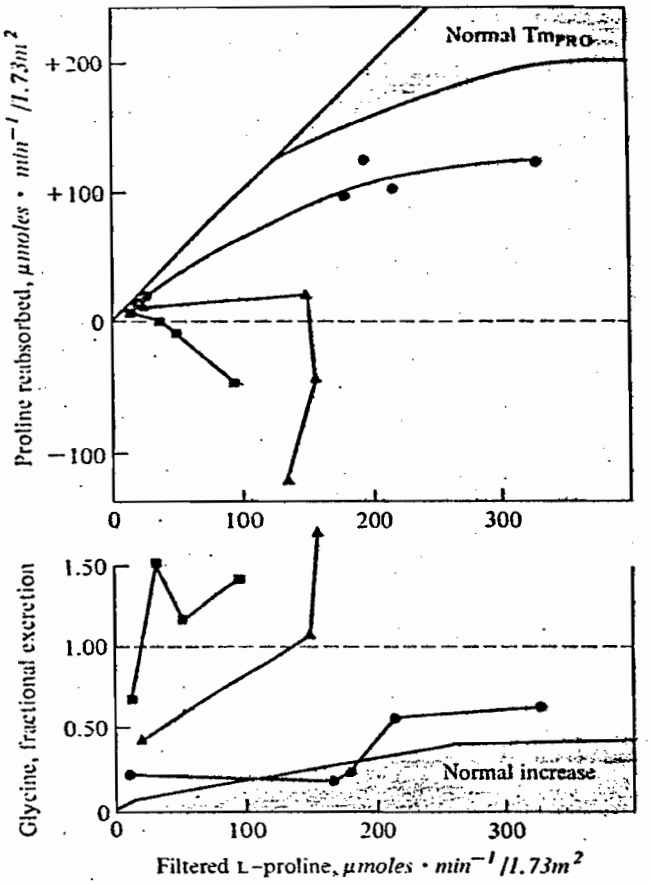



**Fig. 5.** *Upper part: Renal titration curves relating proline reabsorption to filtered load in normal subjects and three patients with the idiopathic Fanconi syndrome.* The lower normal range for $Tm_{PRO}$ [109] is shown by the *shaded area.* Reabsorption is depressed at all concentrations of proline in ultrafiltrate in the Fanconi syndrome. In two subjects, excreted proline exceeded the filtered load when the plasma proline concentration was raised indicating "negative" reabsorption under these conditions. *Lower part: Proline inhibits glycine competitively on a shared, high-capacity system for uptake in the normal subject [109, 111].* The upper normal range for fractional excretion of glycine in the presence of proline is shown by the *shaded area.* Glycine excretion is abnormally increased, even at normal endogenous levels of proline in the Fanconi syndrome, indicating excessive inhibiton of reclamation, or excessive backflux, of glycine on its tubular transport systems [109, 111]. The response to proline inhibition is unusual in two subjects and negative reabsorption occurred. The latter finding indicates retention of membrane sites at which counter-flow inhibiton of uptake may be occurring simultaneously. Negative reabsorption of other amino acids was not observed indicating a selective interaction between proline and glycine at a site whose ability to retain solute has been compromised in the Fanconi syndrome.

"Negative reabsorption" in the human Fanconi syndrome (Fig. 5) and transtubular backflux of solute with reduced cellular accumulation in the maleic acid model in the rat suggest that two fundamental mechanisms are potentially adrift in the syndrome. The

defect in tubular transport may encompass enhanced exodus at the luminal surface after uptake of solute at either pole (see defect 1A, Fig. 2). The mechanism may be a loss of dynamic asymmetry in the carrier secondary to a disorder of energy metabolism and its coupling to the carriers. The other defect concerns integrity of punctate contacts between cells; integrity is maintained by a variety of factors including cellular metabolism.

*Hereditary fructose intolerance* (HFI) [126] is a disease which illustrates how metabolism can be compromised in the Fanconi syndrome. The metabolic abnormality induced by fructose in HFI is initiated by cellular storage of fructose-1-phosphate (F1P) in liver, kidney and bowel [126]. The Fanconi syndrome accompanies this metabolic event [127] waxing and waning with exposure to and withdrawal of D-fructose in the diet. Tissues normally assimilate fructose and vigorously convert it to glucose and lactate. Fructokinase catalyzes the phosphorylation of fructose to F1P; disposal of F1P requires fructose-1-phosphate aldolase which is present in splanchnic tissues and muscle. The aldolase (B-isoenzyme) of splanchnic tissues has strong cleavage activity toward F1P and strong condensing activity toward dihydroxyacetone phosphate and D-glyceraldehyde phosphate [129]. HFI is characterized by deficient aldolase-B activity.

The critical enzymes required for fructose metabolism in human kidney are found in the cortex but not in medulla [128]. This finding constitutes strong indirect evidence that renal cortex metabolizes fructose by the F1P route, and that storage of F1P would occur in this region of kidney in HFI. The association of a precise anatomical location of fructose metabolism in kidney, and provocation of the Fanconi syndrome by exposure to fructose in HFI, is intriguing. The normal activity of fructokinase permits large amount of F1P to accumulate in HFI upon exposure to fructose. This response is accompanied by a fall in serum inorganic phosphorus while cellular adenosine triphosphate (ATP) is consumed to form the F1P [129]. This relationship in HFI constitutes a "futile" hydrolysis of ATP, with depletion of high-energy phosphate and inorganic phosphate pools and it is analogous to the situation in galactosemia in which the Fanconi syndrome also occurs [130].

Parathyroid hormone plays an important modulating role in the pathogenesis of the Fanconi syndrome in HFI [131]. Administration of D-fructose may not provoke the Fanconi syndrome in the absence of the hormone [131]. Parathyroid hormone stimulates renal adenyl cyclase and ATP consumption. The "threshold" defect in ATP metabolism, which appears to exist in HFI, highlights the energy requirements of mechanisms serving transtubular transport. From this perspective it is worth recalling that solutes of different species which do not share common carriers in kidney membranes can still impede each others' reabsorption [132] or uptake [133] by mechanisms which do not involve competitive interaction [133]. Competition for available energy may occur under such circumstances, a mechanism which may explain the unusually intense inhibition of amino acid reabsorption upon exposure to solute loads in the Fanconi syndrome (Fig. 5).

### Disorders of net tubular secretion

There is no known primary hereditary disorder affecting the active tubular secretion of organic solutes, with the possible exception of some forms of *hyperuricemia.* However, one can speculate that organic anions which escape excretion by nonionic diffusion [134], and which require conjugated transport systems for their tubular excretion, are eligible substrates for inborn errors of tubular transport. The recent finding that the proximal tubule contains ligandin [135], an organic anion-binding protein which is antigenically similar to the hepatic Y protein, yields a candidate for an organic anion carrier in kidney. An inherited deficiency of ligandin would provide a significant test of its importance in tubular secretion of organic anions. In the meantime, we can question whether ontogeny of renal ligandin activity, similar to that of hepatic Y protein [136], is an explanation for the abnormally low secretion of substances such as PAH, chloramphenicol and penicillin in the human newborn. An alteration in ligandin-dependent organic acid excretion might even explain why a portion of gouty individuals with normal uric acid production have reduced net renal urate clearance with resultant hyperuricemia [137]. Finally, if it is an important organic anion carrier, we could understand better why hyperuricemia occurs in *type-I glycogenosis, diabetes mellitus, lactic acidosis, maple syrup urine disease* and *fructose-1,6-diphosphatase deficiency* when there is endogenous accumulation of the organic acids peculiar to each of these conditions [133].

Inherited defects of distal tubular acidification are likely to represent true disorders of net tubular secretion. *Classical renal tubular acidosis* (type I or cRTA) occurs when the normal hydrogen ion gradient between tubular urine and plasma is not maintained. Accordingly, there is an inability to lower the urine pH no matter how marked the systemic acidosis [139]. Three abnormalities of hydrogen ion secre-

tion could occur: *1)* $H^+$ secretion may be normal but with excessive back diffusion, so that the normal $H^+$ gradient (800:1-1000:1) between urine and plasma is dissipated; *2)* $H^+$ secretion may be normal but only up to a limited capacity, above which no further acidification can occur; *3)* $H^+$ secretion may be decreased at any urine pH. Recently findings suggest that the third mechanism is an attractive explanation of cRTA [140]. Long ago, Pitts and Lotspeich [141] postulated that the elevated urinary $P_{CO_2}$ in alkaline urine, which is generally 35 mm Hg higher than in plasma, is the result of distal $H^+$ ion secretion followed by delayed, noncatalyzed, dehydration of $H_2CO_3$ to form $CO_2$ and $H_2$. The gradient between urine and plasma was assumed to be dependent on impermeability of the collecting duct to $CO_2O$. Pak Poy and Wrong [142] were the first to observe that the normal $P_{CO_2}$ gradient was absent in patients with distal RTA. This finding was extended by Halperin et al [140] who examined the urinary $P_{CO_2}$ gradient after loading their patients with bicarbonate to maximize $H^+$ secretion. In their opinion [140] the most tenable explanation for the negligible $P_{CO_2}$ gradient which they found is deficient $H^+$ secretion, without production of carbonic acid for delayed dehydration to $CO_2$. Others [143] have provided counter-evidence to this hypothesis, and suggest that the low urinary $P_{CO_2}$ in cRTA may represent excessive backflux of $H_2CO_3$ in alkalosis, and of $H^+$ during acidosis.

Absence of erythrocyte carbonic anhydrase B has been described in a family with *distal RTA and deafness* [144]. This observation is intriguing, in view of the suggestion of Maren [145] that distal bicarbonate reclamation is entirely dependent on intracellular carbonic anhydrase activity, which provides $H^+$ ions to combine with $HCO_2^-$. Distal tubular carbonic anhydrase has not yet been shown to be abnormal in these patients.

### Disorders accompanied by altered tubule-hormone interaction

Hormones regulate several tubular transport functions. Such transport activities will be modified if the tubule is hyperresponsive to normal (or elevated) serum concentrations of the relevant hormone; or if the tubule receptor and translation system are unresponsive to the normal regulatory action of the hormone. Both phenomena can be illustrated by inherited disorders which involve parathyroid hormone (PTH). PTH-responsive, membrane-dependent activities appear to be localized to the basilar surface of outer cortical tubule segments [146].

*Autosomal recessive vitamin D dependency* (ARVDD) [70] is a disorder in which the renal synthesis of 1$\alpha$,25-dihydroxycholecalciferol, a hormonal form of vitamin D, is apparently defective. A "calciopenic" form [70] of postnatal rickets develops, accompanied by secondary hyperparathyroidism; a generalized defect in proximal tubular transport follows. All abnormalities disappear when pharmacologic doses of vitamin D, or quasi-physiologic doses of 1$\alpha$-hydroxyvitamin D analogues, are given. The disorder of tubular transport in ARVDD is related, in some manner, to the constellation of vitamin D hormone and calcium depletion and of PTH excess. Depression of extracellular calcium ion [147] or elevation of cytoplasmic calcium [148] increases plasma membrane permeability. Calciotropic hormones which can modify cytoplasmic calcium alter tubular transport of amino acids and other solutes [149]. Depression of cytoplasmic calcium also increases tight-junction permeability [150]. Depression of cytoplasmic calcium is likely to occur in ARVDD, the result being a change in membrane and transtubular permeability, so that reclamation of solute may be depressed or backflux enhanced. It is also apparent from recent work [96] that the excess of PTH and the depletion of vitamin D hormone, which characterized ARVDD, will combine to deplete mitochondrial and cellular calcium and further alter calcium-dependent cellular permeability.

*Pseudohypoparathyroidism* of the *classical type* (PHP, type I) is an example of tubular unresponsiveness to PTH. In this disease, there is a failure of PTH infusion to augment urinary cyclic 3', 5'-adenosine monophosphate (AMP) [151] and fractional excretion of phosphate is diminished. Endogenous serum PTH levels are typically elevated while serum calcium remains low and phosphorus is elevated. Recent studies [152] have shown that the PTH receptor and adenyl cyclase in renal cortex are apparently intact in PHP-type I; it is the mechanism of cellular response to PTH and the effect on solute transport which are defective. On the other hand, cyclic AMP-dependent mechanisms in the tubules responsive to hormones other than PTH seem to be intact although this facet of the problem has not been rigorously examined to our knowledge. It is of interest that the renal adenyl cyclases responsive to PTH, calcitonin and vasopressin are preferentially located in outer cortex, inner cortex and medulla, respectively [146]. The basis for the insensitivity of adenyl cyclase to PTH in the intact tubule in PTH-type I remains obscure.

A variant of PHP known as *type II PHP*, has been reported [153, 154]. In this trait, fractional excretion of phosphate remains insensitive to PTH infusion,

but a brisk increase in urinary cyclic AMP testifies to a responsive renal adenyl cyclase. PHP type II is further distinguished by a normal serum concentration of inorganic phosphate, a feature which remains unexplained at present. Many facets of the tubular metabolism and handling of vitamin D, phosphate and calcium, as well as of cyclic AMP metabolism regulated by PTH, are pertinent to the interpretation of PHP, but are beyond the scope of this review.

### Commentary

In this review we have used some of the inborn errors of tubular transport listed in Table 1 to illustrate, in a specific manner, the various components of transtubular movement of solute. These experiments of nature, as well as the steadily increasing body of laboratory experimentation in man and animals, support what others have proposed recently [31, 97, 114, 115]; that accumulation of solute by renal tubule cells and its transtubular transport are each essentially different processes, the former being most evident in the straight protion, the latter in the convoluted portion of proximal tubule [115]. The normal topology of absorbing epithelium provides the framework for these concepts which, being of fairly recent origin, are still likely to be a source of controversy as well as a stimulus for further investigation. On the other hand, if they are valid, it should not be surprising that tissues lacking the topological orientations of epithelium (e.g., erythrocytes, blood leukocytes and cultured skin fibroblasts) fail to show any abnormality of solute transport wherever the investigation has been performed, as in cystinuria [155], Hartnup disease [33], iminoglycinuria [156], glucose-galactose malabsorption [57] and X-linked hypophosphatemia [157], for example.

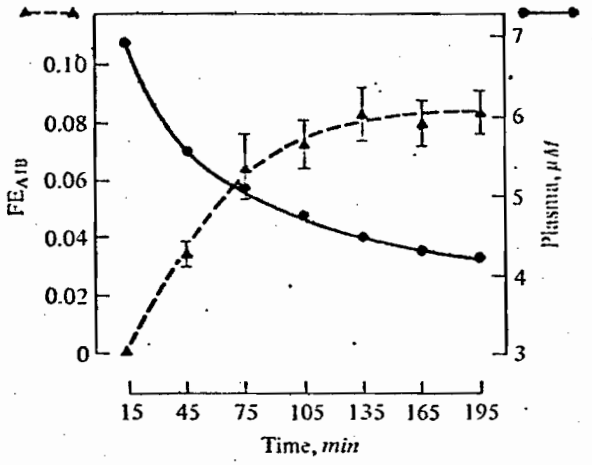



**Fig. 6.** *Renal clearance studies in the rat after bolus injection of $^{14}$C-labelled α-amino-isobutyrate (AIB), an inert synthetic amino acid.* Fractional excretion (*left ordinate*) *rises* above the 15-min value (set at zero to accommodate interindividual variation between 0.03 and 0.05 at 15 min), to approach a steady state after 90 min while the plasma concentration of AIB is falling (•—•, (*right ordinate*). $^{3}$H-inulin clearance remained stable during the experiment. The reciprocal fall in fractional reabsorption of AIB with time could reflect saturation of an intrarenal binding process; this explanation is unlikely since reinjection of AIB, or elevation of AIB plasma concentration, did not alter the phenomenon. AIB equilibration between peritubular fluid and epithelium followed by backflux into the lumen is a more likely explanation. AIB concentration fell in the cortex between 30 and 195 min (from 1284 ± 25 cpm/mg of wet wt of prepared slice, mean ± SD [$N$ = 16] to 953 ± 26 cpm/mg [9$N$ = 16], whereas the concentration remained unchanged in *medulla* (from 830 ± 218 cpm/mg [$N$ = 8] to 923 ± 274 cpm/mg [$N$ = 8]). When considered with the plasma AIB levels, these figures indicate that although the distribution ratio of AIB is the same in cortex and medulla at 3½ hr, it is significantly less in the medulla than the cortex at 30 min. These tissue changes were not an artefact of changing urine or plasma AIB concentration. The findings suggest that a slowly accumulating tissue pool of AIB in the medullary portion of the nephron is a potential source for enhanced luminal exodus (backflux) and, thus, of the observed increase in fractional excretion (McInnes RR, Scriber CR, unpublished data, [149]).

Various elegant experimental methods have provided the important evidence for normal backflux across the luminal membrane, transtubular fluxes in both directions and differential handling of solute uptake and migration in different regions of the kidney (e.g., [19, 25, 31, 83, 114, 115].) Even the traditional "black box" clearance method appears to honor this theme (Fig. 6). As a result of this new awareness, our interpretation of altered Tm values, fractional reabsorbtion rates relative to solute concentration and other clinical indices of altered net tubular transport in the inborn errors of tubular function is likely to undergo refinement in the future. All of this will benefit diagnosis, counselling and treatment of patients with hereditary disorders of tubular transport.

The expression of mutant alleles which affect the gene products controlling the various stages of transtubular transport is also complex [3, 6]. The gene loci which determine the structure and quantity of the relevant proteins are likely to exhibit one or more mutant alleles per inborn error of transport. Genetic heterogeneity underlies a great many monogenic diseases [158] and the hereditary disorders of tubular transport are no exception. Consequently, it is not surprizing that, for example, three different mutant alleles, each apparently at the same "cystinuria" gene locus, occur among subjects with cystinuria [72]; and that four mutant alleles, all apparently at the locus specifying the shared transport system for imino acids and glycine, are now required to explain the different forms of familial renal iminoglycinuria [6, 109]; and that different alleles at two independent gene loci controlling glucose transport are needed to

explain glucose-galactose malabsorption and renal glucosuria [39,40]. The discovery of two different phenotypes in the pseudohypoparathyroidism trait, one with blunted renal cyclic nucleotide formation and one without, again argues for mutant alleles which probably occur at different gene loci and which control different components of the relevant transport processes. It follows that recognition of genetic heterogeneity will also help to direct the physician toward more precise diagnosis, counselling and treatment.

Appropriate dissection of tubular function in man through genetic "probes" has revealed an important general underlying theme. Loss of a specific transport function rarely leaves the homozygous proband devoid of all function for the particular transport in question [160]. For example, homozygotes with renal iminoglycinuria retain a significant fraction of proline reclamation under endogenous conditions [109]; and homozygotes with severe type-A glucosuria retain one-third of their maximum glucose reabsorptive capacity in kidney and have lost none in the intestine [39, 58]. These manifestations of "residual" transport in mutants reflect functional redundancy in the membrane carriers and in the genes which specify transport options for solutes. The normal ontogeny of tubular function in the postnatal period also reveals diversity among carriers, some being present at birth, others disappearing or appearing on schedule postnatally during renal maturation [112, 161]. Genetics and ontogeny, in combination with kinetic and chemical probes [3, 6], have revealed a "horizontal" diversity of membrane sites which allows them to distinguish the chemical identity of solutes; and a "vertical" diversity which allows them to recognize a solute differently when it is present at high or at low concentration.

This diversity among solute transport mechanisms thus provides an adaptive function which protects the organism from the effect of transport mutations [160]; it also provides alternate systems which can be utilized in treatment. Replacement of solute has been accomplished in several of the hereditary tubulopathies. Phosphate replacement is a valuable component of treatment in X-linked hypophosphatemia and the Fanconi syndrome; bicarbonate replacement can be achieved in several forms of RTA. To know both the biological basis and the symptomatic manifestations of the hereditary tubulopathies ought to provide present-day students of Mendel, Darwin and Garrod with the means to relax selection effectively against these intriguing mutations in man.

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SECTION IV

## Renal Handling of Phosphate in Vivo and in Vitro by the X-linked Hypophosphatemic Male Mouse (Hyp/Y). Evidence for a Defect in the Brush Border Membrane.

This study examined the renal transport of phosphate in normal mice and in mutants (Hyp/Y) with X-linked hypophosphatemia (XLH). This murine mutant appears to be homologous to one of the classical human disorders of solute transport, vitamin-D resistant X-linked hypophosphatemic rickets. The argument for homology, or at least that the mutations involve the same gene product, is based on the close similarity of the phenotypes and on the stability of the X-chromosome during evolution (Ref. 15, this section). Investigation of this mouse mutant has allowed the first assignment, using direct evidence, of an inborn error of renal transport to a specific component of the net reabsorptive process.

A defect in net renal phosphate transport was demonstrated in the Hyp/Y mouse in in vivo experiments, and this solute loss at least partially accounts for the hypophosphatemia. No evidence indicated that hyperparathyroidism is present in Hyp/Y mice, so a detailed analysis was made of phosphate transport by normal and mutant mouse kidney in vitro. Although phosphate transport across the basolateral membrane of renal cortical cells is intact in vitro, a specific component of phosphate uptake at the luminal membrane was shown to be defective using brush-border vesicles.

In addition to defining the defective transport system at a subcellular level, this work illustrates that mechanisms serving transport of a solute at the luminal vs. basal surfaces of the absorptive epithelium can have different properties because they are under the control of independent

genes. Furthermore, it reinforces the theme that the renal transport of any one solute may be served by more than one transport system, in a specific membrane and that the deletion of one of the systems by mutation serves to expose the presence of another.

The specific part of this research executed by the candidate was the measurement of fractional phosphate excretion in the normal and mutant animals. Evidence for an intrinsic defect in vivo warranted further investigation in vitro by colleagues.

The manuscript describing this work has been accepted for publication in Kidney International in 1978 (see letter of acceptance from the editor, next page).

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Dear Dr. Tenenhouse, M.D.

The revised version of your manuscript entitled "Renal handling of phosphate in vivo and in vitro by the X-linked hypophosphatemic male mouse (Hyp/Y). Evidence for a defect in the brush border membrane" has been received and considered by the Editors. In their opinion, the major comments of the referees have been handled satisfactorily, and the manuscript is now felt to be acceptable for publication in this Journal. It will be scheduled to appear in an early issue.

You should receive the page proofs for your article within approximately 12 to 16 weeks. Please correct the proofs and return them to me directly within 72 hours of their receipt by you (along with the copy-edited typescript that will accompnay the proofs). Otherwise, it may be necessary to return the proofs to the printer with only those corrections as made by the Editorial Office alone.

Again, thank you for the opportunity to consider your interesting manuscript.

Sincerely yours,

Vincent W. Dennis, M.D.

VWD:rc

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# RENAL HANDLING OF PHOSPHATE IN VIVO AND IN VITRO BY THE X-LINKED HYPOPHOSPHATEMIC MALE MOUSE (Hyp/Y). EVIDENCE FOR A DEFECT IN THE BRUSH BORDER MEMBRANE

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Running Title: Renal Phosphate Transport in Hyp/Y Mouse

## SUMMARY

We have examined renal phosphate transport (in vitro) and reabsorption (in vivo) in hypophosphatemic Hyp/Y (male mutant) mice carrying a gene believed to be homologous with that responsible for X-linked hypophosphatemia in man. Normophosphatemic male littermates (+/Y) were used as controls. Fractional excretion of phosphate in Hyp/Y is increased (0.351 ± 0.073, mean ± SD (n=7) vs 0.210 ± 0.0885 (n=15) in +/Y ($p < 0.01$)), at serum phosphorus concentrations of 34.6 ± 7.6 mg/L and 56.8 ± 9.16 mg/L respectively ($p < 0.001$). Phosphate clearance exceeds creatinine clearance in Hyp/Y mice when their drinking water contains added phosphorus (2 g/L). Urinary cyclic 3'5'-adenosine monophosphate is increased in Hyp/Y mice when compared with +/Y littermates; but this finding which is different from the human phenotype is not accompanied by evidence of hyperparathyroidism in the Hyp/Y mouse. Serum PTH rises during phosphate loading in Hyp/Y as it does in the human patient.

The phosphorus concentration in kidney cortex is similar in mutant and normal mice (Hyp/Y, 46.6 ± 1.1 matoms per g protein; +/Y, 46.6 ± 1.0 matoms per g protein). Uptake of $^{32}P$-labelled phosphate, at 0.1 and 1.0 matoms per L in the medium, by slices of cortex or medulla, and labelling of intracellular inorganic and organic pools of slices observes first-order kinetics and is not significantly different in the two strains of mice. Modulation of calcium in the diet influences these observations in both strains but does not disclose differences between them.

Purified brush border membrane vesicles prepared from mouse kidney cortex homogenates accumulate phosphate by a $Na^+$-dependent, arsenate-inhibited transport system. Total ($Na^+$-dependent plus $Na^+$-independent) uptake of phosphate by +/Y vesicles is 488 ± 23 patoms/mg protein per 60s (mean ± SD) and 302 ± 10 patoms/mg protein per 60s by Hyp/Y vesicles ($p < 0.001$); $Na^+$-independent uptake is similar in the two types of vesicles (79 and 78 patoms/mg protein per 60s respectively). Uptake of D-glucose and relative purity of the brush border membrane fraction are not different in the two strains.

The combined in vivo and in vitro data indicate that impaired net transepithelial transport of phosphate in the X-linked phenotype is the result of an intrinsic defect in net flux of phosphate at the brush border membrane.

## INTRODUCTION

X-linked hypophosphatemia (XLH) in man has long been classified as a form of rickets "refractory to vitamin D" (1-3). The tradition has been to postulate a disorder involving vitamin D-dependent metabolism in this condition. On the other hand, there is evidence indicating that XLH is an inborn error of phosphate transport (3-6). X-linkage of this Mendelian trait (2) confirms that it is a typical inborn error of metabolism, of one type or another.

The discovery of a murine homologue (Hyp) of the mutant human gene (7) provides an invaluable resource for investigation of the mechanism for hypophosphatemia in the X-linked trait. O'Doherty, DeLuca and Eicher (8) have published their evidence for a primary defect in phosphate transport in the intestine of the mutant male (Hyp/Y) mouse. In the following report, we describe net tubular reabsorption (net transepithelial transport) of phosphate in the Hyp/Y mouse in vivo, uptake and efflux of phosphate by thin slices of renal cortex or medulla in vitro, and uptake of phosphate by purified brush border membrane vesicles. The findings indicate that a selective, intrinsic transport defect is present in the brush border membrane of the Hyp/Y mouse.

## METHODS AND MATERIALS

Mice: The Hyp gene (7) was maintained in the inbred C57Bl/6J mouse; Hyp/Y and +/Y litter mates were used in our studies. Breeding pairs were obtained from Jackson Laboratory, Bar Harbor, Maine. The animals were fed mouse chow (0.89% calcium, 0.63% phosphorus, Ralston Purina of Can. Ltd., Mtl)

or Old Guilford 96W (0.22% calcium, 0.74% phosphorus, Jackson Lab., Bar Harbor, Maine).

In Vivo Investigations:

Infusion Studies - Mice were fed overnight, before receiving Inactin, 40 µg/g intraperitoneal (BYK Gulben, Konstanz, West Germany). The mouse was placed supine in the pan of a beam balance and body temperature kept at 37°C under a 75-watt incandescent bulb controlled by a rectal thermistor probe. The trachea was opened below the thyroid cartilage and the lower tracheal ring sutured to the skin. The right external jugular vein and the urinary bladder were catheterized with polyethylene tubing. A solution (5.22 ml) was prepared for venous infusion containing: KCl (2.3 mM), NaCl (60 mM), glucose 1% (w/v) and inulin (0.78 ml, Armor-Stone Lab., Inc., Mount Prospect, Ill.) to which was added 20 µCi inulin ([methoxy-$^{3}$H], 50-150 mCi/gm, New England Nuclear, Boston, Mass.). Radiochemical purity of the inulin was confirmed by chromatographic methods. Rapid infusion of the solution (50 µl over 5 min.) was followed by slow infusion (14 µl per g·hr) delivered by a Sage pump to achieve a steady-state plasma inulin concentration of at least 20 mg/dl. When urine flow had stabilized (in 4-6 hr), urine collections (60-85 min. duration) were made through the indwelling bladder catheter; dead space is about 25 µl in the preparation. Whole blood (25 µl) was collected into heparinized capillary tubes from the tail vein near the mid-point of each urine collection period. After centrifugation to determine hematocrit, the plasma fraction was recovered for analysis of

inulin and phosphorus. Tritium content of plasma and urine was measured in a Packard scintillation spectrometer; inorganic phosphorus was measured by a micro-method kit available from Pierce Chem. Co., Rockford, Ill.

Metabolite Excretion Studies. Urinary excretion of cyclic 3'5'-adenosine monophosphate, phosphate, amino acids, glucose and creatinine were measured in fasting Hyp/Y and +/Y litter mates placed in Gelman cages. Urine collections were obtained during the morning, after feeding ad libitum on selected diets for one week. The urine samples were analyzed for cyclic AMP by a competitive binding assay (available as a kit from Diganostic Products Corp., Los Angeles, CA 90064). The excretion data are expressed as a coefficient of creatinine excretion. Amino acids, glucose and creatinine excretion were measured in Hyp/Y and +/Y mice by standard qualitative and quantitative methods.

Phosphate excretion was measured in +/Y and Hyp/Y mice fed a phosphate supplemented diet for 75 days beginning the 4th-6th week after birth. Phosphate was provided in the drinking water (2 g/L). On the 146h-16th week of the study, mice were placed in Gelman cages for 15 hr and blood was collected by retro-orbital puncture at the end of the urine collection period. Phosphorus and creatinine were measured in plasma and urine and expressed as mg/ml. Phosphorus excretion was expressed by the equation,

$(Urine_{Pi} \times Plasma_{cr}) \div (Plasma_{Pi} \times Urine_{cr})$ which is equivalent to $FE_{Pi}$.

Serum PTH was measured by a standard radioimmune displacement assay using CH-14 Mayo antiserum (kindly provided by Claude Arnaud) and 1-34 bovine PTH standard.

In Vitro Investigations

Preparation of Slices - Mice were stunned and decapitated, the kidneys removed, the capsules stripped and each kidney divided into ventral and dorsal halves. Thin cortex slices (about 0.2 mm thick) were obtained with a Stadie-Rigg microtome, and then placed on filter paper moistened with chilled saline over ice and trimmed to about 4 mg weight; medulla slices were prepared in similar fashion from kidneys hemisectioned into fore and hind halves and trimmed of cortex. Slices were used in the experiments within 20 min. after removal of the kidney from the animal.

Incubation of Slices - The incubation buffer contained: NaCl, 0.12 M; KCl, 5 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2 mM; Tris-HCl, 20 mM; glucose, 5 mM; final pH, 7.4, and final osmolarity, 300 mOsm. Labelled phosphate (as $NaH_2{}^{32}PO_4$ New England Nuclear, Boston, Mass.) was added to sodium phosphate buffer pH 7.3 (0.1-1.0 matom/L, final conc.) to yield about $10^7$ cpm/ml. $^{14}C$-labelled inulin was used to estimate extracellular water, excluding water in luminal lacuna; total tissue water was determined by dessication to constant dry weight (9).

For the measurement of phosphate uptake, slices were added to a flask containing 2 ml incubation medium at $37^oC$ in a Dubnoff shaking incubator gassed with 100% oxygen at 7 L per min. After the appropriate period, the tissue was removed, blotted, weighed and homogenized immediately in 1 ml

cold trichloracetic acid (10% w/v). Homogenate was then centrifuged at 4°C for 10 min. at 8,000 g. An aliquot of the supernatant was counted in Aquasol-2 (New England Nuclear, Boston, Mass.) in a Packard Liquid Scintillation counter to determine the total (Trichloracetic acid-soluble) phosphate pool ($P_T$); another aliquot of the supernatant was extracted according to the method of Vestergaard-Bogind (10) to determine inorganic phosphate (Pi). Reliability of the method was evaluated with $\gamma$-$^{32}$P-labelled ATP standard; contamination of theinorganic pool by Pi released from ATP is less than 1% in our experience.

Measurement of Efflux - Ten cortex slices were incubated in 10 ml incubation medium (the uptake medium), containing labelled phosphate. Two pairs of slices were removed when an isotopic steady-state had been achieved (about 60 min.) and then processed to measure $^{32}$P uptake into $P_T$ and Pi pools. The remaining slices were blotted, weighed and transferred into 10 ml incubation medium at 37°C (the efflux medium), containing unlabelled phosphate at the concentration used in the uptake medium. The flask was shaken at 300 oscillation per min. in a Dubnoff incubator during efflux; 0.3 ml aliquots of efflux medium were removed at 1 minute intervals, after the first 6 minutes of incubation, for 30 min., and placed in scintillation vials; Aquasol-2 was added for counting. At the end of incubation, the slices were processed to determine the number of counts remaining in the tissue. Results are expressed as $^{32}$P appearing in the efflux medium in relation to time; and also as $^{32}$P remaining

in the slices. The latter is expressed as percent of the total cpm in the medium and tissue, and plotted as a function of time in the traditional manner on semilog graph paper to calculate rate of efflux from the slopes.

Preparation of Brush Border Membrane Vesicles - Brush border membrane vesicles were prepared from fresh renal cortex by the method of Booth and Kenny (11). About 2.0 mg of brush border protein were obtained from 9 mice. Hyp/Y and +/Y littermates were studied in parallel on the same day. Purity of brush border membranes was evaluated by monitoring trehalase and alkaline phosphatase activities; succinic cytochrome C reductase was used as a marker for mitochondrial contamination. Transport of labelled phosphate (0.1 matom/L) was measured with a preparation of brush border membrane vesicles according to the methods of Hoffmann et al (12). The isotope used was $^{32}$P-phosphoric acid in 0.02 N HCl, (New England Nuclear catalogue #NEX-054, Boston, Mass.). Each experiment was monitored to offset the known variation in uptake of solute between batches of membranes by measuring transport of $^{14}$C-D-glucose (13) at the same time in the same tube. Radiolabelled D-glucose was obtained from New England Nuclear, Boston, Mass. (Cat.#NEC-042X). A detailed description of the mouse kidney brush border membrane preparation and a comparison of phosphate uptake by normal mouse membrane vesicles and normal rat membrane vesicles is available elsewhere (14).

## RESULTS

### Fractional Excretion of Phosphate ($FE_{Pi}$) In Vivo

We measured $FE_{Pi}$ in +/Y and Hyp/Y animals under the infusion protocol. $FE_{Pi}$ is significantly increased in Hyp/Y mice (Hyp/Y = 0.351 ± 0.073; +/Y = 0.210 ± 0.083, mean ± SD, $p < 0.01$, Student's *t* test) (Table 1) even though plasma phosphorus level is lower in Hyp/Y mice (Hyp/Y, 34.6 ± 7.6 mg/L; +/Y, 56.8 ± 9.16 mg/l, mean ± SD, $p < 0.001$). When tubular reabsorption of phosphorus is expressed as a percent of filtered load, net reabsorption is 80% in +/Y mice and 65.3% in Hyp/Y, at their usual serum phosphorus level.

Fractional excretion of phosphate by animals housed in metabolism cages was elevated in Hyp/Y mice compared to +/Y animals whether they were fed Old Guilford 96W or mouse chow (see Ref. 7). Hyp/Y mice (n=4) receiving a phosphate supplement in the drinking water increased their $FE_{Pi}$ from 0.486 ± 0.045 mean ± SEM to 1.35 ± 0.07 ($p < 0.001$) while +/Y mice (n=12) on the same regimen increased their $FE_{Pi}$ from 0.244 ± 0.029 to 0.709 ± 0.034 ($p < 0.001$). The interstrain differences were also statistically significant ($p < 0.001$).

Urinary excretion of cyclic 3'5'-adenosine monophosphate is increased in Hyp/Y mice (Table 2) whether they are fed low-calcium or normal-calcium diets. Cyclic AMP excretion is higher when Hyp/Y and +/Y littermates are fed a low-calcium diet. Urinary excretion of amino acids and glucose is not abnormal or elevated in Hyp/Y animals.

Parathyroid gland histology has not indicated hyperparathyroidism in these animals (7) and +/Y and Hyp/Y

animals have comparable serum iPTH levels (7,21). Phosphate supplementation of the diet caused serum iPTH to increase in Hyp/Y animals (n=3) from 84 pg/ml (pre-treatment) to 775 pg/ml in the 10th week of treatment.

Uptake of Phosphate by Slices, In Vitro

We examined uptake of $^{32}P$ by kidney cortex slices obtained from +/Y and Hyp/Y mice maintained on two different diets. At steady-state (60 min. incubation) phosphate is taken up into cellular pools against an isotopic gradient ($^{32}P$ distribution ratio > 1.0) (Table 3). The isotopic distribution ratio varies inversely with extracellular phosphate concentration. Labelling of intracellular inorganic phosphorus (Pi) is similar in +/Y and Hyp/Y mice at 0.1 and 1.0 matom per L extracellular phosphorus; the same is true for the labelling of intracellular acid-soluble phosphorus ($P_T$) indicating that incorporation of Pi into organic phosphate is normal. We also found no difference in the labelling of phosphorus pools in +/Y and Hyp/Y of slices prepared from renal medulla. Anaerobic conditions abolished concentrative uptake of labelled phosphate into inorganic and total phosphate pools.

We also measured the inorganic phosphorus in the intracellular pool, directly, by a chemical method. There is no significant difference between kidney slices obtained from +/Y and Hyp/Y mice at zero time or under various conditions of incubation (Table 4) even though blood phosphorus levels are different in +/Y and Hyp/Y mice. The inorganic phosphorus content of cortex slices declines exponentially with time

during incubation in +/Y and Hyp/Y mice (Figure 1).

No significant interstrain difference in tissue phosphorus was observed between Hyp/Y and +/Y mice. The $^{32}P$-labelled inorganic phosphate and total phosphate pools in kidney are lower, on the average, when mice are fed the Old Guilford (low calcium) diet compared to the mouse chow diet (Table 3). On the other hand the chemical inorganic phosphorus content (Table 4) is slightly higher in mice fed Old Guilford. Intrastrain isotopic distribution ratios (Table 3) and chemically determined inorganic phosphorus (Table 4) measured at 15 min. in slices from mice fed on the various diets did show some variation. We have no explanation for these diet-dependent, intra-strain differences. They do not perturb the principal finding of essentially similar tissue phosphorus levels in Hyp/Y and +/Y mice under a variety of conditions.

Efflux of Phosphate Anion from Kidney Cortex Slices

We studied the characteristics of phosphate efflux from kidney cortex slices of +/Y and Hyp/Y mice (Figure 2). Efflux of $^{32}P$, under conditions of transequilibrium with respect to phosphate concentration, proceeds at similar rates from Hyp/Y and +/Y slices; the efflux process observes first-order kinetics. $^{32}P$ in the efflux medium is exclusively inorganic phosphate; chemical data (Table 4 and Figure 1) and isotopic data (Fig. 2) are, therefore, concordant. Detailed examination of efflux by regression analysis, calculation of the Y intercept and estimation of the correlation coefficient for the slopes shown in figure 2B again indicate

no difference in the characteristics of $^{32}$P-efflux from Hyp/Y and +/Y cortex slices (Table 5).

Phosphate Transport by Brush Border Membrane Vesicles.

Our investigation of phosphate uptake by +/Y and Hyp/Y mouse kidney brush border membrane vesicles utilized control experiments with vesicles prepared from rat kidney. These experiments are reported in more detail elsewhere (14). Our findings in the rat, are comparable to those reported by Kinne's group (12). The behaviour of +/Y mouse kidney vesicles was then compared with rat kidney vesicles. The general characteristics of phosphate uptake by brush border membrane vesicles is comparable in the two species (14). We further observed that the phosphate volume of the vesicles under conditions approaching equilibrium and in the presence of sucrose is inversely proportional to sucrose osmolarity; the intercept on the Y axis (phosphate uptake) is close to zero. These findings indicate that significant binding of phosphate to brush border membranes does not occur (14). Measurement of phosphate in total (trichloracetic acid-soluble) and inorganic pools after uptake of $^{32}$P-labelled orthophosphate at 60s, revealed that over 99 percent of $^{32}$P remains as orthophosphate from the mouse brush border membrane vesicles (14).

Our mouse membrane preparation is characterized by 10-fold enrichment of trehelase activity and 8-fold enrichment of alkaline phosphatase; succinic cytochrome-C activity is one tenth compared to the initial cortex homogenate. These findings indicate selective isolation of brush border

membranes. Purification was comparable with +/Y and Hyp/Y kidneys.

Phosphate transport by +/Y vesicles at 60s, 20°C, pH 7.4 and 0.1 matom/L is stimulated by sodium and inhibited by arsenate (Figure 3); the overshoot phenomenon (12,14) is observed and it is maximal at 2 min. Phosphate transport by Hyp/Y vesicles measured under identical conditions, prepared on the same day and examined in parallel is characterized by a partial decrease ($p < 0.001$) in the $Na^+$-dependent, arsenate-inhibited activity (Figure 3). A time course study (14) revealed that partial loss of the $Na^+$-dependent component of phosphate transport was observed at 30s (60 percent loss) 1 min. (45 percent loss) 2 min. (49 percent loss) and at 30 min. (32 percent loss). Measurement of simultaneous D-glucose transport revealed no difference between +/Y and Hyp/Y vesicles (Figure 3).

## DISCUSSION

The X-chromosome has retained stability during evolution (15), and for this reason we believe the X-linked mutation causing hypophosphatemic rickets in mouse and in man involves the homologous gene (7). Even if the mutation is not homologous the same gene product would be involved. Therefore the Hyp mouse should provide insight into the general mechanism of hypophosphatemia in XLH.

The prior evidence for the hypothesis that XLH in man is a selective inborn error of transepithelial transport of phosphate anion is as follows. Mechanisms for solute reabsorption by the renal tubule are normal in XLH, with the

exception of that maintaining phosphate transport. Net reabsorption of phosphate is only partially impaired but the defect is apparent even at the low filtered load of anion characteristic of the mutant phenotype. Indeed fractional excretion of phosphate is inappropriately large,

even exceeding unity under conditions of phosphate loading (6). Residual transport of phosphate cannot be increased at elevated levels of substrate in the affected hemizygote (6). Female XLH patients are heterozygotes by definition, and their TRPi values, over a wide range of serum phosphorus concentration, are intermediate between those for homozygous normal subjects and mutant hemizygotes (6). The defect in phosphate reabsorption in mutant hemizygotes or heterozygotes is not associated with a consistent elevation of serum PTH except in the presence of phosphate loading (16). The renal tubule might be abnormally responsive to PTH in XLH (17), however the urinary excretion of cyclic 3',5'-adenosine monophosphate is not elevated in relation to the serum calcium level in the human patients (6,18), and this relationship suggests that the tubule-PTH relationship is normal (19).

The Hyp/Y (male) mouse has elevated $FE_{Pi}$, (decreased net reabsorption) exaggerated $FE_{Pi}$ which can exceed unity in response to phosphate loading, and serum PTH levels that are not elevated except under conditions of phosphate loading. These findings are homologous to those in human XLH. Moreover, phosphate supplementation repairs the bone lesion and growth failure in mouse (7) as it does in man (18). The only significant difference between the phenotype in Hyp mouse and XLH patients is an elevated urinary cAMP in the former. Serum calcium is slightly depressed in Hyp mice (7); accordingly, there might be concomitant modification of renal calcium activity and regulation of renal adenyl cyclase (20).

The foregoing favors a similar defect that is intrinsic to the process of transepithelial transport of phosphate in XLH and Hyp phenotypes. That this hypothesis is applicable to the Hyp mouse is supported further by evidence for an intrinsic defect in the intestinal transport of phosphate (8); and in micropuncture studies (21,22), which reveal phosphate wasting along the proximal tubule, independent of PTH regulation.

Asymmetry of flux at the luminal membrane accounts for net tubular reabsorption of phosphate from ultrafiltrate (23). Net influx can be achieved by a process which keeps the effective concentration (or activity) of anion in cytosol low at the luminal pole. Two mechanisms for "run-out" of phosphate from this topological pool can be postulated: either a metabolic event, in which net phosphate disposal occurs by incorporation into organic pools; or a membrane event in which there is net outward flux of anion at the basolateral membrane. We found no abnormality in either tissue phosphorus, which resembles published data for rodent kidney (24), or the labelling of organic and inorganic phosphate pools in the Hyp mouse. These findings suggest that metabolic "run-out" of phosphate is not aberrant in the mutant phenotype. Although dietary modulation of calcium does influence renal phosphate, we observed no differences between Hyp/Y and +/Y mice in renal handling of labelled phosphate, or maintenance of cellular phosphate pools. It is of particular interest that tissue phosphorus is similar

in Hyp/Y and +/Y mice, even when extracellular phosphorus level is low in Hyp/Y. This finding implies that an intact component of phosphate transport maintains renal phosphorus levels in the mutant phenotype. We suggest that influx at the basolateral membrane is this component.

We found no abnormality in net uptake of phosphate from the medium or in efflux of phosphate from cortex or medulla slices in the Hyp phenotype. In the slice preparation the basolateral membrane of renal epithelium is exposed to the medium and the brush border participates in uptake of solute only minimally if at all (25,26). Accordingly we deduce that phosphate fluxes across the basilar pole of renal epithelium are not abnormal in the Hyp phenotype.

Purified brush border membranes exhibit a partial loss of the phosphate transport process in the Hyp phenotype. This defect involves a portion of the $Na^+$-dependent, arsenate-inhibited process; the $Na^+$-independent, non-saturable mode of transport is intact. This finding in the Hyp mouse is concordant with the partial loss of a saturable component of phosphate transport in XLH (6). Recently Dennis et al (27) have shown that phosphate reabsorption in the proximal tubule involves more than one mechanism; a high capacity system in the proximal convoluted tubule is not directly influenced by PTH whereas a lower capacity activity in the pars recta responds to the hormone. Further characterization of phosphate transport, including the effect of PTH and cAMP on the process in Hyp and normal brush border membrane preparations will be of

interest in the continuing delineation of the mutant phenotype.

At the present stage of our investigation, the findings account for impaired net renal reabsorption of phosphate in the mutant phenotype. They will also explain "negative reabsorption" ($FE_{Pi}>1.0$), if one assumes that loss of a steeply concentrating brush-border transport system permits efflux (backflux) of phosphate from intact intracellular pools on a residual less-steeply concentrating system. When extracellular phosphate is raised in the mutant phenotype by whatever means, cellular phosphate will rise by virtue of uptake at the basolateral membrane. Backflux from the augmented cellular pool will then increase into the moving column of urine. Equilibrium at the brush border membrane is not achieved under this condition, and therefore $FE_{Pi}$ must increase and negative reabsorption may be observed.

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TABLE 1

Fractional excretion of inorganic phosphate ($FE_{Pi}$)

in Hyp/Y mice and +/Y litter mates

| Mouse | Period | Plasma Pi µg/ml | Clearance of Inulin µl/min | Urine Pi µg/min | $FE_{Pi}$ |
|---|---|---|---|---|---|
| +/Y | | | | | |
| 1 | 1. | 49 | 282 | 4.29 | 0.32 |
| | 2. | 44 | 260 | 3.07 | 0.27 |
| | 3. | 54 | 197 | 1.38 | 0.13 |
| 2 | 1. | 77 | 155 | 2.26 | 0.19 |
| | 2. | 65 | 132 | 1.20 | 0.14 |
| 3 | 1. | 65 | 260 | 3.57 | 0.21 |
| | 2. | 64 | 234 | 2.70 | 0.18 |
| | 3. | 61 | 187 | 2.96 | 0.26 |
| 4 | 1. | 58 | 146 | 3.06 | 0.36 |
| 5 | 1. | 42 | 347 | 2.34 | 0.16 |
| | 2. | 54 | 124 | 0.54 | 0.08 |
| | 3. | 61 | 85 | 0.88 | 0.17 |
| 6 | 1. | 49 | 306 | 4.35 | 0.29 |
| 7 | 1. | 57 | 420 | 7.30 | 0.30 |
| | 2. | 52 | 328 | 1.53 | 0.09 |
| | Mean | 56.8$^{\Delta}$ | 230.9 | | 0.210$^{\Delta}$ |
| | SD | 9.16 | 95.3 | | 0.085 |
| Hyp/Y | | | | | |
| 1 | 1. | 34 | 147 | 1.85 | 0.37 |
| | 2. | 32 | 114 | 1.68 | 0.48 |
| | 3. | 35 | 89 | 1.18 | 0.38 |
| 2 | 1. | 51 | 114 | 1.57 | 0.26 |
| 3 | 1. | 31 | 357 | 3.85 | 0.35 |
| | 2. | 31 | 250 | 2.69 | 0.35 |
| | 3. | 28 | 243 | 1.81 | 0.27 |
| | Mean | 34.6$^{\Delta}$ | 187.7 | | 0.351* |
| | SD | 7.6 | 98.2 | | 0.073 |

Animals were anaesthetized, cannulated and infused with inulin (see Methods). Collection periods (60-85 min) were initiated 4-6 hr after animal was cannulated. Fractional excretion ($FE_{Pi}$) = urine phosphate (µg/min) ÷ filtered phosphate (µg/min).

* Results are significantly different ($p < 0.01$, Student's $t$ test).
$^{\Delta}$ Results are significantly different ($p < 0.001$, Student's $t$ test).

TABLE 2

URINARY EXCRETION OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE (cAMP) CYCLIC AMP CONCENTRATIONS

| DIET | Ca (% w/w) | P | cAMP[a] (μmoles/mg creatinine) Hyp/Y | +/Y |
|---|---|---|---|---|
| Mixed Chow | 1.05 | 0.75 | $41.41 \pm 7.96^{\Delta}_{*}$ | $32.31 \pm 5.89^{o}_{*}$ |
| Old Guilford | 0.22 | 0.74 | $77.44 \pm 7.34^{\Delta}_{**}$ | $46.69 \pm 13.32^{o}_{**}$ |

a) Urinary cAMP concentrations are the mean and standard deviation of individual urine samples from 6 mice in each group, except for +/Y on old Guilford where 7 mice were used.

$^{\Delta}_{**}$ Paired values are significantly different at the 0.001 level by Student's *t* test.

$^{o}_{*}$ Paired values are significantly different at the 0.05 level by Student's *t* test.

TABLE 3

Isotopic Distribution Ratio of $^{32}P$-Phosphorus in Kidney Cortex Slices Following Uptake from Incubation Medium: Effect of Time, Concentration of Phosphate Anion and Dietary Pretreatment of Animal

| Pool measured[b] | Time (min) | $^{32}P$ Distribution Ratio on Mouse Chow[c] | | on Old Guilford | |
|---|---|---|---|---|---|
| | | +/Y | Hyp/Y | +/Y | Hyp/Y |
| | | (at Phosphorus Conc, 0.1 matoms/L) | | | |
| Pi | 15 | 1.72±0.39 | 1.49±0.14* | 1.21±0.19 | 0.94±0.19* |
| | 60 | 5.04±0.74 | 4.83±1.91 | 3.86±0.51 | 3.65±0.56 |
| $P_T$ | 15 | 3.84±0.87 | 3.32±0.30* | 2.76±0.57 | 2.21±0.36* |
| | 60 | 12.02±1.58 | 12.27±3.99 | 11.16±1.75 | 11.49±1.26 |
| | | (at Phosphorus Conc, 1.0 matoms/L) | | | |
| Pi | 15 | 1.29±0.26 | 1.18±0.17* | 0.87±0.24 | 0.71±0.17* |
| | 60 | 3.08±0.38 | 2.80±0.45 | 2.49±0.35 | 2.12±0.19 |
| $P_T$ | 15 | 2.37±0.63 | 2.07±0.32 | 1.77±0.26 | 1.52±0.35 |
| | 60 | 5.74±0.86 | 5.31±1.30 | 5.81±0.61 | 5.26±0.44 |

a) Distribution ratio = $\frac{\text{cpm/ml intracellular water}}{\text{cmp/ml extracellular water}}$; mean & SD of six determinations

b) $^{32}P$ measured in the inorganic phosphate pool (Pi) and total phosphate pool ($P_T$)

c) Mouse Chow diet composition: Calcium 0.89% (W/W), phosphorus 0.63%; Old Guilford 96W composition: calcium 0.22%, phosphorus 0.74%

* Paired results are significantly different ($p < 0.001$, Student's *t* test

TABLE 4

Concentration of Inorganic Phosphorus in Kidney Cortex Slices[a)]

(matoms phosphorus per g protein)

| Incubation period | on Mouse Chow[c] +/Y | on Mouse Chow[c] Hyp/Y | on Old Guilford[c] +/Y | on Old Guilford[c] Hyp/Y |
|---|---|---|---|---|
| zero time[b] | | | 46.6 ±1.0 (n=8) | 46.6 ±1.1 (n=6) |
| | | (at 0.1 matoms/L) | | |
| 15 min | 30.27±5.25[c*] | 30.99± 9.61** | 40.04±8.81* | 41.04±2.15** |
| 60 min | 24.35±5.04 | 24.54± 7.62 | 27.86±7.70 | 26.91±6.38 |
| | | (at 1.0 matoms/L) | | |
| 15 min | 33.86±4.97 | 37.52±10.13 | 40.36±9.37 | 38.89±7.44 |
| 60 min | 27.95±4.54 | 29.54± 3.04 | 30.42±4.78 | 31.92±3.76 |

a) Extraction performed by method of Vestergaard and Bogind (11). Slices are same as those reported in Table 2. Data are mean and SD for six determinations.

b) Tissue phosphorus measured immediately after sacrifice of animals (7).

c) Mouse Chow diet composition: Calcium 0.89% (W/W), phosphorus 0.63%; Old Guildord 96W composition: calcium 0.22%, phosphorus 0.74%.

* Difference in tissue phosphorus of +/Y mice on the two diets significant at 0.05 level (Student's paired *t* test).

** Difference in tissue phosphorus of Hyp/Y mice on the two diets significant at 0.05 level (Student's *t* test).

TABLE 5

REGRESSION PARAMETERS FOR RETENTION OF $^{32}P$ BY KIDNEY CORTEX SLICES FROM +/Y and Hyp/Y MICE DURING ESTIMATION OF EFFLUX[a)]

| | +/Y Slices | | Hyp/Y Slices | |
|---|---|---|---|---|
| | Expt.1 | Expt.2 | Expt.1 | Expt.2 |
| Slope | -0.0290 | -0.0302 | -0.0297 | -0.0295 |
| Y-intercept | 4.2391 | 4.0706 | 4.0971 | 4.1775 |
| Corr. Coeff. | -0.9794 | -0.9714 | -0.9878 | -0.9991 |

a) Slope and intercepts calculated from log n transformation of y axis of Figure 2b between 10 and 25 min.

LEGEND

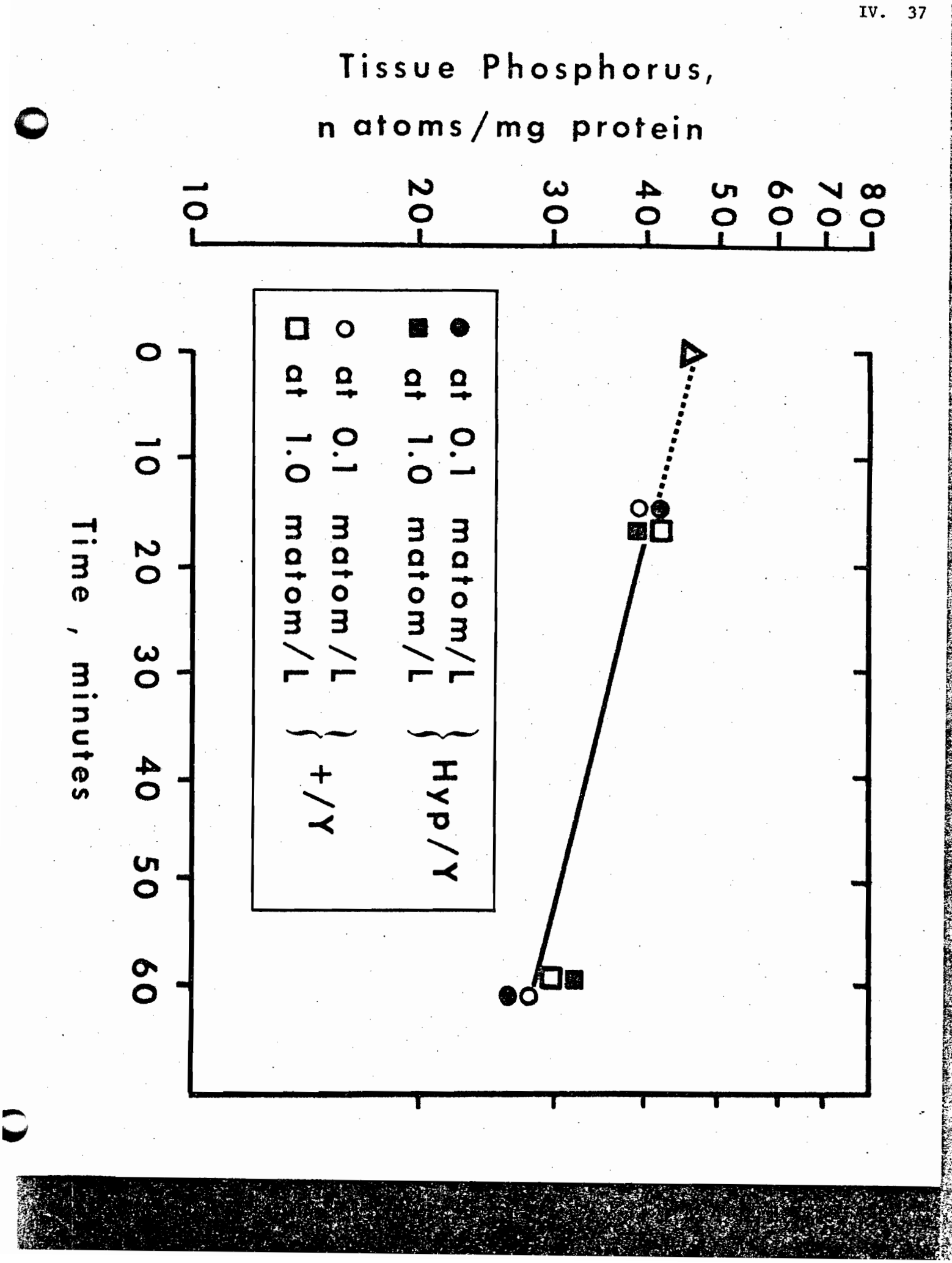
Figure 1 Time course of tissue inorganic phosphorus during incubation of slices from Hyp/Y mice (filled symbols) and +/Y litter mates (open symbols) in the presence of extracellular phosphorus concentrations as indicated on the graph.

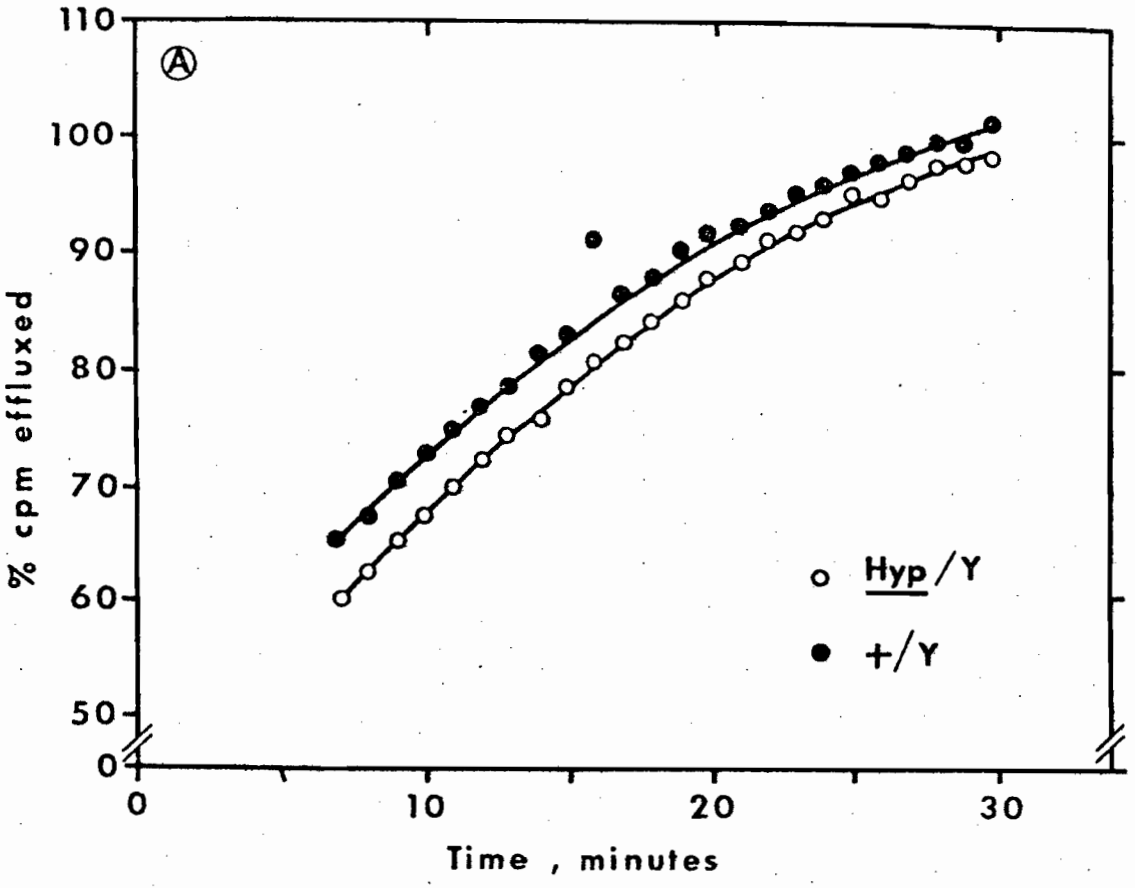
LEGEND

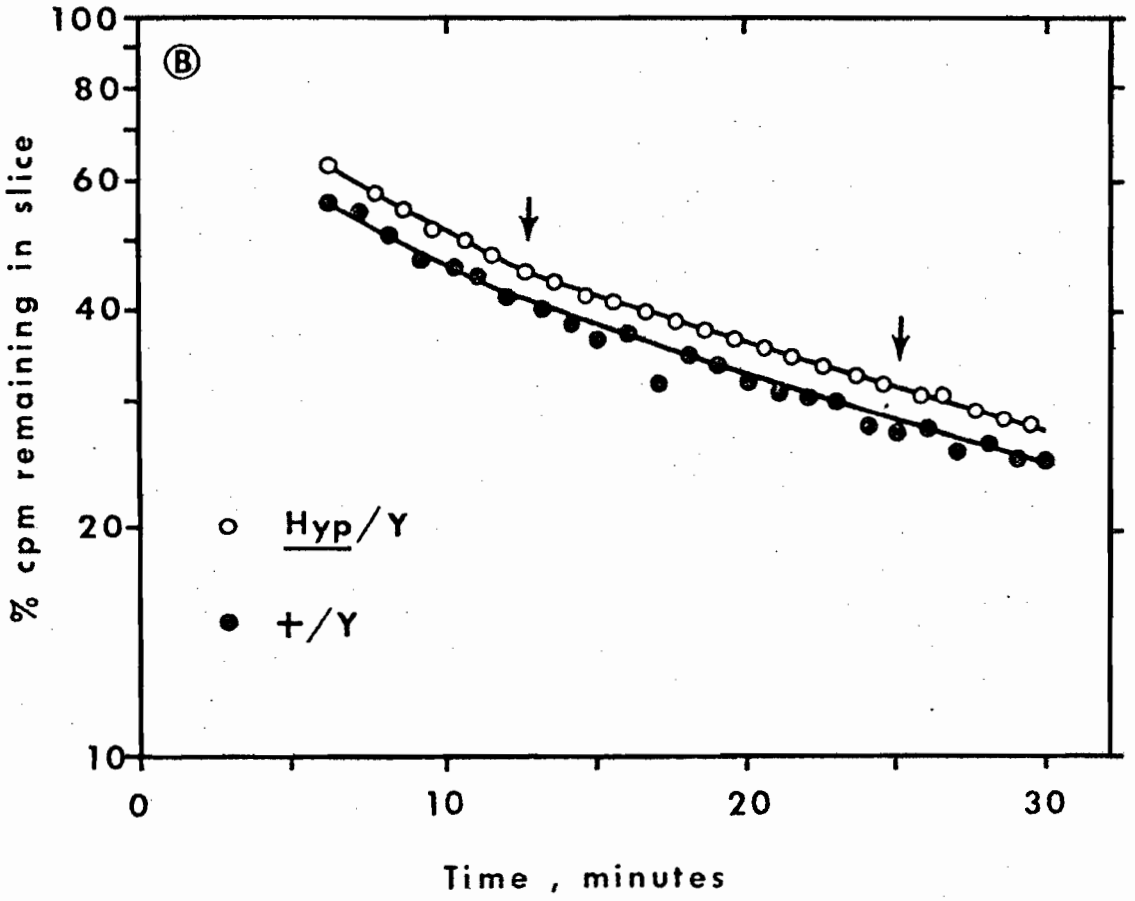
Figure 2 Time course for appearance of $^{32}P$ in medium (upper graph) and disappearance from slice (lower graph). Renal cortex slices from Hyp/Y mice (open circles) and +/Y litter mates (filled cirles) were pre-loaded with $^{32}P$ labelled phosphorus to equivalent specific activity and concentration. Measurement of efflux was initiated at 6 minutes after emptying of extracellular fluid space was complete and when efflux reflects movement from intracellular space.

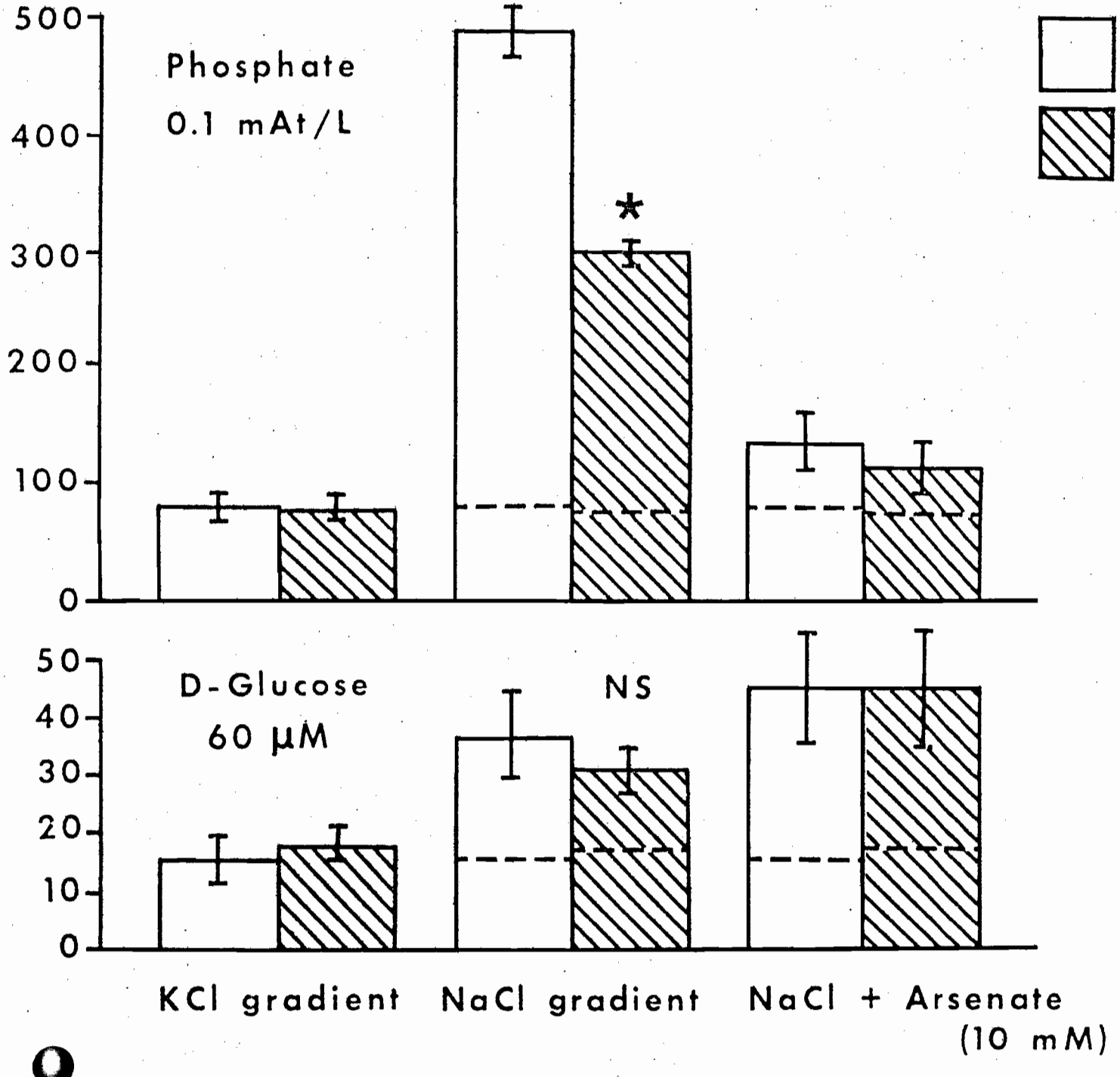
LEGEND

Figure 3 Transfer of phosphate and D-glucose into purified brush border membrane vesicles prepared from +/Y (wild type, open bars) and Hyp/Y (mutant, hatched bars) renal cortex homogenate. Uptake measured at 60s, 20°C, pH 7.4. Data are the mean and SD of quintuplicate measurements on membranes prepared from nine mice of each genotype.


Tissue Phosphorus,
n atoms/mg protein
10
20
30
40
50
60
70
80
0
10
20
30
40
50
60
Time, minutes
● at 0.1 matom/L
■ at 1.0 matom/L
Hyp/Y
○ at 0.1 matom/L
□ at 1.0 matom/L
+/Y


Ⓐ
% cpm effluxed
110
100
90
80
70
60
50
0
0
10
20
30
Time , minutes
o Hyp/Y
● +/Y


Ⓑ
% cpm remaining in slice
100
80
60
40
20
10
0
10
20
30
Time , minutes
o Hyp/Y
● +/Y


Phosphate
0.1 mAt/L
+/Y
Hyp/Y
*
D-Glucose
60 µM
NS
Uptake, pmoles/mg protein
500
400
300
200
100
0
50
40
30
20
10
0
KCl gradient
NaCl gradient
NaCl + Arsenate
(10 mM)

SECTION V

## Role of Epithelial Architecture and Intracellular Metabolism in Proline Uptake and Transtubular Reclamation in PRO/Re Mouse Kidney

The use of non-metabolizable amino acid analogues in mammalian cells, and the study of blocked catabolic mutants in microorganisms has indicated that cellular accumulation of a solute and its intracellular metabolism are essentially independent activities. The discovery of mice (PRO/Re) with hyperprolinemia, hyperprolinuria and less than 1% of normal renal cortex proline oxidase activity (Ref. 3 & 4, this section) afforded the opportunity to rexamine this hypothesis with reference to the transepithelial translocation of a natural solute (proline) in a mammalian tissue (kidney). Using clearance techniques in vivo, and kinetic analyses of proline uptake into, and efflux from renal cortex slices in vitro, it was possible to demonstrate integrity of the transport systems for proline at the luminal and basolateral membranes of the tubular epithelial cell in PRO/Re mice. Nevertheless, renal excretion of proline was greater than normal at any given substrate (filtered) load. The solution to this paradox depended on consideration of the metabolic component of net transepithelial transport. A solute taken up from the tubular lumen can be disposed of metabolically, or through efflux from the cell. In the absence of normal rates of metabolic disposal, intracellular concentrations tend to rise unless efflux across the luminal or peritubular surfaces occurs. Increased solute flux into the tubular lumen produces increased excretion, and we propose this mechanism as the cause of the increased proline clearance in PRO/Re mice.

This study provides an additional illustration of the role that backflux of solutes play in net reabsorption. It also indicates how the metabolism

of a solute may influence its net transcellular transport without violating the concept that the uptake and metabolism of a solute are basically independent processes.

In addition to contributing to the conceptual basis of this research, the candidate did all of the research on amino and transport in vivo in PRO/Re and control mice.

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MEDICAL REPORT ON

"ROLE OF EPITHELIAL ARCHITECTURE AND INTRACELLULAR METABOLISM IN PROLINE UPTAKE AND TRANSTUBULAR RECLAMATION IN PRO/RE MOUSE KIDNEY"

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# Role of Epithelial Architecture and Intracellular Metabolism in Proline Uptake and Transtubular Reclamation in *PRO/Re* Mouse Kidney

**(transport/renal clearance/cortex slice technique/proline oxidase)**

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*Communicated by Elizabeth S. Russell, February 10, 1975*

ABSTRACT **The homozygous *PRO/Re* mouse has less than 1% of the very high proline oxidase activity that characterizes normal kidney cortex. In *PRO/Re* mouse the endogenous proline concentration is eight times normal in plasma and four times normal in kidney cortex cells, but 50 times normal in urine. The integrity of the membrane transport systems for proline uptake at the antiluminal surface of absorbing epithelium is retained in *PRO/Re* kidney, as determined by the slice method. Clearance studies *in vivo* under steady-state conditions indicate that the integrity of the luminal uptake system shared by glycine and proline, and serving proline absorption, is also intact. The exaggerated renal clearance of proline in *PRO/Re* mice (50 times normal) is explained when its raised intracellular concentration, caused by impaired proline oxidation, is considered. Backflux into urine flowing down the nephron will occur under these conditions, thus impairing net reclamation of proline in *PRO/Re* kidney. The findings reveal that membrane transport and intracellular metabolism of a substrate are, indeed, independent functions, but that metabolism of a substance can influence its transcellular transport.**

Transport across the plasma membrane and intracellular metabolism of an amino acid should be considered as independent functions (1). Awareness of this essential dichotomy has been heightened by the use of blocked-catabolic mutant microorganisms and metabolically inert substrates to study membrane transport of various amino acids (1, 2). The opportunity to examine L-proline transport in kidney of the homozygous *PRO/Re* mouse (3, 4), a mutant with less than 1% of the normal renal proline oxidase activity, was of particular interest to us. Proline is avidly taken up from urine (5) and from plasma (6) by mammalian kidney, where it is then vigorously oxidized, with resultant glucogenesis under conditions of fasting (6). It follows that the *PRO/Re* mouse offers a unique opportunity to examine the inter-relationships between intracellular metabolism of proline and the cellular architecture of renal absorbing epithelium; and how they influence proline uptake from extracellular fluid *in vivo* and *in vitro* and the net reclamative flux of proline from urine to blood *in vivo*.

We used renal clearance methods (7) to evaluate tubular reclamation of proline across the luminal membrane, and the kidney cortex slice technique to measure transport across the antiluminal surfaces of epithelial cells (8). Normal mouse kidney transports L-proline *in vitro* by saturable energy-dependent membrane mechanisms, and can reclaim virtually all of the filtered proline from urine. This organ also oxidizes proline at exceptionally high rates. When proline oxidation is blocked, the intracellular proline pool is expanded in *PRO/Re* mouse kidney. Whereas, proline transport as a membrane function remains intact, net reclamation from urine is impaired in *PRO/Re* mice, presumably because of proline backflux at the luminal side of the epithelium.

## METHODS AND MATERIALS

Homozygous *PRO/Re* mice were provided by Dr. Elizabeth Russell of the Jackson Laboratory, Bar Harbor, Maine; control mice (A/J and C57BL/6J) were obtained from Dr. F. Clarke Fraser at McGill University. Blood for proline determination was obtained by orbital sinus puncture, and urine for measurement of proline and creatinine was collected from six to nine animals placed in hanging metabolic cages. Homogenates of dissected renal cortex and of renal cortex slices were prepared from kidneys immediately after their removal and analyzed as described previously (9–13).

L-Proline was infused into lightly anesthetized mice through an exposed cannulated external jugular vein. The bladder was also catheterized. Blood was withdrawn from an exposed tail vein. The animal was kept at constant body temperature and fluid loss was replaced to keep body weight, hematocrit, and serum osmolarity constant. The glomerular filtration rate was measured by [$^{14}$C]inulin clearance. Proline levels in plasma were elevated step-wise by priming doses of L-proline (0.25–5 g/dl) and maintained by a sustaining infusion. Equilibration for 30–60 min was allowed prior to measurement of renal excretion and reabsorption of proline at each plasma level attained. Proline uptake by renal cortex slices was measured after determination of the appropriate tissue water spaces; the distribution of proline in metabolic pools was measured with L-[$^{14}$C]proline and by direct chemical analysis, using methods described previously (9–12).

L-[$^{14}$C]Proline (uniformly labeled; specific activity, 175 Ci/mmol) and carboxyl-labeled [$^{14}$C]inulin (specific activity, 2–5 mCi/g) were obtained from New England Nuclear. Radiochemical purity was verified in the usual manner (9). Unlabeled amino acids were obtained from Schwarz/Mann (New York). Reagent grade chemicals, obtained locally, were used in buffers and solutions.

## RESULTS

### *In vivo* proline concentration: plasma, urine and kidney cortex

*PRO/Re* mice maintain the concentration of proline in plasma and urine well above the range observed in age-matched controls fed on comparable diets (Table 1). Plasma proline is about 8-fold elevated in *PRO/Re* mice while their urine pro-

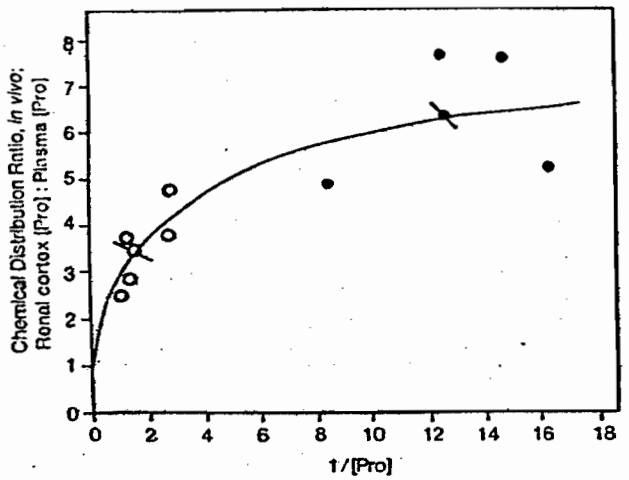


FIG. 1. An Akedo-Christensen plot of the reciprocal of *in vivo* steady-state proline concentration in plasma versus the proline distribution ratio between intracellular water and plasma in kidney of control (●) and *PRO/Re* (○) mice. Symbols with a diagonal bar are the mean values for the two populations. Proline is taken up by kidney against a chemical gradient *in vivo*, by a saturable mechanism.

line is 50 times normal. Proline excretion in control mice is minimal, being about one tenth that of glycine. Intracellular proline is elevated about 4-fold in whole-cortex homogenates of *PRO/Re* kidney. The corresponding proline distribution ratio *in vivo* (proline in kidney intracellular water:proline in plasma water) is depressed when compared with that of control mouse kidney (Table 1); the explanation of this finding is conventional. When the *in vivo* distribution ratio is plotted against the reciprocal of the corresponding plasma proline concentration (the so-called Akedo-Christensen plot), a steady-state transport relationship between plasma and tissue is described which undergoes saturation at elevated concentrations of the substrate (Fig. 1).

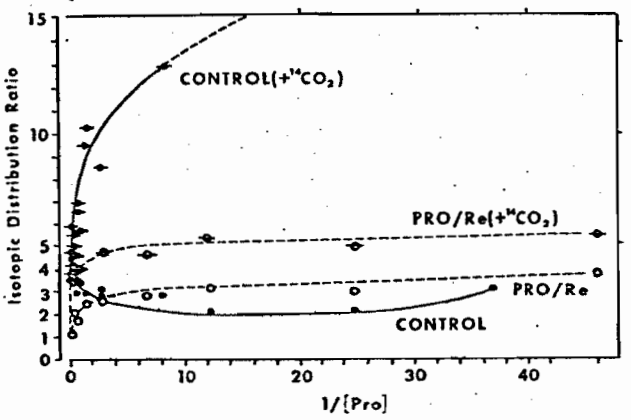


FIG. 2. Akedo-Christensen plots for 30-min uptake of L-proline under steady-state conditions by control and *PRO/Re* kidney cortex slices. The soluble–isotopic distribution ratio, and the uptake ratio which accounts for proline oxidized to $CO_2$ (9, 11) are related to the reciprocal of the proline concentration in the medium. Saturable uptake is observed in *PRO/Re* slices deficient in proline oxidase activity. Control kidney oxidizes proline so rapidly that saturable transport is not observed unless the total amount of proline taken up by slice and lost by oxidation to $CO_2$ is taken into account.

TABLE 1. *Proline concentrations in plasma, urine, and kidney of PRO/Re and control mice**

| | Control mean ± SD | *PRO/Re* Mean ± SD | *P* value‡ |
|---|---|---|---|
| Plasma (μmol/liter) | 81 ± 26 | 670 ± 210 | <0.01 |
| Kidney cortex (μmol/liter of ICW) | 545 ± 146 | 2220 ± 500 | <0.01 |
| Urine (μmol/g of creatinine) | 91 ± 21 | 4960 ± 1850 | <0.01 |
| Renal:plasma distribution ratio† | 6.20 ± 1.51 | 3.46 ± 0.91 | <0.01 |

* All values ($n$ = 6–10) were obtained in mice fasted for no more than 3 hr. Longer fasting reduces proline concentrations.

† The distribution ratio is calculated from the formula (proline concentration in kidney intracellular water, ICW) ÷ (proline concentration in plasma water) taking 0.55 × wet weight for intracellular water, 0.23 × wet weight for extracellular fluid, and 0.92 × volume for plasma water. Proline was determined by elution chromatography on ion exchange resin columns with a modified Beckman amino-acid analyzer (13).

‡ Determined by Student's $t$ test.

We also investigated whether urine trapped in the lumen of tubules, containing a great excess of proline, might contribute to the high *in vivo* tissue:plasma proline distribution ratio in *PRO/Re* kidney. The proline content of whole-cortex homogenates was compared with that of thin cortex slices with a collapsed tubular lumen (8). The latter were prepared quickly, placed briefly on chilled filter paper soaked in buffer, then dried by blotting and homogenized. The proline content in slices was one tenth to one third less than in whole-cortex homogenates. We believe the artefact of trapped intraluminal urine explains why "intracellular" proline is higher *in vivo* at various levels of plasma proline (Fig. 1 and Table 1), than in cortex slices incubated *in vitro* under analogous steady-state conditions at corresponding concentrations of extracellular proline (namely Fig. 2 and Table 2).

### *In vitro* uptake and metabolism of proline by kidney cortex slices

The steady-state relationship between influx and efflux, and oxidation of L-[$^{14}$C]proline, was examined *in vitro* with kidney cortex slices. The isotopic distribution ratio achieved a steady-state after 30 min incubation. The chemical distribution ratio and the soluble–isotopic distribution ratio, corrected for conversion of $^{14}$C-labeled proline to other soluble derivatives, were also determined. The latter two are less than unity in control mice with intact proline oxidase activity (Table 2); the corresponding ratios are greater than unity in *PRO/Re* kidney. Chromatographic development of the soluble $^{14}$C-labeled material, extracted from boiled slices after 30 min incubation, revealed that normal mouse kidney readily converts L-proline to non-proline metabolites. Only 23% of the label accumulated from extracellular proline at 1.2 mM is retained as intracellular proline by control mouse kidney; the majority of $^{14}$C label appears in $CO_2$ (Fig. 2). On the other hand, 77% of the label is retained as proline by *PRO/Re* kidney under similar conditions and oxidation to $CO_2$ is greatly attenuated, as expected, in the kidney deficient in proline oxidase (Fig. 2).

TABLE 2. *Various measurements of the distribution ratio for* L-*proline in kidney cortex slices incubated in vitro*

| Type of distribution ratio* | Control | *PRO/Re* |
|---|---|---|
| Chemical* | 0.81 ± 0.31 | 1.57 ± 0.24 |
| Chemical–isotopic† | 0.80 ± 0.18 | 1.42 ± 0.13 |
| Soluble–isotopic‡ | 3.58 ± 0.8 | 1.91 ± 0.18 |
| Soluble–isotopic§ + $CO_2$ | 9.7 + 2.6 | 4.6 ± 0.6 |

All ratios are the mean and standard deviation of observations in triplicate calculated from dpm accumulated in 1.0 ml of intracellular water of slices incubated in the presence of uniformly labeled L-[$^{14}$C]proline at 1.2 mM for 30 min and 37°; the denominator in the ratio is dpm at zero time in 1.0 ml of extracellular water.

* Chemical analysis of proline in cortex slice homogenate by elution chromatography on anion exchange resin columns on a modified amino-acid analyzer (13).

† Derived by correcting for loss of $^{14}$C label from proline taken up by slice and converted into other soluble metabolites as determined by partition chromatographic methods (9, 11).

‡ Derived from uncorrected $^{14}$C radioactivity in intracellular water.

§ Sum of dpm in $^{14}CO_2$ and dpm in soluble pool (9, 11).

The *PRO/Re* mutant unequivocally transports L-proline against a chemical gradient (Fig. 2). Proline uptake by *PRO Re* kidney slices is inhibited by cyanide and anaerobiosis and by $Na^+$ depletion in the medium, as in normal mammalian kidney (9–12).

### Concentration-dependent uptake and reclamation of proline.

Rat and human kidney cortex slices take up L-proline under steady-state conditions, by more than one saturable mechanism (9–12, 14, 15). Mouse kidney accumulates proline in a similar manner (Fig. 3). The apparent $K_m$ values for proline uptake by normal mouse kidney cortex slices (about 10 mM on the "high" system and about 0.1 mM on the "low" system) are in the range of those reported for rat (9–12), and human (14) kidney under similar conditions. The affinity of L-proline for its transport system(s), particularly at the concentrations of proline experienced *in vivo*, is not significantly modified by proline oxidase deficiency (Fig. 3).

We also examined proline efflux from *PRO/Re* and control cortex slices, at comparable intracellular concentrations, by methods described previously (12). Proline efflux rates are similar in the mutant and control mice; 90% of the initial proline has been lost across the antiluminal membranes within 15 min.

### Net tubular reabsorption *in vivo*

Renal clearance of proline (urinary excretion in μmol/min ÷ plasma proline in μmol/ml), and net tubular reabsorption of proline [(inulin clearance in μl/min × plasma proline in μmol/ml) − (urinary proline in μmol/min)], were measured in control and *PRO/Re* mice. The renal clearance of proline in three different *PRO/Re* mice was about 50 times higher than in age- and weight-matched controls at all comparable levels of plasma proline observed (Fig. 4). Net tubular reabsorption (reclamation) of proline is accordingly, much diminished in the *PRO/Re* renal tubule.

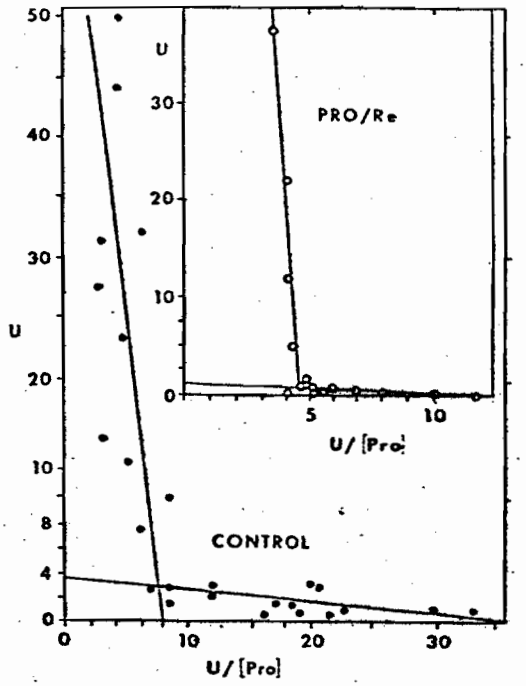


FIG. 3. Eadie-Hofstee plots of 30-min proline uptake (u) by kidney cortex slices prepared from control and *PRO/Re* mice. More than one mode of uptake is present in both strains. The apparent $K_m$ values for the "low" and "high" uptake systems in control and *PRO/Re* kidney slices, are essentially similar.

It is of interest that we were unable to identify any significant saturation of proline reabsorption *in vivo* in the normal mouse, even at plasma concentrations more than 200 times normal (R. R. McInnes and C. R. Scriver, unpublished data).

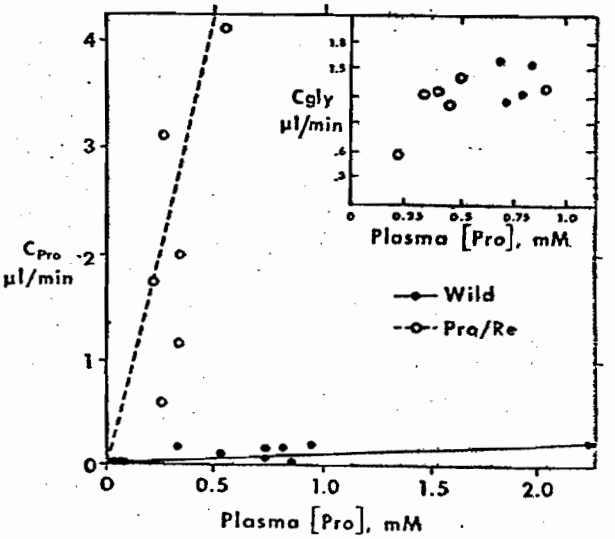


FIG. 4. Renal clearance of proline from blood ($C_{Pro}$) in control (wild) strain and in the mutant (*PRO/Re*) strain at various steady-states monitored by inulin clearance. Proline clearance is approximately 50-fold greater in the *PRO/Re* mouse compared to the controls. Calculation of tubular reabsorption data confirms that net reclamation of proline is depressed in *PRO/Re*. The inset shows glycine clearance at endogenous concentrations in relation to the plasma proline concentration; glycine clearance is not different from normal in *PRO/Re*, indicating integrity of the luminal transport system shared by glycine and proline.

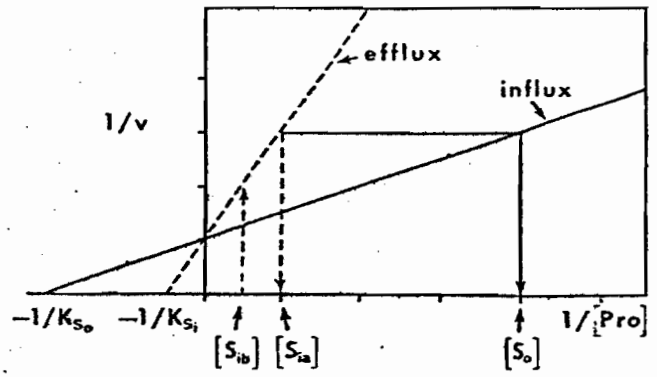


FIG. 5. Theoretical double-reciprocal plots for flux (inward and outward) of proline when concentrated from an external pool $[S_o]$ to an internal pool $[S_{ia}]$ against a chemical gradient, at steady-state. If an intracellular event (e.g., decreased proline oxidation) causes the internal proline pool to expand $[S_{ib}]$ independently of the amount derived from influx, efflux across the membrane will increase. When the latter occurs at the luminal membrane of an epithelial cell, into a moving column of liquid (e.g., urine), the enhanced efflux (backflux) will yield diminished net reabsorption of the solute at that surface.

Proline shares a transport system with glycine, and the former will inhibit reclamation of the latter during tubular reabsorption in man (5) and rat (15, 16) at the concentrations of both substrates encountered in the present study. Proline and glycine share transport in the mouse also (R. R. McInnes and C. R. Scriver, unpublished data).

Therefore, we examined interaction between proline and glycine during reabsorption to determine whether this system is intact in the *PRO/Re* mutant. Glycine reclamation is impaired to the same degree in the *PRO/Re* and control mouse at various concentrations of L-proline (inset, Fig. 4), implying that integrity of the shared luminal membrane system serving reclamation is retained in *PRO/Re* kidney.

## DISCUSSION

The kidney of mouse is quite different from that of man or rat in its capacity to oxidize proline. After 30-min incubation at 0.1–0.2 mM L-proline, the fraction of transported proline oxidized to $CO_2$ by kidney cortex slices is about 50% in the rat (9) and in man (14); it exceeds 80% in normal mouse. The *in vivo* concentration of proline in kidney cortex, as determined by the slice method, is also different in the three species. In man (14), and in rat (11), it exceeds 0.6 mM, whereas in mouse kidney, it is less. The average proline concentration in plasma is above 0.2 mM in rat and in man (9, 11, 19), whereas, it is less in the mouse. These findings imply that the rate of intracellular proline oxidation modulates its concentration in body fluids.

Net tubular reabsorption of proline reaches saturation in man when the plasma proline concentration exceeds 1 mM. The reabsorptive function is dependent on the integrity of membrane transport systems which serve low (physiological) and high intraluminal concentrations of proline (5). Saturability of a high-capacity mechanism for proline reabsorption has also been identified, with some difficulty, by microperfusion methods in rat kidney (18). On the other hand, we could not define a saturable mode for proline reabsorption in the mouse at proline concentrations that evince saturation in man and rat. It occurred to us that the great capacity of mouse kidney to oxidize proline might keep intracellular proline at low levels and that the capacity for proline uptake into the cell does not normally exceed the capacity for disposal of proline in the absorptive cell. In this case, an intracellular enzyme acting on the substrate comes to influence, although not to serve directly, the transcellular movement of the amino acid; that is, metabolism of proline is a determinant of its intracellular concentration and thus of its transepithelial reclamation. The *PRO/Re* mutant proved to be a useful probe of this hypothesis.

Proline concentration is modestly elevated in the body fluids of *PRO/Re* mice whereas, urinary proline is exceptionally high; we suggest that expansion of the intracellular proline pool in tubular cells is largely responsible for the latter finding. Prolinuria will presumably occur in *PRO/Re* mice, in part because the luminal transport system undergoes normal saturation at the higher concentrations of proline in tubular urine (5, 7, 15–18); a greater fraction of filtered proline will, therefore, escape tubular reclamation. However, our studies in control mice reveal that this normal saturation of the transport mechanism does not significantly impede net reabsorption when proline oxidation is intact. The normal nonsaturable mode of entry (20) apparently permits quite efficient reclamation provided proline can be oxidized after uptake and rapid diffusion is possible. Normal integrity of the saturable, energy-dependent proline transport systems which are exposed in glomeruli (21) and at the antiluminal surface of epithelial cells (9) was also demonstrated in *PRO/Re* cortex slices.

The hyperprolinuria relative to extracellular proline concentrations which is characteristic of *PRO/Re* mouse can be interpreted by the application of Michaelis kinetics to the transepithelial reabsorptive function (Fig. 5). We must assume that the relationships for proline efflux in *PRO/Re* kidney, as identified at the antiluminal membranes *in vitro*, pertain also to the luminal membrane *in vivo*, and that proline uptake kinetics at physiological concentrations across luminal and antiluminal membranes are independent but similar. Earlier studies in rat kidney (11, 12) suggest that such assumptions are reasonable. We utilized the evidence that an asymmetry of trans-cellular flux must exist (22), presumably achieved by the disparity in absorptive membrane area of luminal and antiluminal surfaces, to achieve net reabsorption of solute from urine; and that cellular accumulation from blood or urine and transepithelial transport from urine to blood, for some amino acids at least, are dissociated in kidney (23, 24). We can then propose that excessive prolinuria in *PRO/Re* is a simple function of the raised intracellular concentration of proline in the presence of proline oxidase deficiency, and also in the presence of continuing uptake from blood and urine. At high concentrations of proline inside the cell, efflux of proline is enhanced on the same mechanism that moves it into the cell against a gradient (Fig. 5). When this event occurs at the luminal surface of the epithelial cell, proline will flow into a moving column of fluid and will appear in the bladder urine, being perceived as diminished tubular reclamation of proline. The normal structural relationships of tubular epithelium permit this phenomenon to be observed *in vivo* in a manner not possible *in vitro*.

The failure to observe impaired net reabsorption of phenylalanine in phenylketonuria (25, 26) and of sarcosine in sarcosinemia (27), both mutant blocked-catabolic states, can be

explained. The role of kidney in the oxidation of these amino acids is negligible or minor, respectively, so that the intracellular concentration of these two amino acids is not modulated by their renal metabolism.

This study would not have been possible without the interest of Elizabeth Russell and Robert Blake. The interest of Dr. Francis Glorieux and Edgard Delvin in the early stages of this work is also appreciated. We are grateful to Russell Chesney, Halver N. Christensen, Michel Bergeron and John Dirks for review and criticism of this paper. The able assistance of Audrey Shannon was invaluable in this study. This research was supported by a grant from the Medical Research Council of Canada and the Cystic Fibrosis Foundation of Canada (Fellowship to R.R.M.)

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SECTION VI

# Net Reabsorption of α-Aminoisobutyric Acid by Rat Kidney in Vivo. I. Clearance Behaviour and Interaction with Natural Amino Acids.

The facility with which the non-metabolizable analogues of natural solutes separate metabolic from transport phenomena has encouraged their use in studies of biological transport. Christensen and his colleagues (Ref. 4, this section) firmly established the extent to which certain analogues, especially α-aminoisobutyric acid (AIB), mimic the behaviour of specific groups of natural solutes in single cells or tissue in vitro. Few efforts have been made, however, to obtain similar evidence concerning the behaviour of these artificial solutes during transepithelial transport in vivo. In this section we describe how clearance studies in the rat define to what extent the renal transport of AIB in vivo resembles that of the natural L-amino acids. The most significant interactions are between AIB and the short-chain aliphatic amino acids. Nevertheless, many of the interactions between AIB and natural amino acids in vivo in rat kidney cannot be accommodated solely by the designation of AIB as a member of the short-chain neutral aliphatic group. A major theme - namely that transepithelial transport and cellular accumulation of a solute are related but independent processes - is also revealed in this section and further developed in the next.

In addition to characterizing the behaviour of AIB transport in rat kidney in vivo, this section gives a precise description of the methodology used in all of the studies of AIB transport presented in this thesis (sections VI, VII, and VIII).

These investigations have been submitted for publication in The American Journal of Physiology. The following manuscript is organized according to the required format of that journal.

# NET REABSORPTION OF α-AMINOISOBUTYRIC ACID BY RAT KIDNEY IN VIVO. I. CLEARANCE BEHAVIOUR AND INTERACTION WITH NATURAL AMINO ACIDS.

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## SUMMARY

Suitability of the non-metabolizable amino acid α-aminoisobutyrate (AIB) as a model for the study of net tubular reabsorption of amino acids was examined using clearance techniques in the anesthetized rat. Sixteen hours after intraperitoneal injection of AIB-1-[$^{14}$C], plasma AIB and $FE_{AIB}$ are constant and renal loss of AIB controls plasma [AIB]. Interindividual variation in $FE_{AIB}$ is wide (0.07 - 0.21) but intraindividual variation is low ( < 0.03 in 63 clearance periods); this fact permits sensitive examination of factors modulating AIB excretion in a given animal. Net reabsorption of AIB resembles the process for natural amino acids in that: (i) $FE_{AIB}$ is not influenced by spontaneous fluctuations in GFR or urine flow rate; (ii) AIB excretion rate and renal cortex accumulation of AIB are not directly related; (iii) AIB does not interact with phosphate during reabsorption; (iv) AIB impairs reabsorption of short-chain, aliphatic amino acids which, like AIB, have high endogenous excretion rates in rat and man. Proline and glutamate exhibit a biphasic plasma and urinary oscillation in response to AIB infusion; (v) AIB reabsorption is altered by changes in the filtered load of various natural amino acids so that at filtered loads of L-lysine, glycine, L-proline and L-alanine 20 - 30 fold in excess of AIB, $FE_{AIB}$ is increased whereas at low proline infusion rates, $FE_{AIB}$ is decreased. We confirm the preferential accumulation of AIB in renal medulla vs. cortex. Renal tissue levels are decreased by infusions of other amino acids; only proline abolishes the medulla-cortex difference.

These findings indicate that: (i) AIB is reabsorbed by amino-acid transport processes; (ii) it may serve as a highly sensitive model of renal amino acid transepithelial transport; (iii) net AIB reabsorption determines its endogenous pool size at steady-state; (iv) specific interactions between AIB and natural amino acid (e.g. with proline) cannot be accounted for simply by the general role of AIB as a short-chain aliphatic amino acid.

α-Aminoisobutyric acid (AIB) is a synthetic non-metabolizable, short-chain neutral amino acid which is reactive with membrane transporters serving uptake of other amino acids in various tissues (4) and it has long been used as a model substrate in the study of amino acid transport by kidney (24,30,35). However, if AIB uptake by kidney slices in vitro (24,30) represents transport at the antiluminal surface of epithelial cells primarily (29,37), such data are not then reliably informative about events at the brush border membrane. The suitability of AIB as a probe of net transepithelial reabsorption, which reflects initial uptake at the brush border membrane (30), requires that its renal handling in vivo be examined carefully.

Reabsorption of AIB across the luminal membrane of the proximal tubular cell in rat kidney in vivo is energy and $Na^+$-dependent (33). Moreover, some form of direct or indirect interaction between AIB and dibasic amino acids cystine, homocystine, and glycine during renal reabsorption in man is indicated by increased renal clearance of these natural amino acids when AIB is present in plasma (8). Nonetheless even at significant concentrations in plasma, AIB has no effect on the excretion of natural amino acids in the dog (35), yet AIB induces increased urinary loss of many natural amino acids in the rat (6). However, the mechanisms for these effects are unclear since plasma amino acid levels were not always reported. Glycine has been shown to impair net reabsorption of AIB in the rat (23).

The following report together with the accompanying paper (R.R. McInnes and C.R. Scriver, 1978b) extends the characterization of net renal reabsorption of AIB in the rat. The interactions between AIB (or α-methyl-L-alanine) and natural amino acids indicate that it does not behave as a simple probe of the alanine-preferring or "A" system of amino acid transporters (20). Moreover, observations on the renal tissue concentration of AIB indicate that complex interactions occur during modulations of its net reabsorption. Our observations were obtained with a method which permits sensitive and precise measurement of net reabsorption of AIB in vivo.

## METHODS

Female Long-Evans hooded rats obtained from Canadian Breeding Farms, Montreal and weighing between 190-220 gm, were given AIB (0.115 mmoles/kg or 1.22 mmoles/kg) and 5 μCi of AIB-1-[$^{14}$C] (2-10 mCi/mmole, from New England Nuclear Corp., Boston, Mass.) by intraperitoneal injection 16 hr before the experiment. The animals were provided with water and Purina Rat Chow overnight. This technique yields concentrations of AIB in arterial plasma of approximately 0.03 mM and 0.24 mM respectively, at the two dosages, at the time of study 15-20 hr later. The animal was anesthetized with Inactin (Na-5-ethyl-5(1-methylpropyl)2-thiobarbituric acid (Henley and Co., New York, N.Y.), 100 mg/kg ip), immobilized supine on narrow cardboard strips, and suspended on a single-pan balance. The trachea was bisected 3-5 rings below the thyroid cartilage, and the most cranial ring of the lower segment was exteriorized by suturing it to the skin.

The right external jugular vein was used for the infusion and the right femoral artery was used for sampling blood; each was catheterized with polyethylene tubing (Clay-Adams), and kept patent with heparinized saline (100 units/ml). The bladder was cannulated with 3-4 cm of polyethylene tubing, tied into the bladder to minimize dead space to less than 30 µl. The body temperature was kept at 37$^{o}$ with a rectal thermistor probe connected to a 75-watt incandescent light bulb 35 cm above the animal. Tracheal lavage was performed every 30 min with 30 µl of sterile 0.9% NaCl, followed by suction. This preparation maintained physiological homeostasis in the rat for 6 hours.

Animals were infused, at 16 ml/kg·hr. with a solution containing KCl, NaCl and inulin; NaCl (6.5 ml of 0.9%) containing 650 mg of purified inulin (Arnar-Stone Labs. Inc., Mount Prospect, Ill.) was added to 43.5 ml of NaCl (40 mM) and KCl (2.65 mM) containing 20 µCi of inulin [methoxy-$^{3}$H] (50-150 millicuries/gm; New England Nuclear). Tritium-labeled inulin mimics the behaviour of unlabeled inulin in the kidney (16). The solution was infused with a Harvard Pump Model 906 (Harvard Appliances Co., Millis, Mass.) at a rapid rate (3 ml/15 min) to initiate diuresis and to prime with inulin. Urine collections were initiated later (at 90-150 min) when body weight returned to within 1 gm of the initial post-dissection weight.

Renal Clearance Protocol. Seven consecutive urine collections each lasting 30-35 minutes, were obtained and acidified

immediately to pH 2 with HCl. At the mid-point of each period a blood sample (80-120 µl) was collected into heparinized capillary tubes from the femoral artery catheter. Cells were separated from plasma by centrifugation and the hematocrit was measured. Inulin, AIB and hematocrit were measured in all blood samples; calcium, phosphate, and the natural amino acids were determined only in periods 1,3,5 and 7. Calcium, phosphate, inulin, AIB and amino acids were measured in all urine samples. The rat was weighed at the end of each clearance period; body weight generally remained within 1.5% of the preinfusion weight.

Infusions of Natural Amino Acids. Amino acids (reagent grade) were obtained from Sigma Chemicals Co., St. Louis, Mo. Glycine, L-alanine, and L-proline were infused during successive 30 min. periods at incremental rates of 2.5, 5.0, 10 and 15 mmole/kg·hr. Lysine was also infused at 1.25 mmole/kg·hr. Each amino acid was dissolved in a solution containing NaCl (60 mM) and KCl (2.3 mM) and infused with a Sage Model 355 Infusion Pump (Orion Research Inc., Cambridge, Mass), inserted into the main infusion line; the combined infusion rate was 16 ml/kg·hr.

Infusion of AIB. Nine rats were infused with AIB-1-[$^{14}$C] (2-4 µCi/hr) mixed with unlabeled AIB (0.034-2.67 millimoles/hr). The specific activity of the infusion was kept constant for each rat but varied between animals so that a wide range of plasma AIB concentration could be examined. Clearance studies were begun after the AIB had been infused for 5

minutes. The infusion rate was kept constant, so that the increase in plasma AIB concentration resulted solely from ongoing accumulation of this non-metabolizable amino acid in excess of its disposal into tissues and urine. The infusion lasted 3 hrs. in each animal.

Measurement of AIB in Renal Tissue. At the end of the seventh collection period, the rat was killed by cervical dislocation; the kidneys were removed within one min, placed in ice-cold saline and decapsulated. Thin (0.2 mm) cortical slices were obtained with a Stadie-Rigg microtome. The capsular slice was discarded and the two outermost cortical slices were used for analysis of cortical AIB. Medullary slices were also obtained avoiding cortex and papilla. The slices were weighed and placed in NaOH (1N, 2 ml) overnight (25). After heating (70$^{o}$C x 90 minutes), the extract (100 µl) was added to 3 ml water containing 50 µl of concentrated acetic acid in a scintillation vial to which Aquasol (New England Nuclear Corp.) was added for counting.

Liquid Scintillation Counting. Plasma (20 µl) or urine (50 µl) were added to water (3 ml) in a glass scintillation vial, containing 10 ml Aquasol (18). The vials were shaken, chilled at 4$^{o}$C overnight prior to counting in a Uniflux II scintillation counter (Nuclear-Chicago). $^{14}$C and $^{3}$H were counted at 40% and 17% efficiency respectively, and the external standard:channel ratio method was used to resolve the $^{14}$C and $^{3}$H components in these double-label studies.

Purity of [$^3$H]-insulin and [$^{14}$C]-AIB. The purity of each lot of inulin methoxy-[$^3$H] was examined by descending partition chromatography on filter paper (Whatman No. 1) developed in a mixture of butanol:ethanol:$H_2O$ (26:9:15); strip-scanning was used to locate the labeled inulin. Material with more than 3% radiochemical impurities was rejected.

Purity of AIB-1-[$^{14}$C] was verified by partition chromatography on thin-layer cellulose chromatograms developed in a mixture of butanol:acetic:$H_2O$ (12:3:5); radiochemical purity exceeded 99%.

Chemical Methods. Na and K were measured by flame photometry. Osmolarity was determined by freezing-point depression. Amino acids were measured by elution chromatography on ion exchange resin columns with a modified Beckman-Spinco 120 amino acid analyser provided with an expanded scale (28). Plasma samples were deproteinized immediately with 5 volumes of sulfosalicylic acid (3% w/v) and held at -20$^{o}$C before amino acid analysis. Total calcium and inorganic phosphorus was measured in acidified urine by the methods of Gitelman (11) and Kraml (13) respectively adapted for the Technicon Autoanalyser (Technicon Corp., Tarrytown, N.Y.). Calcium and phosphorus were measured in plasma (volumes 10 µl and 5 µl respectively) with Rapid Stat$^R$ kits (Pierce Chemical Co., Rockford, Ill.) using Dade$^R$ Monitrol-1 solution (American Hospital Supply, Miami) as the standard. The reliability of this method of calcium measurement has been established (2).

Statistical Methods and Calculations. Tests of significance were performed by analysis of variance (31). Glomerular filtration rate (GFR) is the equivalent of the inulin clearance ($C_{In}$). Fractional excretion of AIB ($FE_{AIB}$) = Excreted AIB (μmoles/min) ÷ Filtered AIB (μmoles/min) where filtered AIB = GFR x plasma [AIB].

## RESULTS

1. Renal Handling of AIB in the Rat

Stability of the Physiological Preparation: Intra-peritoneal injection of AIB 16 hrs previously achieves stable plasma levels and fractional excretion of AIB in the rat (Figure 1). The plasma inulin level obtained with the infusion protocol (55.1 ± 0.15 mg/dl, mean ± SEM, 7 periods, 9 animals) was sufficient to estimate GFR reliably (9). The plasma level and fractional excretion of phosphate anion (Pi), selected to evaluate renal handling of a non-amino acid solute, was also stable under these conditions.

Sodium and potassium in serum of infused rats were 141.1 ± 1.8 mEq/L and 4.63 ± 0.18 mEq/L respectively in the 7th period (mean ± SEM for 9 animals), and not significantly different from those of the non-infused rat at zero time (143.4 ± 0.7 mEq/L and 4.06 ± 0.1 mEq/L respectively). The hematocrit was 47.67 ± 0.41 percent and 46.44 ± 0.53 at the beginning and end of the renal clearance and infusion protocol (seven periods). Body weight was 199.14 ± 3.17 g and 199.42 ± 3.35 g respectively. Serum osmolarity was 284.9 ± 0.15 mOsm in the seventh period. These data indicate stability of water and electrolyte metabolism during the infusion procedure.

$FE_{AIB}$ Urine Flow Rate and GFR. Urine flow rate and GFR were not related to $FE_{AIB}$ in a total of 112 clearance periods in 16 rats. The observed increase in urine flow rate in period 7 (46.2 ± 3.7 µl/min, mean ± SEM in period 7 vs 37.63 ± 3.83 µl/min, mean ± SEM, for the first six periods; $p < 0.01$) was not associated with a change in $FE_{AIB}$ (Figure 1). The cause for this increase was not investigated.

Relationship between $FE_{AIB}$ and Plasma [AIB]. Six rats were injected with AIB at low dosage (0.115 mmoles/kg, ip) 16 hrs prior to study while nine rats were given a 10-fold higher dose of AIB (1.22 mmoles/kg, ip). Steady-state plasma [AIB] after injection was 0.037 mM ± 0.006 (mean ± SEM) with the low dose and 0.242 mM ± 0.07, or 6.5-fold higher, with the high dose. Since renal excretion is the principal route for disposal of AIB in the rat (19), increased $FE_{AIB}$ could explain the relative deficiency of plasma [AIB] in the high-dose group. $FE_{AIB}$ was two-fold greater (0.126 ± 0.033, mean ± SEM) in the high-dose group, than in the low-dose group (0.061 ± 0.020; $p < 0.001$).

A negative correlation was found between plasma [AIB] and $FE_{AIB}$ among animals in the high-dose group (corr. coeff. 0.734, $p < 0.05$) (Figure 2A). This phenomenon is not unique to the clearance period depicted (the 6th); significantly negative correlations ($p < 0.05$) were present in five of the seven clearance periods, and in the remaining two periods (#3 and #4) the correlation was still negative but less significant ($p < 0.10$). The phenomenon appears to reflect

interindividual differences in renal handling of AIB in the rat (Figure 2), animals maintaining a higher plasma [AIB] have less renal loss under identical conditions of dosage and infusion.

Interindividual and Intraindividual variation in $FE_{AIB}$:

When $FE_{AIB}$ was plotted against time for each rat infused with AIB at the high-dose level (Figure 2B), little intra-individual variation is found. Variation between clearance periods did not exceed 0.033 (about 10% around the mean) in any single rat; it was less than 0.02 in 54 consecutive periods and less than 0.01 in another 37 periods. Experiments were rejected if the intraindividual variation in $FE_{AIB}$ exceeded 0.03 in three consecutive control periods. Inter-individual variation in $FE_{AIB}$ is 3-fold greater than intra-individual variation (Figure 2B); accordingly, each animal served as its own control in subsequent studies.

2. Effects of Natural Amino Acid Infusion.

On Plasma [AIB]: L-proline and L-alanine increased steady-state plasma [AIB] significantly ($p < 0.005$) (Figure 3, top row), as the infusion rate was increased from 2.5 mmoles/(kg·hr) to 15 mmoles/(kg·hr). Glycine infusion had a similar but smaller effect ($p < 0.01$) while L-lysine decreased plasma [AIB] ($p < 0.005$). The plasma concentration of the natural amino acids under these conditions were 9-11 mM (30-100 times normal) at the highest infusion rate; their filtered loads exceeded that of AIB by 20-30 fold.

On $FE_{AIB}$, GFR, and $FE_{Pi}$: Infusion of L-proline and L-alanine, at or below 5 mmoles/kg·hr, decreased $FE_{AIB}$ ($p < 0.005$ and $p < 0.001$ respectively), while L-lysine and glycine both increased it ($p < 0.001$) (Figure 3, middle row). At higher infusion rates the four amino acids increased $FE_{AIB}$.

GFR and $FE_{Pi}$ were altered only by alanine infusion. GFR was increased: control GFR, 1.93 ± 0.04 ml/min (mean ± SEM); experimental GFR, 2.32 ± 0.12 ml/min; $p < 0.005$. $FE_{Pi}$ was decreased: control $FE_{Pi}$, 0.200 ± 0.008; experimental $FE_{Pi}$: 0.165 ± .002; $p < 0.025$ at alanine infusion rates < 10 mmoles/kg·hr. The change in GFR and the parallel effects on $FE_{AIB}$ and $FE_{Pi}$ probably reflect alanine-induced alterations in renal hemodynamics.

On Renal Tissue [AIB]: The accumulation of AIB in renal cortex and medulla in vivo is expressed as a "tissue ratio" ($TR_{AIB}$), derived from the equation

$$\frac{\text{dpm/mg wet tissue weight} \times 1000}{\text{dpm/ml plasma}} .$$

This ratio, of necessity, includes AIB trapped in the proximal tubule lumen during preparation of the tissue. Accordingly, $TR_{AIB}$ is not equivalent to the distribution ratio for AIB achieved in slices incubated in vitro, where the amino acid appears to accumulate only in the cells (37).

Cortical $TR_{AIB}$ (n=9) is similar bilaterally (Figure 3, bottom row), (right kidney, 12.17 ± 0.62, mean ± SEM; left kidney 12.34 ± 0.65; NS). Medullary $TR_{AIB}$ (15.3 ± 0.7, mean ± SEM) is significantly higher than cortical $TR_{AIB}$ ($p < 0.025$), in keeping with earlier observations reported by Wedeen and Thier (36).

Glycine, alanine, proline and lysine each decrease $TR_{AIB}$ in both cortex and medulla when infused at 15 mmoles/kg·hr ($p < 0.001$ for each amino acid) (Figure 3). The effect of proline is unique in that it alone abolishes the difference between cortical and medullary $TR_{AIB}$ (Figure 3). Regardless of whether $FE_{AIB}$ is diminished or increased by proline at a low or high rate of infusion, the tissue ratio is decreased. Thus, AIB excretion and cortical accumulation are dissociated.

3. Effect of AIB Infusion on Plasma Levels of Natural Amino Acids.

The plasma concentration of sixteen natural amino acids was measured before and during intravenous infusion of AIB (see Methods). The concentrations of plasma amino acids during control periods were similar to published values for the rat (10,15). A negative correlation exists between plasma [AIB] at < 1 mM, and plasma concentration of most natural amino acids; the exceptions are phenylalanine, tyrosine and ornithine. The negative correlation is significant ($p < 0.05$) for eight amino acids, (leucine, isoleucine, valine, proline, glycine, alanine, lysine and glutamic acid). Representative data for neutral, cationic and anionic amino acids are shown in Figure 4. This figure also reveals a particular type of interaction for AIB with proline and glutamate. Their plasma concentration is increased above control when plasma [AIB] is < 1 mM. This finding was not apparent with any other amino acid.

Fractional Excretion: The relationship between fractional excretion of several natural amino acids and plasma [AIB]

is plotted in Fig. 5. When AIB is absent from plasma the excretion of amino acids is similar to that reported for rats by Lingard et al (15). The fractional excretion of most natural acids increases when plasma [AIB] is raised[(a)].

The AIB-induced increase in fractional excretion of natural amino acids in general is directly proportional to plasma [AIB] (Figure 5). $FE_{AA}$ is increased for threonine, serine, asparagine plus glutamine and glycine when the ratio of filtered AIB: filtered natural amino acid, is below 4.0; for glutamate and histidine when it is about 10.0; for phenylalanine, tyrosine, lysine and ornithine when it is above 40. Representative data are shown in figure 5. These observations indicate that any interaction between AIB and natural amino acids which affects net reabsorption is not related only to the chemical structure of the natural amino acid.

The glutamic acid excretion response is biphasic (Figure 5). When filtered AIB is below that of glutamate ($F_{AIB}/F_{glu}$, 0.69 ± 0.20, mean ± SD, n = 5), $FE_{glu}$ decreases 6-fold (to 0.68 ± 0.17) compared to control (4.22 ± 0.72 at zero plasma [AIB] $p < 0.05$): but when filtered AIB exceeds filtered glutamate, $FE_{glu}$ then rises to match or exceed control values.

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(a) Because AIB interferes with the chromatographic analysis of alanine, renal excretion of the latter could not be determined accurately.

4. Effect of AIB Infusion on Phosphate and Glucose Reabsorption;

AIB infusion had no effect on $FE_{Pi}$ (Figure 6) and plasma [Pi] remained constant. At no time during infusion of AIB did we detect glucosuria in the rat.

## DISCUSSION

We have developed a stable preparation for the study of factors affecting renal transport of AIB in vivo in the rat. Plasma AIB concentration, $FE_{AIB}$, GFR, urine flow rate, serum electrolyte concentration and osmolarity and blood hematocrit maintain an acceptable steady-state without blood volume contraction or expansion. The use of a non-metabolizable amino acid in vivo circumvents the problem of circadian fluctuation in plasma concentration and filtered load of natural amino acids (10) and avoids the influence of intrarenal metabolism on transepithelial transport (29). We chose AIB for our investigation, in contrast to cycloleucine whose reabsoption is greater than 99.9% in the rat (5; McInnes and Scriver, unpublished observations), because its reabsorption is about 85% at "physiological" concentrations in plasma (0.1-0.4 mM). Therefore variations in its reabsorption, both increased and decreased, are more easily observed. Furthermore, it is responsive to some of the factors which regulate tubular transport of amino acids (23).

We injected AIB 16 hr in advance to allow time for its equilibration between plasma and other endogenous pools. Under these conditions $FE_{AIB}$ is 7-20 fold greater than

previously reported at similar plasma levels but without the interval for equilibration (23). The apparent explanation for this finding is related to the equilibration of AIB with tubular epithelium as described in the accompanying paper (R.R. McInnes and C.R. Scriver, 1978).

We observed threefold interindividual variation in $FE_{AIB}$ but little intraindividual variation in the rat. Individuality of epithelial transport activity (17,26) and renal excretory activity (38) has been demonstrated previously and is part of the larger theme of biochemical individuality. AIB is eliminated from the body mainly by the kidney at increased rates at higher plasma levels. In rats achieving high plasma [AIB] in response to a fixed dose of the amino acid, $FE_{AIB}$ is low compared to that in rats with relatively low plasma [AIB] at the same dose. Thus, individuality of renal handling of AIB determines its plasma profile under a given environmental condition (i.e. dose of AIB).

The suitability of AIB for the study of transepithelial transport by mammalian kidney depends on the extent to which its behaviour parallels that of the natural amino acids. In addition to the previously-documented sodium-dependence of its proximal tabular reabsorption (33) we have made the following observations.

AIB reabsorption is not affected by spontaneous fluctuations in GFR or urine flow rate, in which respect it resembles the natural amino acids in man (14).

Net reabsorption of AIB is not directly proportional to its renal tissue concentration. A similar relationship

between tissue content and net reabsorption has been noted for lysine (1) and also for taurine (3). Tissue AIB is higher in the medulla than in cortex at steady-state. Although the in vivo tissue ratio that we adopted (25) to evaluate AIB accumulation by kidney measures solute in both the lumen and the intracellular space (32), our finding is compatible with intracellular accumulation of AIB as demonstrated by quick freeze radioautography studies by others (36,37). Moreover the modulation of tissue AIB in the presence of other amino acids presumably reflects specific interaction between AIB and the natural amino acids during movement across plasma membranes, as demonstrated in vitro (30).

AIB is capable of inhibiting net reabsorption of cationic, anionic and neutral amino acids. When one compares the ratio of filtered AIB-filtered natural amino acid, the short-chain aliphatic neutral amino acids are the most readily inhibited by AIB. These are the amino acids with least efficient reabsorption at endogenous levels in the rat (15) and man (26). Amino acids which are reabsorbed more avidly under endogenous conditions are least readily inhibited by AIB. The particular lack of effect of AIB on leucine excretion is compatible with exclusion of AIB from the leucine preferring site as described by Oxender and Christensen in Ehrlich cells (20). The interaction between AIB and cationic (dibasic) amino acids is not novel (8) and resembles that observed in vivo with natural amino acids (34). This phenomenon may reflect non-specific or non-competitive interactions during reabsorption.

Some natural amino acids stimulate AIB reabsorption in rat kidney. The increase in plasma AIB following alanine or proline infusion at low rates is associated with a stimulation of AIB reabsorption. Stimulation of AIB reabsorption in vivo at the low rate of proline infusion mimics enhancement of AIB uptake by cortex slices in vitro when exposed to low external concentrations of L-proline (30). It is also noteworthy that proline depletes renal tissue AIB less than other amino acids during in vivo infusion in the rat . This stimulation of net reabsorption of one amino acid by another has been noticed previously in vivo in the effect of sarcosine infusion on glycine reabsorption in human subjects with hypersarcosinemia (12). It may reflect transmembrane exchange of intracellular amino acid for luminal amino acid on a shared carrier.

The diminished fractional excretion of glutamic acid at low plasma concentrations of AIB is yet another example of the presence of one amino acid enhancing the net reabsorption of another. Since the plasma concentration of glutamic acid is elevated at low levels of AIB, this effect on fractional excretion is not an artifact of an AIB-induced fall in the filtered load of glutamate.

The stimulation of AIB reabsorption at low rates of alanine infusion, on the other hand, may be secondary to an alteration of renal hemodynamics, as the concomitant rise in GFR suggests. A true stimulant effect of alanine on AIB transport in vivo would be contrary to its inhibitory action

on AIB uptake by cortex slices in vitro (30).

AIB resembles the dibasic amino acids and is in contrast to several neutral amino acids in its failure to enhance phosphate excretion when infused into mammals (7). The constancy of phosphate excretion in our study of interactions between AIB and other amino acids, except in the presence of L-alanine suggests that the effect of one amino acid on another is the result of interactions at specific amino acid transport sites during their tubular reabsorption. On the other hand, AIB-induced decreases in the plasma levels of the natural amino acids cannot always be accounted for by an increase in their fractional excretion. For example, Christensen and Cullen (6) observed that AIB, at plasma levels comparable to those achieved in our study, reduced the hepatic content of many amino acids including branched-chain amino acids. In our study, however, the branched-chain amino acids, together with methionine, experienced no change in fractional excretion. Since the plasma levels of the branched-chain amino acids are lowered by insulin (21), and since AIB has been reported to stimulate insulin release from cultured foetal pancreas (22), the AIB-induced fall in the plasma level of some amino acids may be associated with an insulin effect on amino acid pools in tissues.

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## LEGENDS

Figure 1: Observations on the plasma concentration of AIB, fractional excretion of α-amino-isobutyric acid (AIB) and inorganic phosphorus (Pi), the renal clearance of inulin-[methoxy-$^{3}H$], and urine flow rate during seven consecutive 30-min clearance periods (9 rats, mean ± SEM). The animals were injected with AIB 16 hr prior to study and prepared as described in Methods.

Figure 2A: Relationships between $FE_{AIB}$ and plasma [AIB] in the sixth clearance period (150-180 min) in rats injected intraperitoneally with AIB sixteen hours previous to zero time. Two dosage levels are shown: low dose, 0.115 mmoles/Kg (open squares); and high dose, 1.22 mmoles/Kg (open circles). The correlation coefficient for regression of $FE_{AIB}$ on plasma AIB in the high dose group is $-0.734$ ($p < 0.05$).

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Figure 2B: Interindividual and intraindividual variation in $FE_{AIB}$. A three-fold inter-individual variation if $FE_{AIB}$ is observed in the group, whereas the intraindividual variation in each rat does not exceed ten percent around the mean for a given rat.

Figure 3: Effect of intravenous infusion of L-lysine (●), glycine (o), L-proline (Δ), and L-alanine (□), on plasma [AIB] (top row), $FE_{AIB}$ (middle row) and renal tissue AIB; the latter is expressed as a tissue ratio (see text) in cortex (◪, right kidney; ◪, left kidney, etc.) and in medulla (■, etc.).

Figure 4: Relationship between the plasma concentration of natural amino acid (ordinate) and plasma concentration of AIB (abscissa). Filled circles indicate concentration of the natural amino acid before AIB infusion; open circles indicate response during AIB infusion. The data are pooled from 9 animals, each studied at a different rate of AIB infusion so as to achieve a range of AIB concentration in plasma. Representative data for neutral, cationic and anionic amino acids are presented.

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Figure 5: Fractional excretion of natural amino acids in relation to plasma concentration of AIB. Representative data are presented for interactions when the filtered AIB: filtered natural amino acid is below 4 (left panel), approaching 10 (middle panel), and above 40 (right panel). Note biphasic response for glutamate (open circles indicate glutamate concentration prior to AIB infusion; closed circles, during AIB infusion).

Figure 6: Fractional excretion of inorganic phosphate phosphate in relation to $FE_{AIB}$ in nine rats infused with AIB by method used for Fig. 4 and Fig. 5. The regression of $FE_{Pi}$ on $FE_{AIB}$ (0.075) is not significant.

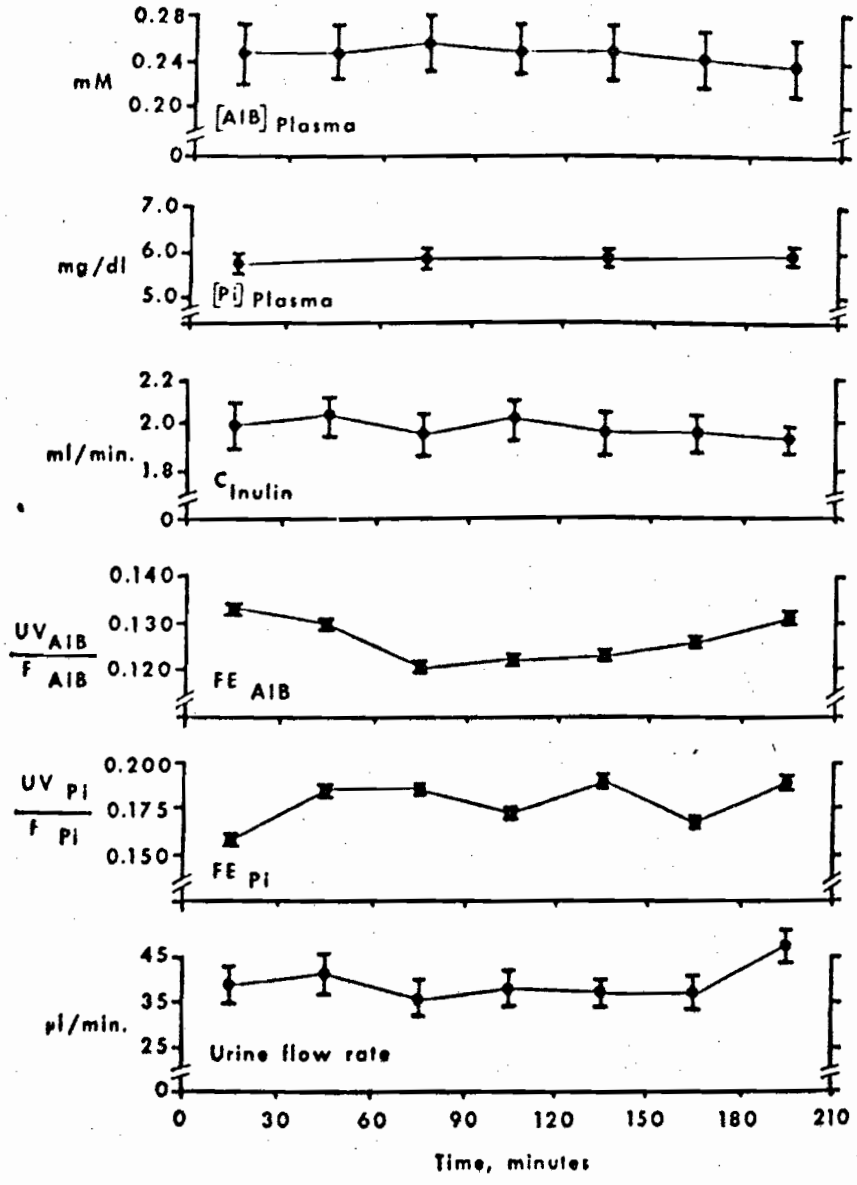
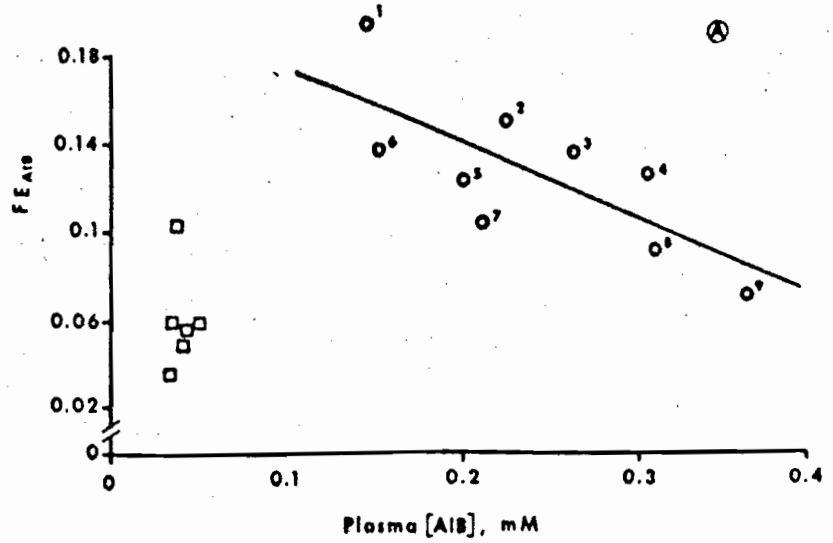
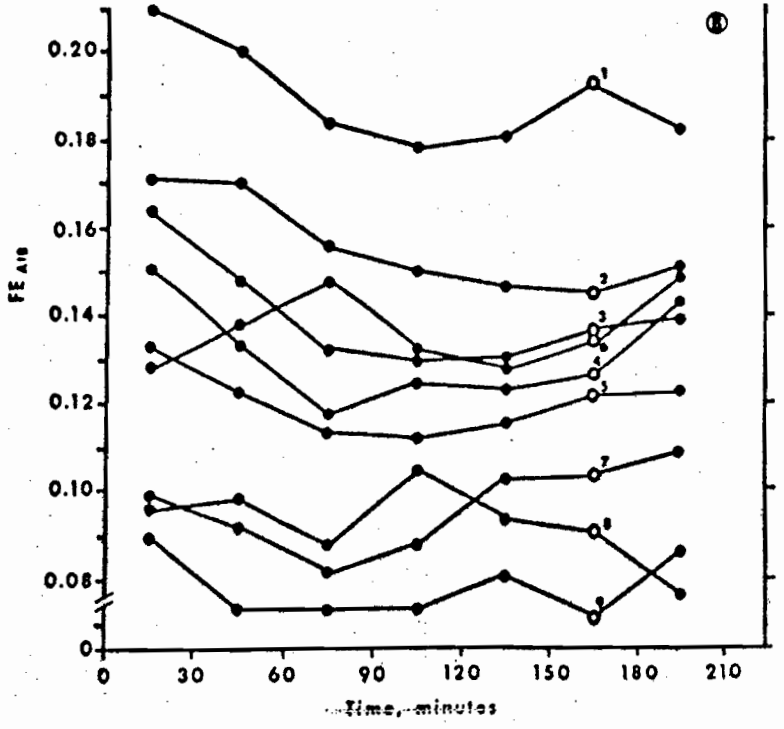

mM
0.28
0.24
0.20
0
[AIB] Plasma
mg/dl
7.0
6.0
5.0
[Pi] Plasma
ml/min.
2.2
2.0
1.8
0
C Inulin
UV AIB / F AIB
0.140
0.130
0.120
FE AIB
UV Pi / F Pi
0.200
0.175
0.150
FE Pi
µl/min.
45
35
25
0
Urine flow rate
0
30
60
90
120
150
180
210
Time, minutes

Figure 1


A
FE AIB
0.18
0.14
0.1
0.06
0.02
0
0.1
0.2
0.3
0.4
Plasma [AIB], mM


B
FE AIB
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0
30
60
90
120
150
180
210
Time, minutes

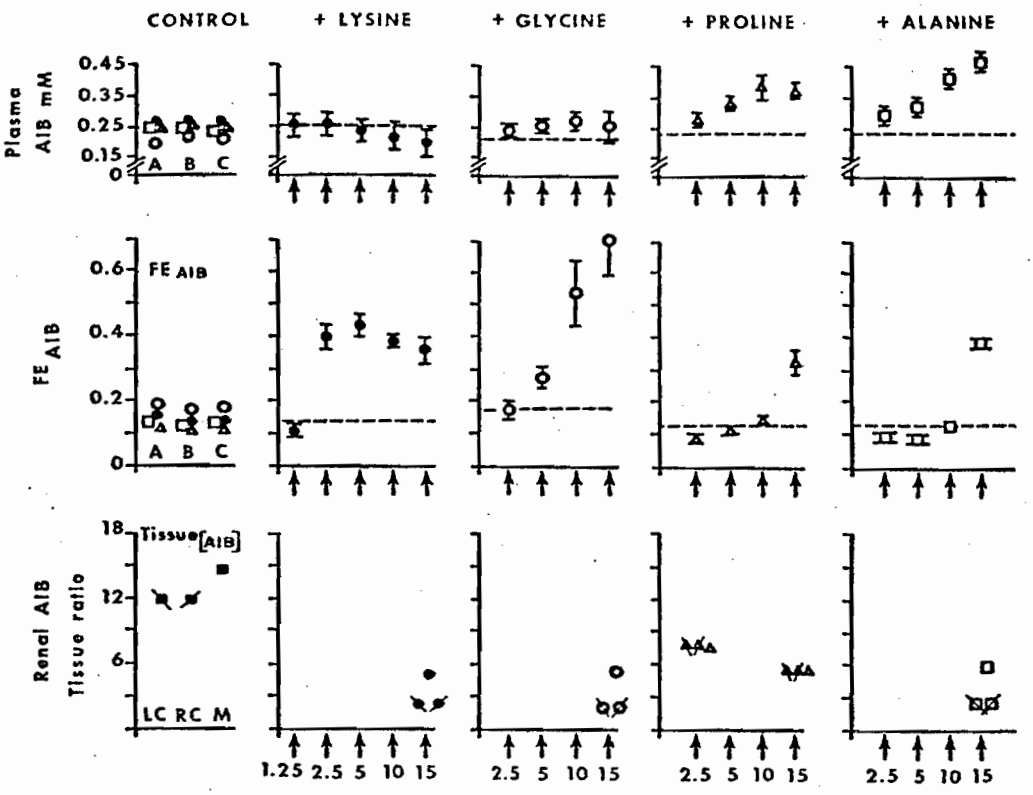



Figure 3

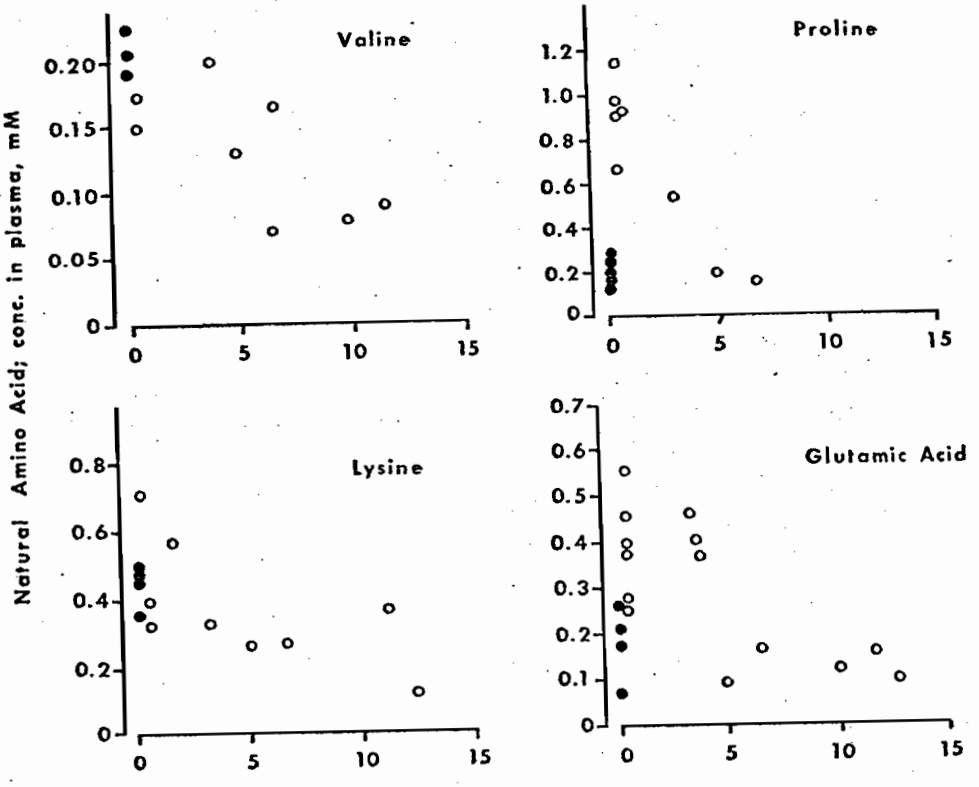



Figure 4

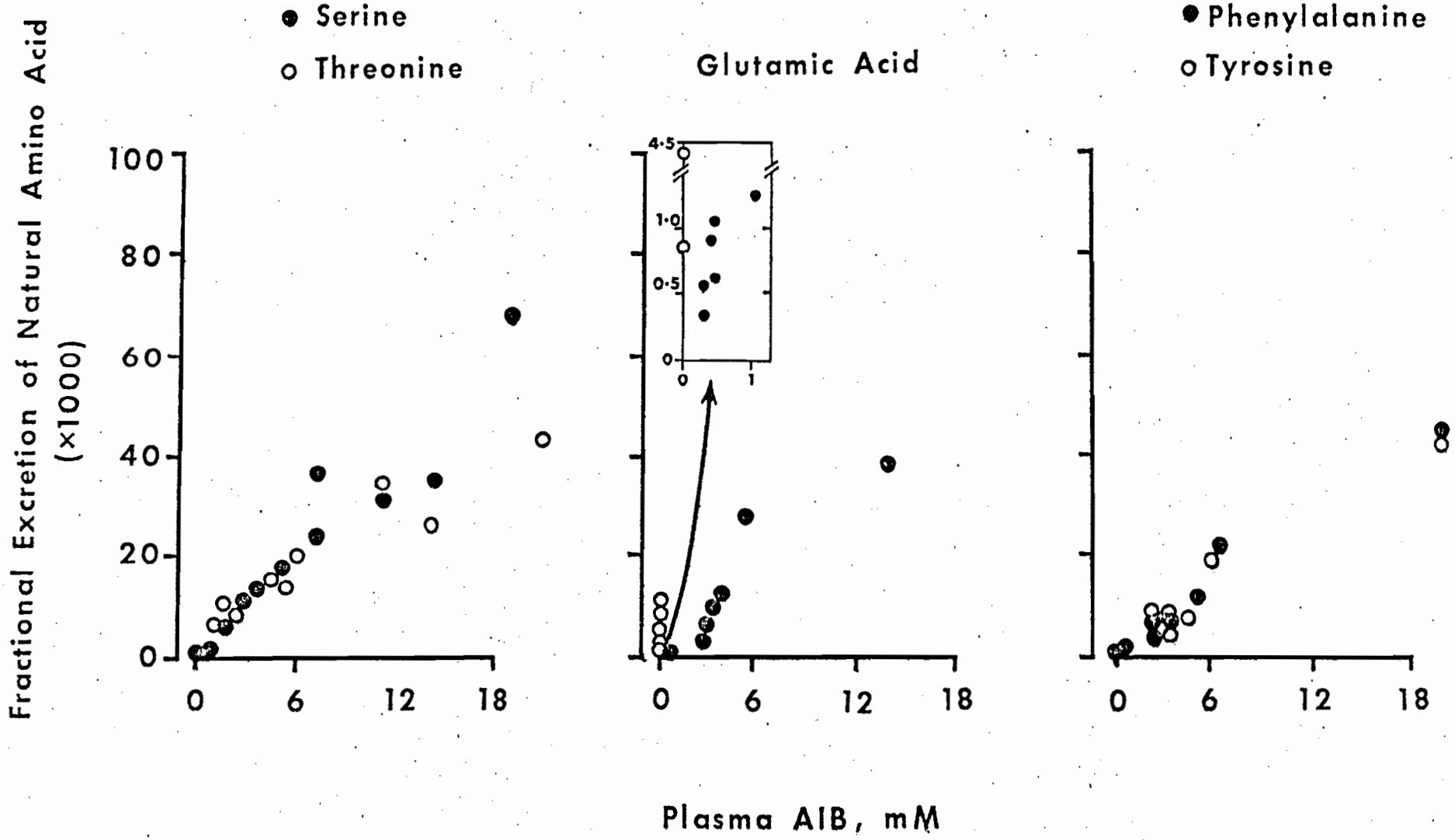

Serine
Threonine
Glutamic Acid
Phenylalanine
Tyrosine
Fractional Excretion of Natural Amino Acid (×1000)
100
80
60
40
20
0
0
6
12
18
4·5
1·0
0·5
0
1
Plasma AIB, mM

Figure 5

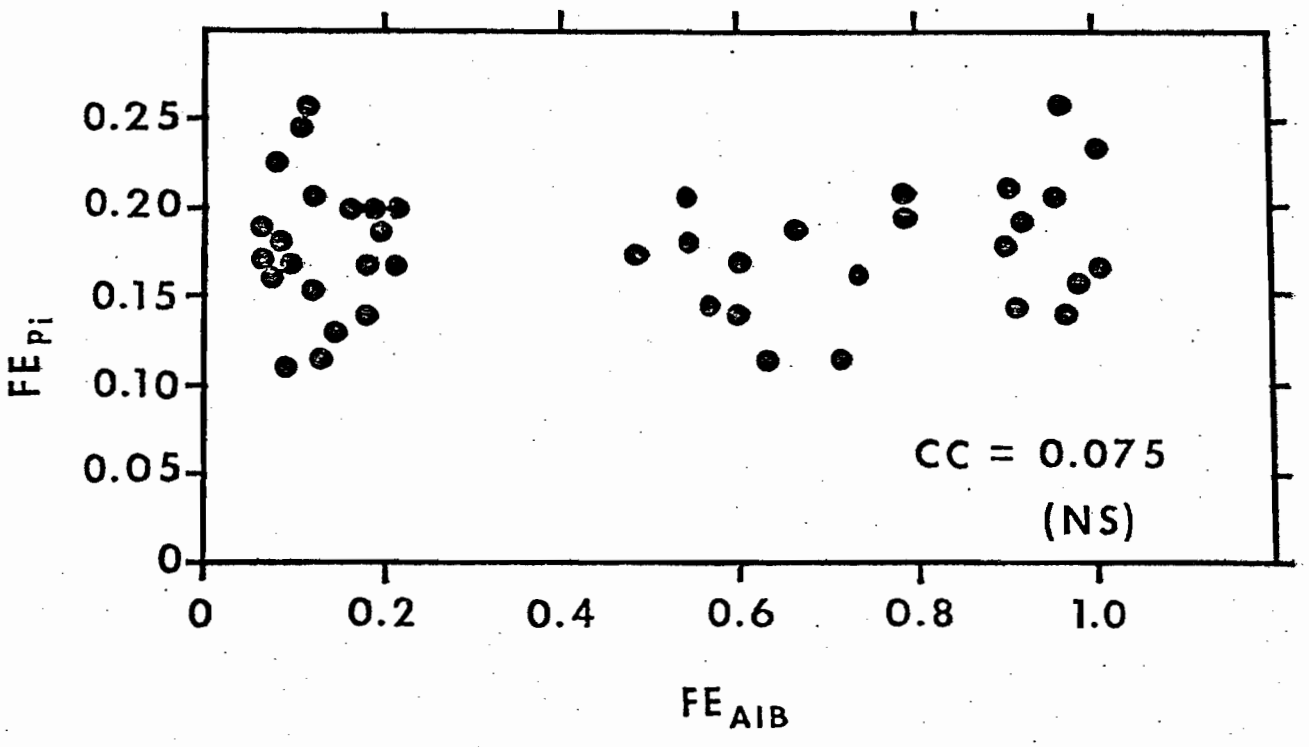



Figure 6

SECTION VII

## Net Tubular Reabsorption of α-Aminoisobutyric Acid. Evidence for a Cell to Lumen Flux

The identification of cell-to-lumen flux (backflux) of non-electrolyte solutes as a component of net tubular reabsorption in kidney was initially made using highly sophisticated technology: micropuncture techniques in vivo and isolated tubules in vitro. Under the usual physiological conditions backflux appears to have only a small influence on net reabsorption of amino acids, although in situations of abnormal solute excretion, this component may be of greater significance.

By means of the clearance techniques described in Section VI, we have been able to demonstrate the presence of a cell-to-lumen flux of α-aminoisobutyrate (AIB) in rat kidney. The contribution of this flux to net tubular reabsorption has been measured and its influence on the determination of the tubular reabsorption maximum (Tm) of AIB has been demonstrated. Although the specific cells giving rise to this flux were not identified, examination of AIB content in renal tissue suggests medullary segments as a potential source. Irrespective of the nephron segment involved, the findings suggest that renal cell accumulation of a solute influences net tubular reabsorption by modulating the cell-to-lumen flux. The ability to quantitate this flux suggests that this method might be used to differentiate between events which impair net AIB reabsorption by reducing luminal uptake from those which increase tissue-to-lumen flux of the amino acid.

These investigations also revealed that the use of artificial substrates for the study of biological transport in vivo, requires complete equilibration of them with body water pools before their renal

handling can be compared with the handling of natural analogues.

The report of these investigations has been submitted to The American Journal of Physiology. The following manuscript is organized according to the required format of that Journal.

# NET TUBULAR REABSORPTION OF α-AMINOISOBUTYRIC ACID.
## II. EVIDENCE FOR A CELL TO LUMEN FLUX

by

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Short Title: Bidirectional movement of AIB during net reabsorption.

## SUMMARY

The renal clearance technique was used to examine net reabsorption and fractional excretion of the non-metabolizable amino acid, α-aminoisobutyric acid (AIB), following rapid intravenous injection in the anesthetized rat. Only 6.5% - 11% of the injected dose (9.75 micromoles·kg$^{-1}$) remains in plasma after 2 min. (n=12 rats). Log-linear plasma decay is observed after 75 min. ($t_{\frac{1}{2}}$ = 440 min; slope - 0.00397 mmole·min$^{-1}$). Plasma inulin, GFR, urine flow rate, and $FE_{Pi}$ are stable in the post-injection period. $FE_{AIB}$ increases ($\Delta$ = + 0.075 ± 0.007, mean ± SEM, n = 6) while plasma [AIB] falls during the first 2 hr post-injection. This paradoxical finding is observed at various AIB injection loads (2.05 - 121 micromoles·kg$^{-1}$) and with reinjection of AIB into the same rat. Delayed equilibration of AIB with urinary tract dead-space does not account for the observation. Cell-to-lumen flux of AIB is the apparent source of the increasing $FE_{AIB}$. Slow uptake of AIB into renal tissue and particularly into medulla is observed during the period that $FE_{AIB}$ is rising and plasma [AIB] is falling. About half of net AIB excretion into bladder urine can be accounted for by a cell-to-lumen flux of AIB. Saturation of net tubular reabsorption of AIB is observed when plasma [AIB] exceeds ∿ 12 mM. When filtered AIB rises from 1 to 2 micromole·min$^{-1}$·100 cm$^{-2}$ in the titration experiments, net tubular reabsorption falls sharply. Differential behaviour of unidirectional fluxes (lumen to peritubular fluid and vice versa) at given concentrations of AIB may account for the finding. Clearance techniques

permit quantitation of the contribution of cell-to-lumen flux to net amino acid reabsorption and reveal the kinetics of renal tissue equilibration with extracellular AIB.

The synthetic non-metabolizable amino acids α-amino-isobutyrate (AIB) and cycloleucine have been used to study net renal tubular reabsorption and endogenous distribution of amino acids in the mammal (7-10,15,19,20). We have observed that fractional excretion of AIB ($FE_{AIB}$) in the rat is 7-20 fold greater (see accompanying manuscript, (11)) than has been reported previously (15). Although this finding may reflect a species difference (Long-Evans vs Sprague Dawley respectively) or the hormonal status (intact vs ovariectomized females respectively), it is more likely that an important methodological difference exists in the two studies. We gave AIB by intraperitoneal injection the night before measurement of $FE_{AIB}$, whereas Riggs and Barber (15) performed their measurements within 50 minutes after a subcutaneous injection of the amino acid. Because there are no endogenous pools of AIB in the mammal, there is an interval as it equilibrates with body water. This interval influences $FE_{AIB}$, as we will show.

Our data indicate that cell-to-lumen flux of AIB may occur in vivo under near steady-state conditions and that this phenomenon influences net tubular reabsorption. Possible sites of the cellular efflux of AIB are considered and the relationship between net reabsorptive activity and AIB pool size in renal tissues is described.

## METHODS AND MATERIALS

Female Long-Evans hooded rats (190-220 gm) (Canadian Breeding Farms, Montreal) were anesthetized with Inactin 100 mg/kg and placed on a pan balance at constant body temperature as described in the accompanying paper (11). The right external jugular vein (for infusions) and the right femoral artery (for blood sampling) were catheterized with polyethylene tubing. The urinary bladder was exposed and a funnel-shaped tip of PE-240 tubing introduced into the bladder fundus and tied with a purse-string suture to reduce bladder volume and dead space to a minimum. The animals were infused at 16 ml/kg·hr with a solution containing NaCl, 60 mM; KCl, 2.3 mM; inulin, 1.3 gm/dl (containing methoxy-$^{3}$H, 50-150 millicuries/gm, 400 μCi/L). Each animal was studied during seven consecutive clearance periods, each 30-35 minutes in duration. At the mid-point of the clearance period, whole blood (80-130 μl) was collected from the femoral artery catheter into heparinized capillary tubes. After separation of plasma by centrifugation, inulin, AIB, phosphate and hematocrit were measured. Urine volume and the content of inulin, phosphate and AIB were determined in each collection. The animal was weighed at the end of each clearance period.

At specified times the animal was killed by decapitation and the kidneys removed in 60 secs. After decapsulation, thin slices (0.2 mm) of cortex or medulla (excluding papilla) were taken with a Stadie-Riggs microtome, weighed and

digested overnight in 2 ml of 1N NaOH, then heated (70°C x 90 min); 100 µl of this extract was added to water (3 ml) containing acetic acid (50 µl) to which Aquasol was added for scintillation counting.

The accumulation of AIB by renal tissue was measured using the formula:

$$\text{Tissue ratio } (TR_{AIB}) = \frac{\text{dpm/mg wet tissue weight x 1000}}{\text{dpm/ml plasma}}$$

Protocol for rapid intravenous injection of AIB

Unlabelled AIB (9.75 micromoles/kg) and AIB-1-[$^{14}$C] were mixed in 100 µl of 60 mM NaCl and infused into the jugular vein (30s). Renal clearance studies were begun exactly 5 min after the injection to allow emptying of 1-2 volumes of urinary tract and catheter dead space. The urinary tract dead-space volume was measured directly and found to be 130 µl on the average. The volume in the catheter was also taken into account.

Determination of maximum rate of tubular reabsorption ($Tm_{AIB}$)

Nine rats were infused with a mixture of AIB-1-[$^{14}$C] (2-4 microCi/hr) and unlabelled AIB (0.034-2.67 millimoles/hr), the specific activity of which was kept constant for each rat. The AIB infusion rate was varied between animals to achieve a wide range of AIB concentration in plasma. Plasma AIB concentration increased because accumulation of this non-metabolizable amino acid exceeded the rate of its disposal into tissues and urine. The infusion lasted 7 periods (210 min) in each animal: renal clearance measurements were begun after AIB had been infused for 5 min.

Chemical and Isotopic Measurements: Liquid-scintillation counting, verification of the radiochemical purity of AIB-1-[$^{14}$C] and inulin-methoxy [$^{3}$H], and the chemical methods used to measure serum and urine electrolytes are described in the accompanying paper (11).

Materials: Inactin was obtained from Henley and Co., New York, N.Y.; isotopes from New England Nuclear, Boston, MA; α-aminoisobutyric acid from Sigma Chemical Co., St-Louis, MO.

Statistical Methods: Tests of significance were performed by analysis of variance, according to established methods (17).

## RESULTS

### Plasma [AIB] After Rapid Infusion

After rapid injection of labelled AIB (9.75 micromoles/kg), only 6.5% - 11% of the dose is recovered in plasma at 2 min, assuming a plasma volume of 31.3 ml/kg (5). The plasma concentration at this time is 25.2 ± 1.7 μM (mean ± SEM, 12 rats) Two-thirds of the injected AIB is in the extracellular fluid, assuming an extracellular fluid volume of 249 ml/kg rat (5). The plasma decay curve for log AIB plotted against time after 15 min is shown in Figure 1. Decay is first-order after 75 min, the estimated $t_{\frac{1}{2}}$ being 440 min (Fig. 1). Plasma decay of AIB reflects equilibration with tissue fluids and renal loss, since AIB is not metabolized (12).

Tubular Reabsorption of AIB After Rapid Infusion

Plasma inulin, renal clearance of inulin, urine flow rate and phosphate excretion remained stable following rapid infusion of AIB in our experiments (Table 1). Fractional excretion ($FE_{AIB}$) is related to plasma concentration of the amino acid (Figure 2A). The relationship is paradoxical. $FE_{AIB}$ increases as plasma [AIB] declines following rapid infusion of the amino acid. This unusual finding is the principal focus of interest in our experiments. The corresponding decline in tubular reabsorption of AIB after rapid injection of the amino acid is plotted in Figure 2B. Extrapolation of the regression to zero time allows one to calculate the theoretical tubular reabsorption of AIB at the instant of injection (Footnote a).

Variation in the dose of injected AIB over a wide range (2.05 - 121 micromoles/kg) results in plasma [AIB] varying between 0.002 μM and 0.12 μM in clearance period #1. Irrespective of plasma [AIB] in the first period, $FE_{AIB}$ always increased in the following two or three clearance periods. Two consecutive injections of AIB (9.75 micromoles/kg each), 120 min apart in the same rat, elicited the typical increase in $FE_{AIB}$ following the second injection (Figure 3).

AIB in Renal Tissue after Rapid Injection

The AIB content of renal cortex after injection in vivo is higher at 30 min than at 3.5 hr, while in medulla it is the same at 30 min and 3.5 hr (Table 2). The tissue ratio of AIB is the same in medulla and cortex at 3.5 hr (Table 2).

Renal Reabsorption and Excretion as a Function of Duration of Infusion and AIB Concentration in Filtrate

Plasma AIB is said to be completely filterable (15). We have confirmed this fact by observing complete recovery of labelled AIB added to rat plasma followed by gel filtration on a Sephadex G-25 column.

Plasma [AIB] was varied 400-fold (0.088 - 33.2 mM) by infusing different concentrations of the amino acid (0.17 - 13.4 millimoles/kg·hr; n=9 rats). $FE_{AIB}$ was then measured and plotted against plasma [AIB] for each rat.

An important feature was observed. In the first three of the seven sequential clearance periods, $FE_{AIB}$ is related not only to plasma [AIB] but also to the clearance period in which it is measured. For example, at plasma [AIB] 0.41 mM in period No. 1 of rat No. 2, $FE_{AIB}$ is 0.02, while at plasma [AIB] 0.40 mM in period No. 4 of rat No. 4, $FE_{AIB}$ is 0.134 (Figure 4). This 7-fold difference in $FE_{AIB}$, at comparable plasma [AIB] exceeds that expected of interanimal variation (11) and is a function of the clearance period in which the measurement is made. The finding is rather analogous to those depicted in Figures 2 and 3.

The time-dependent characteristic of $FE_{AIB}$ in relation to the plasma [AIB] achieved in the infusion experiments is shown in detail in Figure 4. Statistical analysis reveals that $FE_{AIB}$ in the first clearance period for each of the eight rats is significantly lower than in periods 3-7 ($p < 0.005$). $FE_{AIB}$ in the second period, at any given

plasma [AIB], is intermediate to that observed in the first period and the third or subsequent clearance periods (Figure 4). This finding indicates that the duration of AIB infusion is a significant determinant of $FE_{AIB}$ when AIB has been infused less than 60-90 min.

At 0.25 mM AIB in plasma, $FE_{AIB}$ in later clearance periods following constant infusion is similar to $FE_{AIB}$ at that plasma concentration after intraperitoneal injection and 16-hr equilibration (11). This finding suggests that equilibration of AIB with body water and renal tissue is approached in about 90 min - as indicated in Figure 2A.

Net tubular reabsorption of AIB under near steady-state conditions apparently saturates at an elevated plasma concentration of the amino acid (Figure 5). However the relationship between filtered load and net reabsorption is complex. At filtered loads between 1 and 2 $\mu moles \cdot min^{-1} \cdot 100\ cm^{-2}$ there is a striking decline in net tubular reabsorption. The anticipated progressive saturation of the process is seen clearly again only at filtered loads exceeding 3 $\mu moles \cdot min^{-1} \cdot 100\ cm^{-2}$ or the equivalent of about 6 mM AIB in plasma. The latter increase and saturation of net reabsorption in relation to the filtered load of AIB has been previously reported by Riggs and Barber (15).

Renal Tissue AIB During Tm and Relative to $FE_{AIB}$

The concentration of AIB was measured in renal cortex at the end of the seventh clearance period and the tissue ratio plotted against the reciprocal of the plasma AIB

concentration and against $FE_{AIB}$ (Fig. 6). Saturation of AIB uptake by kidney cortex in vivo is observed and there is a reciprocal relationship between the tissue ratio of AIB and its fractional excretion.

## DISCUSSION

We have observed (11) that rats injected with AIB 16 hr prior to study have fractional excretion rates seven to twenty-fold greater than the rates measured by Riggs and Barber (15), who injected their animals less than one hour prior to measurement of AIB reabsorption. Although this discrepancy in $FE_{AIB}$ at different time intervals may reflect the use of ovariectomized Sprague-Dawley rats by the earlier investigators, in contrast to the intact Long-Evans hooded female rats used in our experiments, a more likely explanation is suggested by the studies reported here. We observe that $FE_{AIB}$ in the same rat is approximately ten-fold less in the first 60-90 minutes of AIB infusion than in subsequent clearance periods. Since Riggs and Barber measured $FE_{AIB}$ in the first hour following its injection into their rats, it is likely that their measurements were obtained before $FE_{AIB}$ had fully equilibrated with body fluids and renal tissue.

The initial disappearance of AIB from plasma, after its rapid intravenous infusion, reflects dilution in extracellular fluid, analogous to that described for extracellular tracers. Oldendorf and Kitano (13) showed that about 80% of any extracellular fluid probe (e.g. mannitol) disappears from

the plasma volume in the first minute after injection, irrespective of renal function. Stabilization of $FE_{AIB}$ proceeds for another 60-90 min and is therefore not a phenomenon that reflects redistribution of the solute. Binding to and release of AIB from saturable tissue binding sites might explain the rise in $FE_{AIB}$ while plasma [AIB] is falling. However, the phenomenon is seen over a wide range of plasma [AIB] and it can be uniformly elicited following two sequential injections of AIB. These observations make binding of AIB to tissue sites an unlikely explanation for the paradoxical rise in $FE_{AIB}$.

Initial filling of urinary tract dead-space is another artefact to be considered. AIB must equilibrate with the dead-space before its rate of urinary excretion can be determined reliably. The glomerulus-to-catheter volume required to account for the delayed rise in $FE_{AIB}$ noted in the early clearance periods can actually be calculated; it is approximately 1350 microlitres (Footnote b). This volume greatly exceeds the true glomerulus-to-catheter volume in our experiments. Thus, filling of dead-space is not an appropriate source of the rising $FE_{AIB}$ in early clearance periods.

Diffusional efflux of AIB from peritubular fluid to lumen via intercellular channels is yet another possibility. This mechanism is also unlikely, since $FE_{AIB}$ should then be in direct linear relationship to plasma [AIB] and it is not. The relationship is inversely proportional and non-linear.

We suggest that renal tissue itself - presumably tubular epithelium - is an important source of urinary AIB and that it contributes significantly to the estimate of net $FE_{AIB}$ and to the change in $FE_{AIB}$ with time. If the uptake and equilibration of plasma AIB with renal tissue is slow, as it is across basolateral membranes in cortical slices in vitro (16,22), then $FE_{AIB}$ should change in direct proportion to AIB accumulation by renal cells in vivo. In this context the rising tissue ratio of AIB ($TR_{AIB}$) in vivo from early to later clearance periods is of interest. Tissue uptake ratios of amino acids in general (1) and of AIB in particular (16) are inversely proportional to extracellular concentration of solute. We observed that the medullary $TR_{AIB}$ in vivo is less than and then equal to, cortical $TR_{AIB}$ at 0.5 hr, 3.5 hr respectively (Table 2); it is greater than cortical $TR_{AIB}$ at 20 hr (11). These findings suggest that a slowly rising medullary pool of AIB relative to the plasma concentration could be the source of a cell-to-lumen flux of AIB. This flux could account for the rising $FE_{AIB}$ while plasma AIB is falling after rapid injections of AIB.

Although medullary segments of the nephron may be the principal source of AIB accumulation in the lumen (21), any other cell in contact with urine could contribute to efflux. But regardless of the specific cells giving rise to the proposed efflux, quantitation of this contribution to net AIB excretion is possible by extrapolation to zero time of the initial decay in net AIB reabsorption (see Footnote a). The demonstration of solute backflux during net reabsorption

in the mammalian urinary tract is not novel (2,3,4,18). Our findings are, however, the first evidence obtained by simple clearance techniques for a cell-to-lumen efflux of an amino acid. Our particular interest is that the finding, in our experiments, permits quantitation of the backflux and its contribution to net excretion of AIB (Footnote a). It has not escaped our notice that this method of estimating cell-to-lumen flux might be used to differentiate between events which impair net AIB reabsorption by reducing luminal uptake, from those that increase tissue-to-lumen flux of the amino acid.

The attainment of constant $FE_{AIB}$ as the filtered AIB approaches $\sim$ 1 micromole$\cdot$min$^{-1}\cdot$100 cm$^{-2}$ (plasma AIB $\sim$ 6 mM) is consistent with saturation of renal uptake into tissue pools. In vitro studies of AIB uptake in renal cortical slices (16) and our measurement of the tissue ratio of AIB in cortex in vivo indicate that saturation does occur. The in vivo finding reveals that transport of AIB into renal cells is mediated by a membrane process, as it is known to be in vitro (16).

The anomalous fall in net reabsorption of AIB (or rise in $FE_{AIB}$) at filtered loads above 1.0 micromole$\cdot$min$^{-1}\cdot$100 cm$^{-2}$ and below 3 micromole$\cdot$min$^{-1}\cdot$100 cm$^{-2}$ is a matter of interest. It does not represent intrinsic general changes in tubular function, since the simultaneous fractional excretion of phosphate does not change (11). We suggest that the independent mediated transports of AIB across cell

membranes from lumen to peritubular fluid and vice versa saturate at different concentrations. A rise in $FE_{AIB}$ relative to filtered load would reflect augmentation of the backflux component, relative to the uptake component from the lumen, at filtered loads of AIB between ∿ 1.0 and ∿ 3.0 micromole·min$^{-1}$·100 cm$^{-2}$. The fall in $FE_{AIB}$ relative to filtered load when filtered AIB is further increased would indicate saturation of the mediated backflux process, with continuing diffusional backflux proportional to the extracellular concentration of AIB. This condition permits saturation of the uptake flux to be observed at higher solute concentrations in filtrate.

Apparent saturation of the reabsorptive capacity for AIB at filtered loads in excess of 3.0 micromoles·min$^{-1}$·100 cm$^{-2}$ (about 10-12 mM AIB in plasma) may have an explanation, additional to filling of the carrier at high substrate concentrations. Riggs and Barber (15), showed that infusions of mannitol or high concentrations of glycine provoked saturation. Since our animals experienced an increased urine flow rate and a loss of about 2.5% body weight (unpublished observations) when infused with AIB at these high rates, (vs. no weight loss at lower AIB infusion rates (11)), it is possible that volume contraction secondary to an osmotic diuresis is responsible for the initial recovery of the reabsorptive capacity for AIB prior to saturation. Active AIB reabsorption has been shown to be $Na^+$-dependent (19), and $Na^+$-reabsorption in the proximal tubule is influenced by physical factors

such as changes in oncotic pressure. A rise in oncotic pressure would be expected to increase $Na^+$-reabsorption (6), and therefore AIB reabsorption, during volume contraction.

In summary, our experiments imply that there is a bidirectional flux of AIB during its tubular reabsorption. The unidirectional fluxes may saturate independently and they may contribute to the ultimate saturation of net reabsorptive flux. Our experiments did not reveal an unequivocal Tm for AIB but an apparent Tm was at least approached. Perturbation of plasma volume may have complicated the demonstration of a classical Tm.

The intercept, 0.943 (from Figure 2B) represents net reabsorption of AIB prior to any contribution of cell-to-lumen flux to net amino acid excretion. Since the fractional reabsorption of AIB at apparent steady-state is 0.853, the cell-to-lumen flux is estimated to be

$$\frac{0.943 - 0.853}{1.0 - 0.853} = 0.6$$

This value represents the theoretical contribution of back flux to net excretion of AIB at steady-state under the conditions of our experiments when plasma [AIB] is 4.3 - 7.3 μM.

The glomerulus to catheter (dead-space) volume required to produce slow equilibiation of $FE_{AIB}$ during the first three periods can be estimated by assuming that the actual $FE_{AIB}$ in clearance periods 1 and 2 is the same as the $FE_{AIB}$ measured during the steady-state clearance periods 4-7.

Using data from a representative animal:

$TR_{AIB}$ (mean, periods 4-7) at equilibrium is 83.9% filtered AIB (period 1) is 104,000 dpm/min. Therefore, theoretical $UV_{AIB}$ (period 1) = 104,000 - (83.9% of 104,000 = 16,760 dpm/min. The observed $UV_{AIB}$ in period 1 is 6966 dpm/min. Therefore, AIB accumulated in dead-space = 16,760 - 6966 = 9795 dpm/min and AIB accumulated in dead-space over 30 min (period 1) = 293,850 cpm. Since the actual entry of AIB into the dead-space = 16,760 dpm/min, the time required for dead-space to accumulate 293,850 dpm is 293,850 dpm ÷ 16,760 dpm/min = 17.53 min. With an observed urine flow-rate of 41 µl/min, the dead-space volume = 17.53 min x 41 = 718.7 µl. Similar calculations for period 2 yield a dead-space volume of 509.6 µl. The combined dead-space volume for 60 min is then 1228.33 µl.

To this volume must be added that entering the dead-space in the 5 minutes after AIB injection before the clearance study began. That volume is calculated by assuming that in the first period, 17.53/30 minutes were needed to fill the dead-space. In the 5 min period, 2.92 minutes would be required to fill dead-space; at a flow rate of 41 µl/min,

this volume would be 119.72 µl. Therefore total theoretical dead-space required to account for the gradual rise in $FE_{AIB}$ in periods 1 and 2 = 1228.33 + 119.72 = 1348.1 µl.

Direct measurement of urinary tract plus catheter dead-space with saline yields an average volume of 130 µl per rat. Owens (14) reports glomerus-to-catheter dead-space to be 300 µl, but attempts to limit bladder and catheter dead-space were not described. Therefore we are confident in saying that the dead-space volume in our experiments is 4-10 times too small to account for the phenomenon of rising $FE_{AIB}$ in early clearance periods after rapid intravenous infusion of AIB.

## ACKNOWLEDGEMENTS

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TABLE 1

RENAL FUNCTION FOLLOWING RAPID INJECTION OF AIB IN THE RAT

(Mean ± SEM)

| Post-Injection Period No | Plasma Inulin (mg/dl) | Inulin Clearance (ml/min) | Urine Flow Rate (µl/min) | $FE_{Pi}$ |
|---|---|---|---|---|
| 1 | 62.8±4.1 | 1.85±0.01 | 42.7±5.7 | 0.210±.03 |
| 2 | 61.2±4.3 | 2.03±0.05 | 49.9±3.3 | 0.158±.02 |
| 3 | 63.1±4.4 | 1.97±0.07 | 45.8±1.5 | 0.162±.02 |
| 4 | 62.0±4.7 | 2.05±0.09 | 45.3±2.9 | 0.141±.02 |
| 5 | 62.7±5.2 | 1.95±0.06 | 45.0±3.3 | 0.140±.02 |
| 6 | 62.1±5.0 | 1.97±0.06 | 44.5±2.2 | 0.161±.02 |
| 7 | 64.0±5.2 | 1.92±0.05 | 47.0±1.5 | 0.161±.03 |

TABLE 2

AIB CONTENT AND TISSUE RATIO IN KIDNEY

AFTER RAPID INJECTION OF AIB (mean ± SD)

| | At 30 min | At 3.5 hr |
|---|---|---|
| 1. Content, dpm/mg wet wt. | | |
| Cortex | 1284.5±24.8 (n=16) | 953.4±26.6** (n=16) |
| Medulla | 830.0±218* (n=8) | 923.8±274.6 (n=8) |
| 2. Tissue Ratio[a)] | | |
| Cortex | 27.65±5.01 (n=16) | 43.74±9.84** (n=16) |
| Medulla | 17.65±3.33* (n=8) | 43.74±15.47 (n=8) |

* vs cortex at 30 min, $p < 0.001$

** vs cortex at 30 min, $p < 0.001$

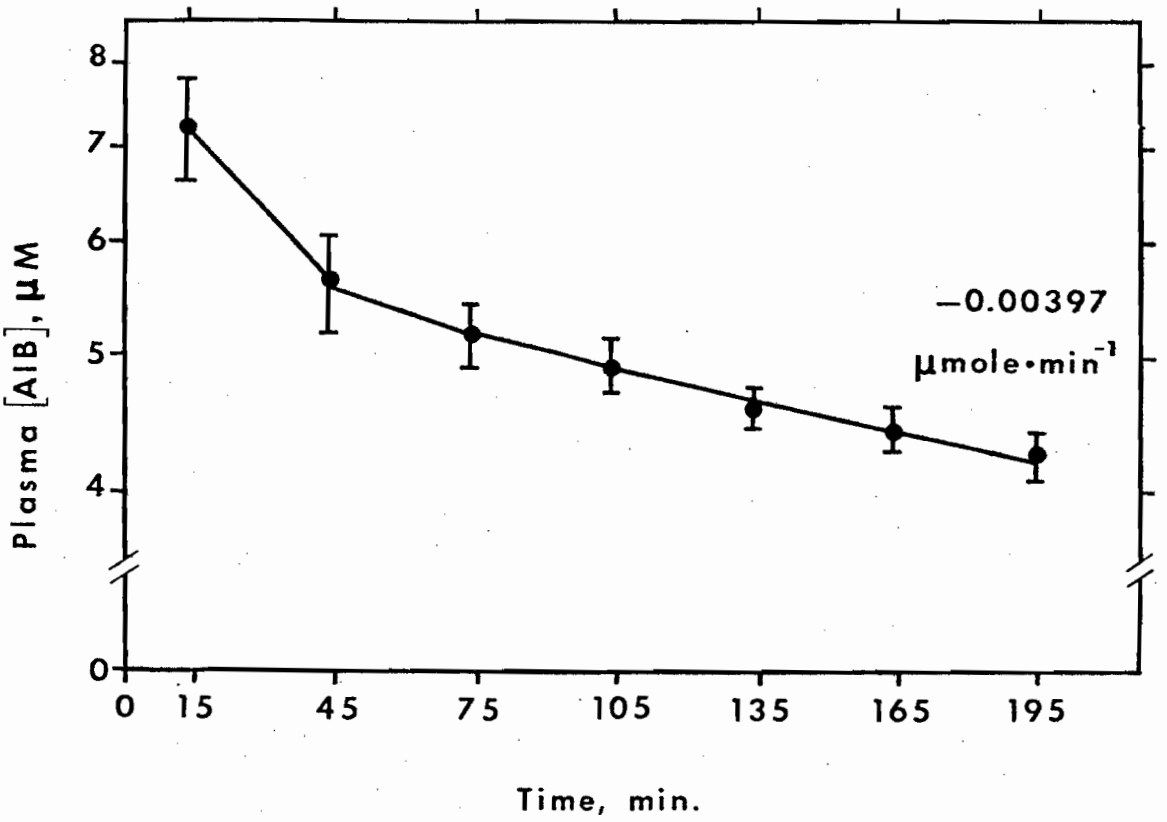
(a) see Methods for description of tissue ratio

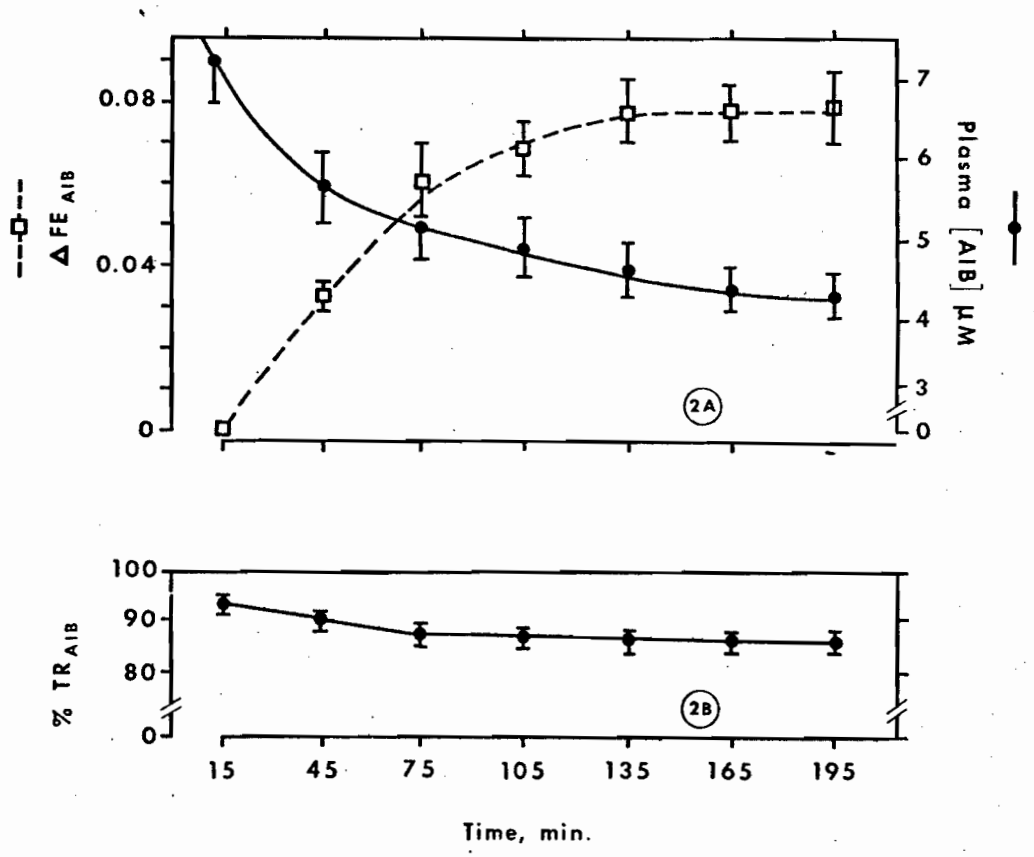
## LEGENDS

Figure 1: Decay of plasma [AIB] (mean ± SEM, n=6) following rapid injection of 9.75 micromoles/Kg. The first clearance period began 5 min after the injection. Arterial blood samples were taken in the mid-point of each 30 min clearance period. The decay is log-linear after 75 min; the slope of the decay from 75 to 195 min is shown (-0.00397 $\mu mole \cdot min^{-1}$).

Figure 2A: The relationship between $\Delta FE_{AIB}$ and plasma [AIB] after a rapid injection of 9.75 micromoles/Kg of AIB (n=6 rats, mean ± SEM). Because of interindividual variation in $FE_{AIB}$ (see ref. 11), data for $FE_{AIB}$ are plotted as the difference between the actual clearance period and the first clearance period, the latter set at zero and differences expressed as $\Delta FE_{AIB}$.

Figure 2B: Net reabsorption of AIB (% of filtered load reabsorbed, log scale) in relation to time. Data obatined from animals described in Figure 2A. Log linear extrapolation of the regression to zero time is used to estimate theoretical net reabsorption of AIB at the instant of injection (see text and footnote A).

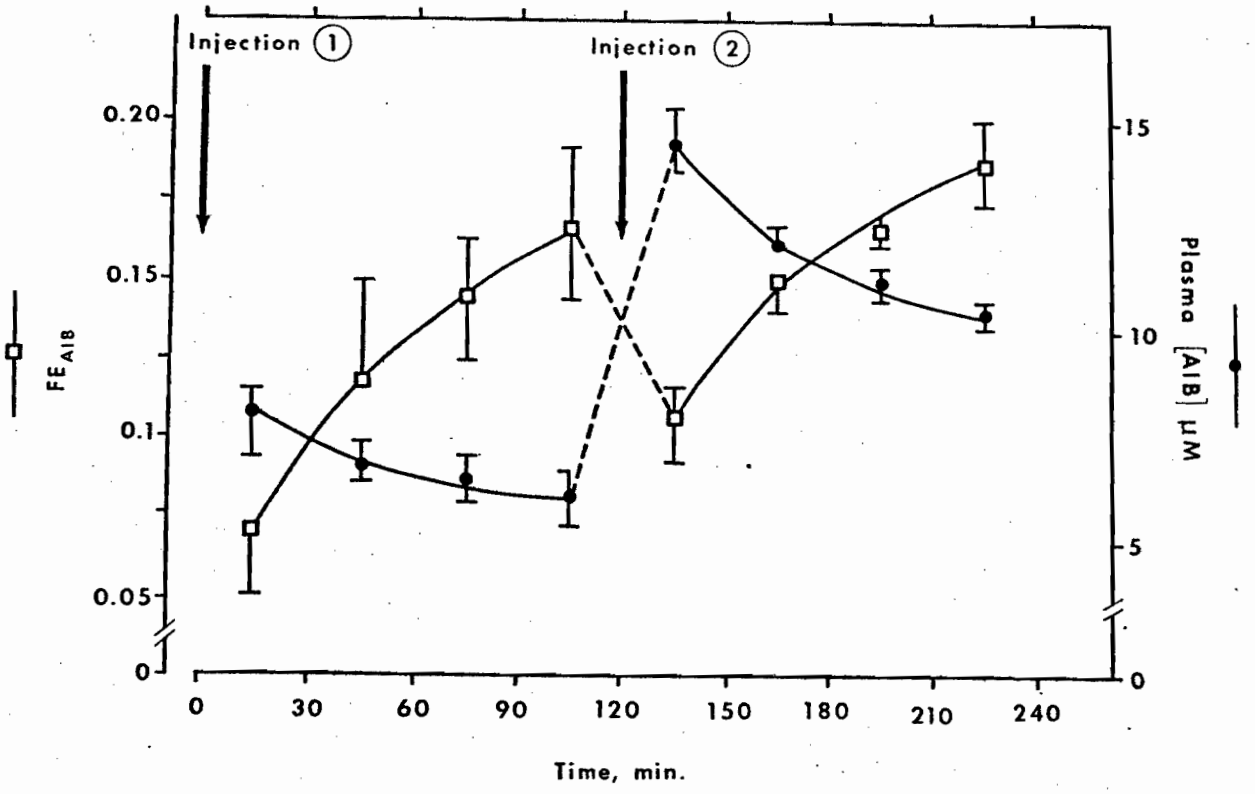

Plasma [AIB], μM
8
7
6
5
4
0
0 15 45 75 105 135 165 195
Time, min.
−0.00397
μmole·min⁻¹

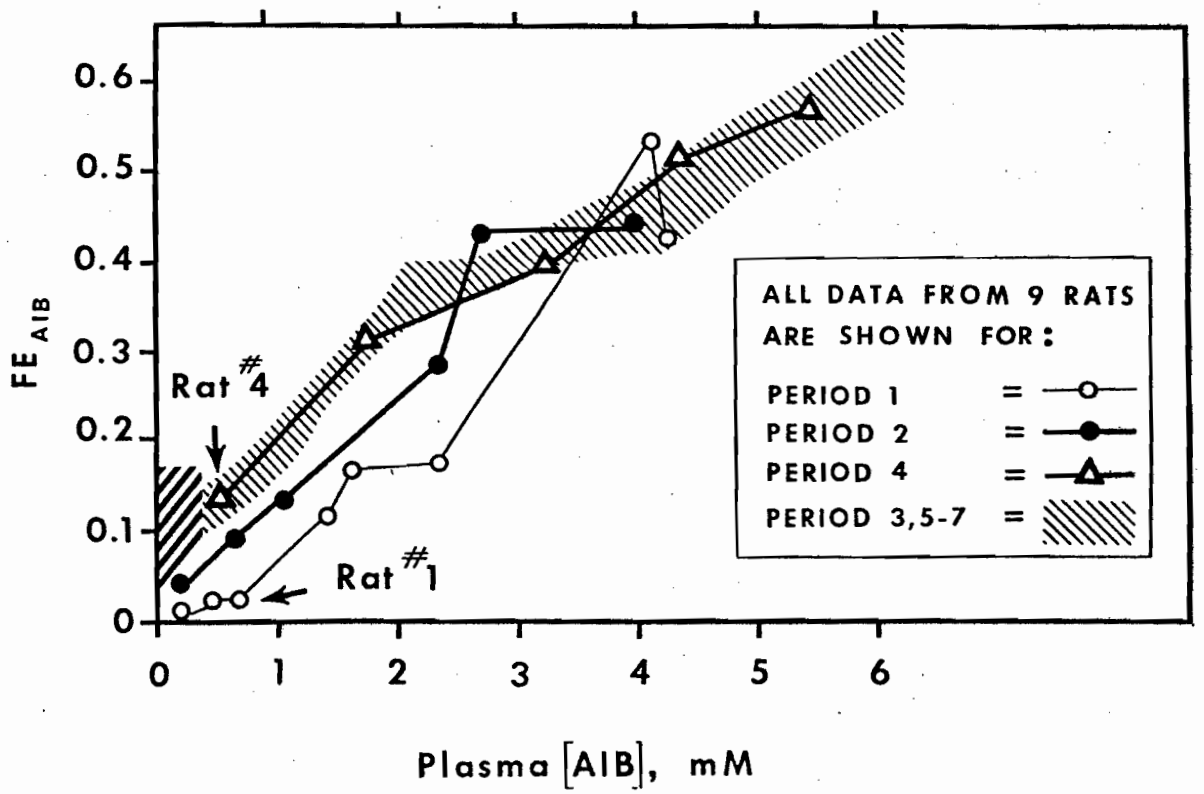

ΔFE AIB
0.08
0.04
0
Plasma [AIB] μM
7
6
5
4
3
0
2A
% TR AIB
100
90
80
0
2B
15 45 75 105 135 165 195
Time, min.

## LEGENDS

Figure 3: $FE_{AIB}$ after two sequential rapid injections of AIB (9.75 micromoles/Kg, 2 rats (mean ± SD)). Because $FE_{AIB}$ in the first clearance period was comparable in the two animals, data were not converted to $\Delta FE_{AIB}$ (vs. Fig. 2A). The rise in $FE_{AIB}$ in the presence of falling plasma [AIB] occurs after each injection of AIB.

Figure 4: $FE_{AIB}$ as a function of both plasma [AIB] and clearance period, for individual rats (n=8) infused to raise plasma [AIB] to various levels. All points for first-clearance periods (O) are joined. Data from the second (●) and fourth (Δ) clearance periods are treated in similar fashion. The shaded area represents the area on graph covered by data for the third to seventh clearance periods inclusive. The first period begins at 5 min; the seventh ends at 215 min approx. Note that first-period $FE_{AIB}$ below 3 mM AIB in plasma is always significantly lower than $FE_{AIB}$ in periods 3-7. Second-period $FE_{AIB}$ is intermediate ($0.02 < p < 0.05$) between first- and third-period $FE_{AIB}$ at plasma [AIB] below 3 mM.


Injection (1)
Injection (2)
0.20
0.15
0.1
0.05
0
FE AIB
15
10
5
0
Plasma [AIB] µM
0
30
60
90
120
150
180
210
240
Time, min.

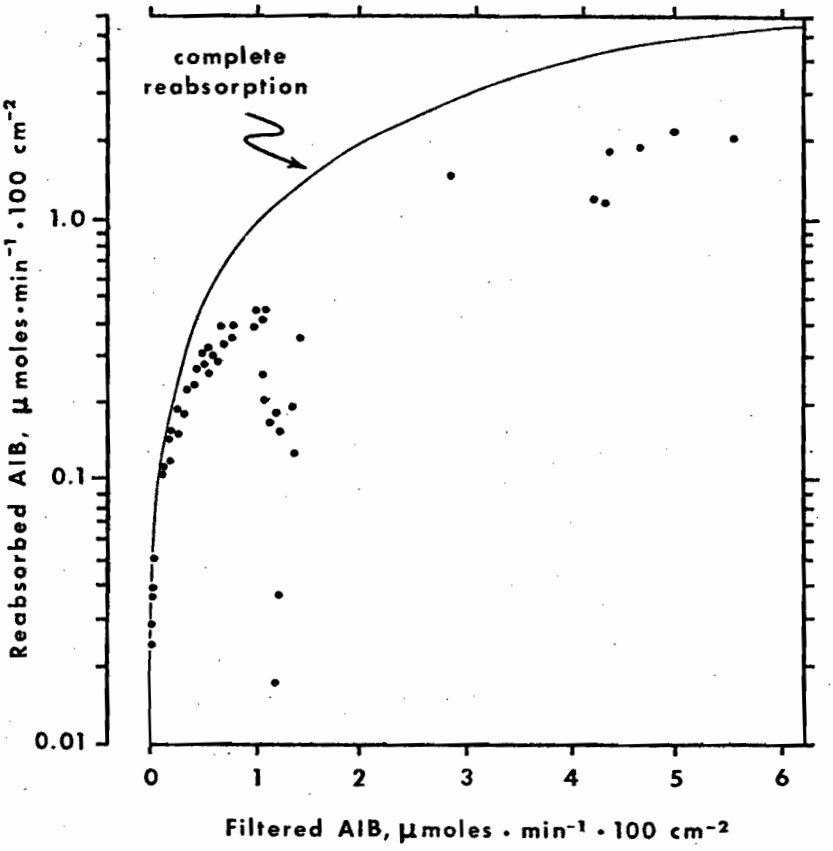

0.6
0.5
0.4
0.3
0.2
0.1
0
FE AIB
Rat #4
Rat #1
ALL DATA FROM 9 RATS
ARE SHOWN FOR:
PERIOD 1 =
PERIOD 2 =
PERIOD 4 =
PERIOD 3,5-7 =
0
1
2
3
4
5
6
Plasma [AIB], mM

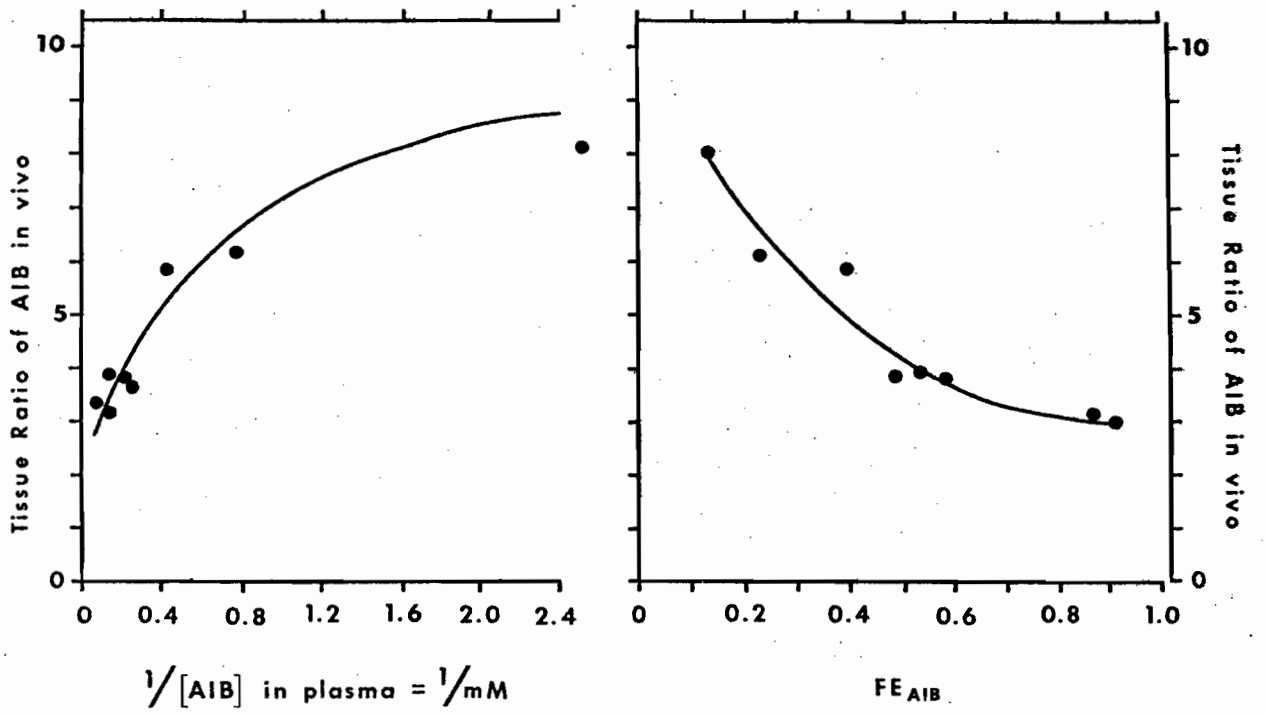
## LEGENDS

Figure 5: Net tubular reabsorption of AIB as a function of AIB in filtrate. AIB is considered to be completely ultrafiltrable. Data for first and second clearance periods are excluded (see Figure 4 for rationale). Animals were infused with AIB at different rates to achieve different plasma concentrations, thus randomizing the plasma AIB concentration achieved in relation to clearance period (third to seventh).

Figure 6A In vivo tissue ratio of AIB ($TR_{AIB}$) in relation the reciprocal of plasma [AIB] (adapted from Akedo and Christensen, 1962). Plasma [AIB] was sampled $\sim$ 90 sec. prior to bilateral nephrectomy for determination of the tissue [AIB]. Each point is the mean of 4 cortex slices per rat (n=8).

Figure 6B: $TR_{AIB}$ from Figure 6A plotted in relation to observed $FE_{AIB}$; the latter was determined in the clearance period immediately preceeding nephrectomy.


complete
reabsorption
Reabsorbed AIB, µmoles·min⁻¹·100 cm⁻²
1.0
0.1
0.01
0
1
2
3
4
5
6
Filtered AIB, µmoles · min⁻¹ · 100 cm⁻²


Tissue Ratio of AIB in vivo
10
5
0
0
0.4
0.8
1.2
1.6
2.0
2.4
1/[AIB] in plasma = 1/mM
0
0.2
0.4
0.6
0.8
1.0
FE AIB
Tissue Ratio of AIB in vivo
10
5
0

SECTION VIII

## Effect of Calciotropic Hormones and Cyclic Nucleotides on Net Reabsorption of α-Aminobutyric Acid and Phosphate by Rat Kidney in Vitro

Several metabolic and genetic insults to the renal cortex result in the increased excretion of a spectrum of solutes known, from other studies, to be transported by separate carriers. Consequently, these disorders, called renal Fanconi syndromes, would appear to be due to the dysfunction of a component(s) common to or independent of the individual transport systems of each actively transported substrate. Alterations in membrane permeability or in renal cell bioenergetics are the two most likely causes of this type of disturbance.

In this section we describe experiments which have facilitated our understanding of the generalized hyperaminoaciduria found in several disorders of calcium metabolism, including autosomal recessive vitamin-D dependent rickets (see Section III for a review of this disease). Using the in vivo rat model described in Section VI, we measured α-aminoisobutyrate (AIB) excretion during perturbations of calcium metabolism by the infusion of calciotropic hormones, cyclic nucleotides, or $CaCl_2$. Only calcitonin decreased net AIB reabsorption significantly in the TPTX rat. Whereas PTH and cyclic nucleotides had the anticipated effect only on phosphate excretion. Alterations in membrane permeability follow changes in cytoplasmic $[Ca^{++}]$ in many tissues. Since calcitonin is

believed to lower cytoplasmic [$Ca^{++}$] of target cells, the impairment of amino acid reabsorption in vitamin D dependency and deficiency states may be a reflection of this particular event.

In addition to proposing a basic mechanism for the renal Fanconi syndrome of several disorders, these studies also indicated that, contrary to much of the literature, phosphaturia is not one of the physiological actions of calcitonin.

This work has been submitted for publication in Pediatric Research, and the following manuscript has been written according to the style of that journal.

# EFFECT OF CALCIOTROPIC HORMONES AND CYCLIC NUCLEOTIDES ON NET REABSORPTION OF α-AMINOISOBUTYRIC ACID AND PHOSPHATE BY RAT KIDNEY IN VIVO

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Short title: Effects of calciotropic agents on amino acid reabsorption

## SUMMARY

Parathyroid extract (PTE), calcitonin (CT), $N^6$ - 2' - O - dibutyryl cyclic 3', 5' monophosphate (db cAMP), adenosine 3', 5' - monophosphate (cAMP) and $CaCl_2$ were infused separately into calcium - and vitamin-D-replete rats either intact or acutely thyroparathyroidectomized (TPTX) to examine their effects on fractional excretion (FE) of phosphate anion (Pi) and non-metabolizable α-aminoisobutyrate (AIB). $Fe_{Pi}$, $FE_{AIB}$ and GFR were equally stable in the intact and TPTX rats (Figure 1) while $FE_{Pi}$ and $FE_{AIB}$ are decreased by TPTX ($p < 0.001$) (Fig. 1). PTE and db-cAMP increased $FE_{AIB}$ in intact rats, (Figure 2); the effect was not observed in TPTX animals (Figure 3). Although PTE decreased, and db-cAMP increased GFR in intact rats (Figure 2), these effects on renal hemodynamics do not account for the rise in $FE_{AIB}$, since analagous hemodynamic changes occurred in the TPTX rats (Figure 3). Despite the rise in GFR in db-cAMP treated rats, filtered AIB fell because plasma [AIB] fell sharply (Figure 4). cAMP and $CaCl_2$ both reduced $FE_{AIB}$ in TPTX rats (Figure 3), but GFR also fell and cAMP also caused a small decline in plasma [AIB]. A CT-mediated permissive role in PTE- and db-cAMP- induced aminoaciduria in the intact animal is suggested because: (i) PTE increased total plasma [$Ca^{++}$] to normocalcemic levels in TPTX rats, but did not alter total plasma [$Ca^{++}$] in intact rats (Table 1); (ii) db-cAMP is a known CT secretagogue; (iii) CT (25 mU/kg·hr) increased $FE_{AIB}$ in TPTX rats without

altering GFR or urine flow rate, despite an associated fall in plasma [AIB]. At this dose CT also reduced total plasma [$Ca^{++}$] and plasma phosphate, but did not increase FE phosphate. These data indicate that CT can impair net reabsorption of amino acids apparently by a direct effect on kidney and suggest that PTH and dibutyryl-cAMP-induced increases in fractional excretion of amino acids in the intact animal depend on increased CT secretion or the change it produces in renal cells. We suggest also that phosphaturia is not a necessary physiological effect of CT.

## INTRODUCTION

An acquired defect in renal tubular reabsorptive activity occurs in man in the presence of vitamin-D or calcium deficiency and in primary hyperparathyroidism (37). The tubular dysfunction can include excessive phosphaturia and aminoaciduria (17,28,33), hyperbicarbonaturia (36, 38) and increased excretion of other solutes normally reabsorbed by the proximal tubule (12). The pathophysiological basis of the tubulopathy is unknown. Alterations in renal hemodynamics (36,37), or in the permeability characteristics of the proximal tubule (30,31) secondary to excess parathyroid hormone (PTH) (1,26) or its intracellular messenger adenosine-3': 5'-monophosphate (cAMP) (50,51) may be responsible. Excess PTH, however, does not consistently induce a reabsorptive defect. For example, there is no hyperaminoaciduria in two-thirds of subjects with primary hyperparathyroidism (14). Furthermore, Muldowney et al (38) noted no increase in amino acid excretion after PTE administration to normal subjects. Of four subjects with endogenous hyperparathyroidism or infused with PTH and studied by Short et al (47), only two had an abnormally low Tm proline which returned to normal after removal of the PTH source.

Therefore, it appears that the sensitivity of the amino acid reabsorptive process to PTH is such that only unphysiological quantities of the hormone elicit any effect and that individual response, even to these quantities, covers a wide range. This variation in response suggests that increased PTH per se is insufficient to cause hyperaminoaciduria, and that additional

factors must be involved.

In order to elucidate the basis of this pathophysiological process further, we examined by methods described previously (35) the effects of infused calciotropic hormones and cyclic nucleotides on tubular reabsorption of the non-metabolizable amino acid, α-aminoisobutyrate (AIB), in calcium- and vitamin-D-replete rats, either intact or acutely thyroparathyroidectomized (TPTX). The results of this investigation follow.

## METHODS

Female Long-Evans hooded rats (190-220 gm) were anesthetized with Inactin (Henley & Co., N.Y., N.Y.) 100 mg/Kg) and following dissection of the neck, the thyroid and parathyroid glands were either left intact, or the rats were thyroparathyroidectomized (TPTX) using an electric cautery. All control rats were thus sham-operated.

In either case all clearance studies were begun between 4 - 6 hours following TPTX or simple neck dissection. The right external jugular vein (for infusions) and the right femoral artery (for blood sampling) were catheterized with polyethylene tubing, and the bladder was cannulated. The animals were infused with a solution containing 60 mM NaCl, 2.3 mM KCl, 1.3 gm/dl inulin and 400 uCi/l inulin-methoxy (methoxy-$^{3}$H, 50-150 millicuries/gm) at 16 ml/kg·hr to achieve a steady state as described previously (35). Each animal was studied during seven to eight consecutive clearance periods of 30-35 minutes

each. Near the mid-point of the clearance, a blood sample (80-130 ul) was collected from the femoral artery catheter into heparinized capillary tubes. Plasma inulin, AIB and hematocrit were measured in all periods; plasma phosphate was measured in periods 1,3,5 and 7. Urine inulin, phosphate, AIB, and urine volume were measured and the animal was weighed in each 30 minute period.

Infusion of Calcium, Calciotropic Hormones, and Cyclic Nucleotides

These infusions were made using a Sage Model 355 Infusion Pump (Orion Res. Inc., Cambridge, Mass.). The syringe containing the treatment compound was "piggy-backed" into the polyethylene tubing delivering the mixture of electrolytes and inulin. The experimental treatment were: (1) calcium chloride, 0.66 millimoles/Kg·hr; (ii) parathyroid extract (PTE) (Lilly Para-Thor-Mone$^{R}$ 100 USP/ml), 40 units/Kg·hr; (iii) calcitonin (homogenous pure porcine calcitonin, 90 MRC units/mg - a generous gift of Dr. Claude Arnaud), 25 or 500 milliunits/ Kg·hr; (v) $N^{6}$ - 2' - O - dibutyryl cyclic 3',5' -monophosphate (cAMP) (Sigma),15 mg/Kg·hr. The nucleotides were dissolved in an aliquot of the infusion mixture less than 30 min. before their use. The PTE was infused undiluted to maintain its stability. Calcitonin was dissolved in a solution of 0.9% saline, and 0.01M acetic acid (1:1) and human albumin (4mg/ml). Less than 200 ul of this solution was infused per study, and vehicle alone was infused in control periods.

Renal Clearance Protocol

The clearance techniques used were identical to those described previously (35). Three baseline clearance period studies were made on each rat, followed by four treatment clearance periods. This allowed each animal to serve as his own control, an approach necessitated by the significant interindividual variation in the fractional excretion of AIB in rats, in contrast to the small intraindividual variability of this parameter (35).

Chemical and Radioactivity and Statistical Measurements

The method used for liquid-scintillation counting, verification of the radiochemical purity of AIB-1-($^{14}$C) and inulin-methoxy (methoxy-$^{3}$H) and the chemical methods used to measure serum and urine electrolytes have been described previously (35).

Analyses of variance were made according to standard methods (48). The rat model that we have used to study the renal tubular reabsorption of AIB and Pi exhibits good intra-individual stability of $FE_{AIB}$, $FE_{Pi}$, GFR and plasma AIB concentration within individual rats under baseline conditions (35). However there is considerable interindividual variation in renal handling of AIB in our experience. In the present investigation, data for groups of rats are presented as the mean ± SEM of the interindividual differences between the interindividual mean of the control (first three) clearance periods, and each clearance period (control) after the

experimental procedure. This method of analysis accomodates the interindividual variation and permits data to be analyzed from groups of rat. Using this approach, significant effects of treatment were revealed.

## RESULTS*

Control rats (intact and TPTX) have stable $FE_{AIB}$, $FE_{Pi}$, GFR and plasma concentration of AIB throughout the period of study (Fig. 1). Fractional excretions of AIB and phosphate are decreased by thyroparathyroidectomy ($p < 0.001$, both situations); the effect of TPTX is considerably greater on phosphate excretion than on AIB excretion. There is no significant difference in GFR between intact and TPTX animals.

### PTE and dbcAMP Infusions

Infusions of PTE into intact rats, after 30 minutes increases $FE_{AIB}$ ($\Delta FE_{AIB}$, + 0.061 ± 0.016, $p < 0.001$) (Fig. 2). The response of the renal tubule to PTE appears to be at least partly mediated by the intracellular generation of cyclic AMP (11). Therefore, the effect of its dibutyryl analogue was examined. $FE_{AIB}$ is increased by dbcAMP in the intact rats ($\Delta FE_{AIB}$, + 0.043 ± 0.006 after 30 minutes; $p < 0.001$) (Fig. 2). As expected, both PTE and dbcAMP also increased phosphate excretion significantly in the intact animal ($p < 0.001$) (Fig. 2).

PTE and dbcAMP have opposite actions on GFR (Figure 2). Despite their similar effects on $FE_{AIB}$ and $FE_{Pi}$, PTE produces

a pronounced fall in GFR in intact rats ($\Delta$GFR, - 0.33 ± 0.06 after 30 minutes, $p < 0.001$) while dbcAMP increases GFR, ($\Delta$GFR, + 0.138 ± 0.06; $p < 0.05$ first period only).

PTE and dbcAMP did not alter $FE_{AIB}$ in TPTX rats, even though GFR is affected (Figure 3). GFR is again decreased by PTE ($\Delta$GFR, - 0.13 ± 0.05 after 30 minutes, $p < 0.001$) and increased by dbcAMP ($\Delta$GFR, + 0.03 ± within 30 min. $p < 0.005$). PTE increased $FE_{Pi}$ significantly in TPTX rats ($p < 0.001$) (Fig. 3). The time course of phosphaturia following PTE was significantly slower in the TPTX animals when compared with intact controls ($\Delta FE_{Pi}$ in the first treatment period of the two groups was + 0.18 ± 0.04 and + 0.51 ± 0.08 respectively ($p < 0.001$)). Dibutyryl cAMP induced phosphaturia in TPTX rats was greater ($p < 0.001$) but observed a time course similar to that seen in intact animals given identical doses of the nucleotide.

Cyclic AMP and $Ca^{++}$ Infusions

cAMP infusion reduced $FE_{AIB}$ (-0.032 ± 0.005 after 30 minutes, $p < 0.001$), in association with a decrease in GFR (-0.16 ± 0.05, $p < 0.005$). As expected, $FE_{Pi}$ was strikingly increased by cAMP infusion ($p < 0.001$) (Fig. 3).

$CaCl_2$ produced effects similar to those that followed infusion of cAMP (Figure 3). $FE_{AIB}$ fell ($\Delta FE_{AIB}$, - 0.035 ± 0.007 after 30 min. $p < 0.01$). GFR also fell ($\Delta$GFR, - 0.125 ± 0.04 after 30 min. $p < 0.01$). A small increase in phosphate excretion resulted after 60 min. in TPTX rats ($\Delta FE_{Pi}$, + 0.017 ± 0.01, $p < 0.001$) (Fig. 3), that could be ascribed presumably to the

concurrent hypercalcemia (13).

Calcitonin Infusion

Calcitonin produced a significant rise in AIB excretion ($\Delta FE_{AIB}$, + 0.064 ± 0.005, $p < 0.001$) after 30 min. of infusion); it had no effect on $FE_{Pi}$. The fluctuations in GFR produced by calcitonin infusion had little effect on $FE_{Pi}$ and $FE_{AIB}$ and was limited to two rats given 500 mU/kg·hr. In 5 rats given 25 mU/kg·hr. the control GFR was 2.04 ± 0.06 mL/min. (mean ± SE, n = 15) vs. 2.06 ± 0.08 µl/min. (mean ± SE, n = 20) (ns) during CT infusion.

Effect of Cyclic Nucleotides and Hormones on:

(A) Plasma $Ca^{++}$ and Pi

Plasma calcium and phosphate levels were stable in both intact and TPTX rats (Tables 1 and 2). The effects of the various treatments are also shown in Table 1 and 2. Thyroparathyroidectomy reduced plasma total calcium ($p < 0.001$) and increased plasma phosphate ($p < 0.001$) as expected. Infusion of PTE and dbcAMP into intact rats had no effect on total plasma calcium. PTE raised plasma calcium in TPTX animals but dbcAMP did not. Calcitonin caused plasma calcium to fall ($p < 0.001$) in TPTX rats, while cAMP and $CaCl_2$ raised their calcium levels.

The anticipated hypophosphatemic effects of PTE, dbcAMP, and cAMP were observed, in the intact and TPTX rat (all $p < 0.001$) (Table 2). Calcitonin reduced plasma phosphate ($p < 0.001$) (Table 2), but $CaCl_2$ did not.

(B) Urine Flow Rate

Cyclic nucleotides and calciotropic infusion altered urine flow rates (Table 3) in parallel with the above-mentioned changed in GFR. Dibutyryl cAMP increased urine flow rate in the intact and TPTX rat ($p < 0.01$ and $p < 0.005$, respectively) while PTE, decreased it. The effect lasted only 90 min. in the former and 60 min. in the latter group ($p < 0.05$ and $p < 0.01$ respectively). Infusions of CT, cAMP and $CaCl_2$ had little or no effect on flow rates.

(C) Plasma [AIB] and Filtered Load of AIB

Plasma [AIB] is stable in untreated control and TPTX rats (Figure 4). Significant changes were produced only by dbcAMP, cAMP and CT. Whereas CT and dbcAMP decreased plasma [AIB] in the intact and TPTX animal ($p < 0.001$) the filtered load did not change equivalently because of the corresponding rise in GFR in both cases. Thus the significant rise in $FE_{AIB}$ referred to above (Figure 3) is of particular interest.

## DISCUSSION

An increase in AIB excretion following administration of parathyroid hormone to intact vitamin-D replete animals was observed in our studies (Figure 1). Gekle (19) also reported this finding with respect to natural amino acids in the intact rat studied by the micropuncture technique. Because the hyperaminoaciduria of vitamin-D-deficiency and calcium deficiency in Man (17,28,33) and the rat (23) seems to be related to hyperparathyrodisim, it was initially puzzling why an excess of PTH is not uniformly associated with impaired net reabsorption of amino acids in man (14,38,47). An explanation for this discrepancy may now be available.

We failed to obtain an increase in AIB excretion in the TPTX rat. Furthermore, prolongation of PTE infusion up to 12 hours does not increase AIB excretion in TPTX rats; Nor does EGTA infusion, sufficient to cause increased endogenous PTH in the intact rat (McInnes and Scriver, unpublished observations). These findings indicate that excess PTH alone is an insufficient condition for impairment of net reabsorption of amino acids.

There is some evidence that alteration in renal hemodynamics may play a role in the tubulopathy of primary and secondary hyperparathyroidism (37). Perturbation of renal hemodynamics by PTE infusion is indicated by the fall in GFR and urine flow rate in our intact and TPTX rats. However it is unlikely that these changes are responsible for increased $FE_{AIB}$ in the intact, PTE-infused rat, since $FE_{AIB}$ in TPTX rats was unaffected

by PTE, despite changes in GFR and flow rate analogous to those found in intact animals. In the case of dbcAMP infusion, an increase in GFR and urine flow rate occurred in the intact and TPTX animal, and AIB excretion still increased in the intact group. Thus, while PTE and dibutyryl cAMP both increase $FE_{AIB}$ in intact rats, they have divergent effects on GFR. A hemodynamic explanation for the former effect is thus insufficient.

Further evidence against a simple relationship between GFR and $FE_{AIB}$ in response to calciotropic agents is found in our studies with cAMP and $CaCl_2$. Decreased renal blood flow and GFR in response to cAMP has been described previously (20,27). Although the $Ca^{++}$ induced fall in GFR may be PTH-dependent (25) we observed the effect in TPTX rats. Since GFR decreased in situations in which the $FE_{AIB}$ fell (calcium or cAMP infusion in TPTX rats), remained unchanged (PTE infusion in TPTX rats) or rose (PTE infusion in the intact rat), and GFR increased in situations in which $FE_{AIB}$ rose (dibutyryl cAMP infusion - intact rats) or remained unchanged (dbcAMP infusion-TPTX rats), We conclude again that hemodynamic changes are insufficient explanation for the changes in $FE_{AIB}$.

It is probable that the alterations in GFR induced by PTE are not the effect of parathyroid hormone itself but rather of contaminants (2). Dibutyryl cAMP has been reported to be without effect on the renal vasculature of the dog (20) but other studies suggest that it has vasodilatory actions (5,27) which could be consistent with the effects we observed.

Plasma [AIB] falls in the intact and TPTX rat treated with dbcAMP. This change cannot be related to increased urinary losses of AIB alone although the rapid decrease in plasma [AIB] in intact rats may reflect its increased renal excretion. Enhanced AIB uptake by liver and other organs (10,39,52) is probably an important cause for the fall in plasma [AIB] following treatment with dbcAMP. This analogue also depletes kidney of AIB under in vivo conditions (18), a decrease which could reflect increased flux of AIB from cells to lumen (36).

The permissive effect of the thyroid gland on the aminoaciduria induced by PTE or dbcAMP may reflect increased endogenous secretion of PTH, thyroxin, or calcitonin. Although PTH release from the parathyroid glands is thought to be adenyl cyclase-dependent, and to be stimulated by dbcAMP, increased PTH secretion is not likely to be of importance here, since we have shown that even pharmacological doses of PTE do not alter AIB excretion in TPTX rats. Although thyroxin secretion is also an adenyl-cyclase-dependent mechanism which can be stimulated by dibutyryl cAMP (16), the rapidity of the effect on AIB secretion is inconsistent with the slow onset of action of the physiological effects of thyroxin (22). In addition, hyperthyroidism in man is not known to cause hyper-aminoaciduria (46). Finally, PTE would not be expected to increase either PTH or thyroxin secretion.

Increased secretion of calcitonin, on the other hand, is a known consequence of exposure of the thyroid to dbcAMP,

both in vitro (4) and in vivo (8). While PTH does not stimulate calcitonin secretion directly, its hypercalcemic action acts as a potent stimulant of CT release experimentally (42). The failure of PTE to produce an increase in plasma total calcium in the intact rat in our studies contrasts significantly with the rise in total plasma calcium obtained in TPTX rats given identical doses of PTE. This preservation of normocalcemia in PTE-infused intact animals is indirect evidence that physiologically significant quantities of calcitonin were released by the thyroid gland during these studies.

The prompt increase in $FE_{AIB}$ provoked by CT infusion in the TPTX rat suggests that it mediates of the rise in $FE_{AIB}$. The small but significant difference between intact and TPTX rats in baseline $FE_{AIB}$ is also consistent with an effect of physiological concentrations of calcitonin on AIB excretion. In the absence of any effect of CT infusion (25mU/Kg·hr) on GFR or urine flow rate, there is no evidence that the increase in AIB excretion induced by CT is secondary to altered renal hemodynamics. CT has not been noted to alter plasma flow in other studies (41).

The lack of any phosphaturic effect of CT in our TPTX rats concurs with some studies (49) but conflicts with others (40). The reasons for these varying results are unclear (2), but some of the differences may be dose-related, an impression supported by Robinson et al (44). For example, while all effects in our studies were obtained with 25mU/Kg·hr of porcine CT, the dose

of the more potent salmon CT used by Popovtzer et al (40) was ∿3200-fold larger. Consequently, CT appears to have important biological effects (hypocalcemia, hypophosphatemia, increased amino acid excretion) at doses which are not phosphaturic, suggesting that this latter action may not be one of its physiological properties.

Although it is not known whether all renal actions of CT are mediated by cAMP, CT-responsive adenyl-cyclase activity has been identified in only the medullary and cortical portions of the thick ascending limb, and the distal convoluted tubule (9). Whether these regions of the nephron represent sites of decreased AIB uptake from the lumen, or increased cell to lumen flux of AIB (35) remains to be determined.

Studies by Borle (6) with cultured kidney cells suggest that a major effect of calcitonin is to lower the cytoplasmic calcium ion concentration. A similar depression of cytoplasmic calcium has been identified in cells of vitamin-D deficient chicks (7). A role for cytoplasmic calcium in controlling membrane permeability has been recognized in other tissues (7,34,43). Membrane permeability to ions and other solutes is increased by elevation of cytoplasmic calcium (3,21,29,45). Consequently, a depression of cytoplasmic calcium, as it may occur in vitamin-D deficiency or calcitonin infusion, should decrease the permeability of the plasma membrane, thereby impairing net reabsorption. Depletion of cell calcium stores, with a fall in cytoplasmic calcium ion and membrane permeability, may therefore be the common denominator underlying the tubular dysfunction of calcium-deficiency, vitamin-D deficiency, and

isolated deficiency of 1,25 dihydroxycholecalciferol (15). Decreased cytoplasmic calcium ion may also occur in some target organs in primary hyperparathyroidism because of increased calcitonin release (24) and relative vitamin-D deficiency (32,53).

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TABLE 1

Effect of Hormones and Nucleotides on Plasma [Ca++] (mg/dl) (mean ± SEM)

| Period # | CONT INTACT | CONT TPTX | dbcAMP INTACT | dbcAMP TPTX | PTH INTACT | PTH TPTX | CT TPTX | cAMP TPTX | Ca++ TPTX |
|---|---|---|---|---|---|---|---|---|---|
| 1. | 9.6 ± .2 | 7.7 ± .2 | 10.0 ± .2 | 6.9 ± .4 | 9.9 ± .3 | 7.1 ± .3 | 7.3 ± .3 | 7.9 ± .2 | 8.2 ± .4 |
| 2. | | | | | | | | | |
| 3. | 9.9 ± .3 | 7.4 ± .3 | 10.0 ± .3 | 7.7 ± .4 | 9.2 ± .4 | 6.9 ± .4 | 7.2 ± .4 | 7.7 ± .3 | 8.2 ± .4 |
| 4. | | | | | | | | | |
| 5. | 9.8 ± .3 | 7.4 ± .3 | 9.8 ± .3 | 7.7 ± .3 | 9.2 ± .5 | 7.5 ± .4 | 6.2 ± .3 | 8.4 ± .3 | 9.9 ± .4 |
| 6. | | | | | | | | | |
| 7. | 10.0 ± .4 | 7.3 ± .3 | 9.78 ± .4 | 7.4 ± .4 | 8.9 ± .3 | 9.4 ± .6 | 5.8 ± .3 | 9.2 ± .5 | 11.9 ± .2 |
| n = | 10 | 10 | 6 | 8 | 8 | 10 | 7 | 7 | 7 |
| eriods & 3 vs. & 7 | n.s. | n.s. | n.s. | n.s. | n.s. | p<.005 | p<.001 | p<.01 | p<.001 |

All tests of significance were analyses of variance.

TABLE 2

Effect of Hormones and Nucleotides on Plasma [Pi] (mg/dl) (mean ± SEM)

| Period # | CONT INTACT | CONT TPTX | dbcAMP INTACT | dbcAMP TPTX | PTH INTACT | PTH TPTX | CT TPTX | cAMP TPTX | Ca++ TPTX |
|---|---|---|---|---|---|---|---|---|---|
| 1. | 5.7 ± .2 | 8.5 ± .3 | 6.2 ± 0.4 | 9.1 ± .7 | 5.7 ± .3 | 8.5 ± .2 | 7.9 ± .3 | 9.1 ± .4 | 8.3 ± .21 |
| 2. | 5.8 ± .2 | 8.9 ± .2 | | 9.1 ± .7 | 5.8 ± .3 | | 8.1 ± .3 | 8.8 ± .4 | |
| 3. | 5.8 ± .2 | 8.6 ± .2 | 6.0 ± 0.3 | 8.7 ± .7 | 5.8 ± .3 | 8.5 ± .3 | 7.9 ± .3 | 8.5 ± .3 | 8.2 ± .4 |
| 4. | 5.9 ± .2 | 8.5 ± .1 | | 7.9 ± .3 | 4.9 ± .3 | | 7.1 ± .2 | 7.9 ± .4 | |
| 5. | 5.8 ± .2 | 8.5 ± .2 | 5.1 ± 0.3 | 6.3 ± .4 | 4.3 ± .3 | 6.9 ± .3 | 6.7 ± .2 | 7.1 ± .5 | 8.6 ± .3 |
| 6. | 5.9 ± .2 | 8.5 ± .1 | | 5.9 ± .4 | 4.2 ± .2 | | 6.4 ± .2 | 6.7 ± .5 | |
| 7. | 5.8 ± .2 | 8.2 ± .2 | 4.3 ± 0.2 | 5.9 ± .4 | 3.9 ± .2 | 6.0 ± .3 | 6.3 ± .2 | 6.1 ± .3 | 8.6 ± .2 |
| Statistical significance* | n.s. | n.s. | p<.001 | p<.001 | p<.001 | p<.001 | p<.001 | p<.001 | n.s. |

*periods 1,2,3 vs. 5,6,7 )
or 1 & 3 vs. 5 & 7 ) depending on whether Pi was measured in periods 2 and 6, as seen in table.

All tests of significance were analyses of variance.

TABLE 3

Effect of Hormones and Nucleotides on Urine Flow Rate (ul/min) (mean ± SEM)

| eriod # | CONT INTACT | CONT TPTX | dbcAMP INTACT | dbcAMP TPTX | PTH INTACT | PTH TPTX | CT TPTX | cAMP TPTX | Ca++ TPTX |
|---|---|---|---|---|---|---|---|---|---|
| 1. | 38.5±4.4 | 42.1±4.4 | 41.8±4.2 | 39.4±4.2 | 37.3±7.7 | 34.9±1.9 | 37.2±2.5 | 41.8±2.5 | 51.8±7.1 |
| 2. | 40.7±4.5 | 38.2±4.8 | 48.0±5.7 | 32.4±5.5 | 40.3±7.3 | 33.9±3.6 | 40.7±4.4 | 39.4±3.0 | 45.2±3.2 |
| 3. | 35.6±3.8 | 45.0±3.4 | 32.8±6.2 | 40.9±4.5 | 41.1±3.4 | 34.5±4.3 | 40.2±1.9 | 53.4±4.1 | 45.1±3.6 |
| 4. | 37.7±4.0 | 51.5±3.9 | 41.9±6.7 | 57.3± 10 | 29.5±3.4 | 23.4±4.6 | 41.9±1.8 | 32.9±4.3 | 39.7±2.6 |
| 5. | 36.7±2.5 | 42.4±5.4 | 57.1±5.0 | 53.9±5.5 | 32.3±3.7 | 26.9±4.1 | 37.5±4.5 | 39.8±4.5 | 52.0±3.7 |
| 6. | 36.6±3.8 | 47.1±5.8 | 50.6±7.6 | 49.7±5.6 | 27.5±2.8 | 37.2±5.7 | 38.7±4.8 | 43.2±2.3 | 49.8±4.6 |
| 7. | 46.2±3.7 | 44.4±7.1 | 55.0±5.9 | 53.1±7.9 | 34.6±3.7 | 39.6±3.8 | 36.9±5.7 | 45.6±6.4 | 52.7±4.4 |
| atistical gnificance | n.s. | n.s. | p<.01* | p<.005* | p<.05** | p<.01*** | n.s. | n.s. | n.s. |

* periods 1,2,3 vs. 5,6,7

** periods 1,2,3 vs. 4,5,6

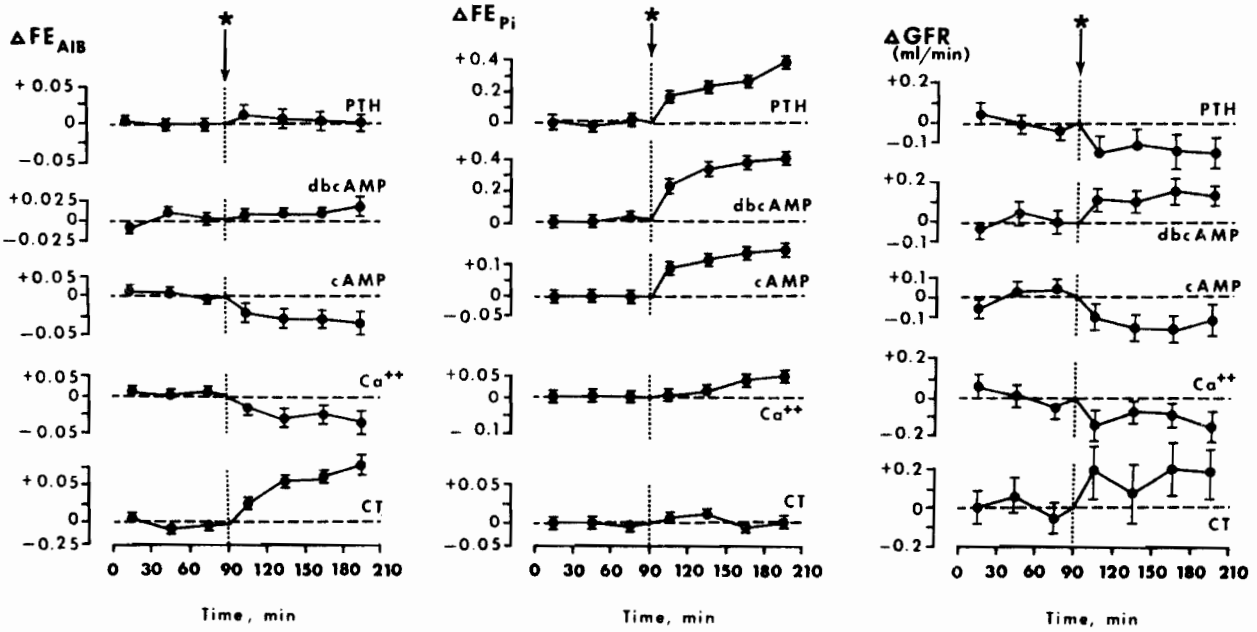
*** periods 1,2,3 vs. 4 & 5

All tests of significance were analyses of variance.

## LEGENDS

Figure 1: The change in fraction excretion of the inert amino acid α-aminoisobutyric acid and phosphate anion ($\Delta FE_{AIB}$, $\Delta FE_{Pi}$), and change in inulin clearance (ΔGFR) as a function of time in the intact and TPTX control rat. Data are presented as the average and SEM for the group of the difference between the mean of all clearance periods and each individual period for the individual rats. Interindividual differences between animals are thus accomodated. The numbers above each set of data are mean ± SEM absolute values for the group in periods 1-3.

Figure 2: $\Delta FE_{AIB}$, $\Delta FE_{Pi}$ and ΔGFR as a function of time in intact rat group, infused with parathyroid extract or dibutyryl cyclic AMP. Data are presented as the difference (mean ± SEM) for the group between the mean of the first three (control) periods (shown by broken horizontal line) and each individual period (for both control or treatment clearance periods). Treatments (parathyroid extract (PTE) or dibutyryl cyclic AMP (dbcAMP) began at 90 min. and continued throughout periods 4-7 (90-210 min); the vertical broken line indicates onset of treatment.


ΔFE_AIB
ΔFE_Pi
ΔGFR (ml/min)
PTH
dbcAMP
cAMP
Ca++
CT
Time, min

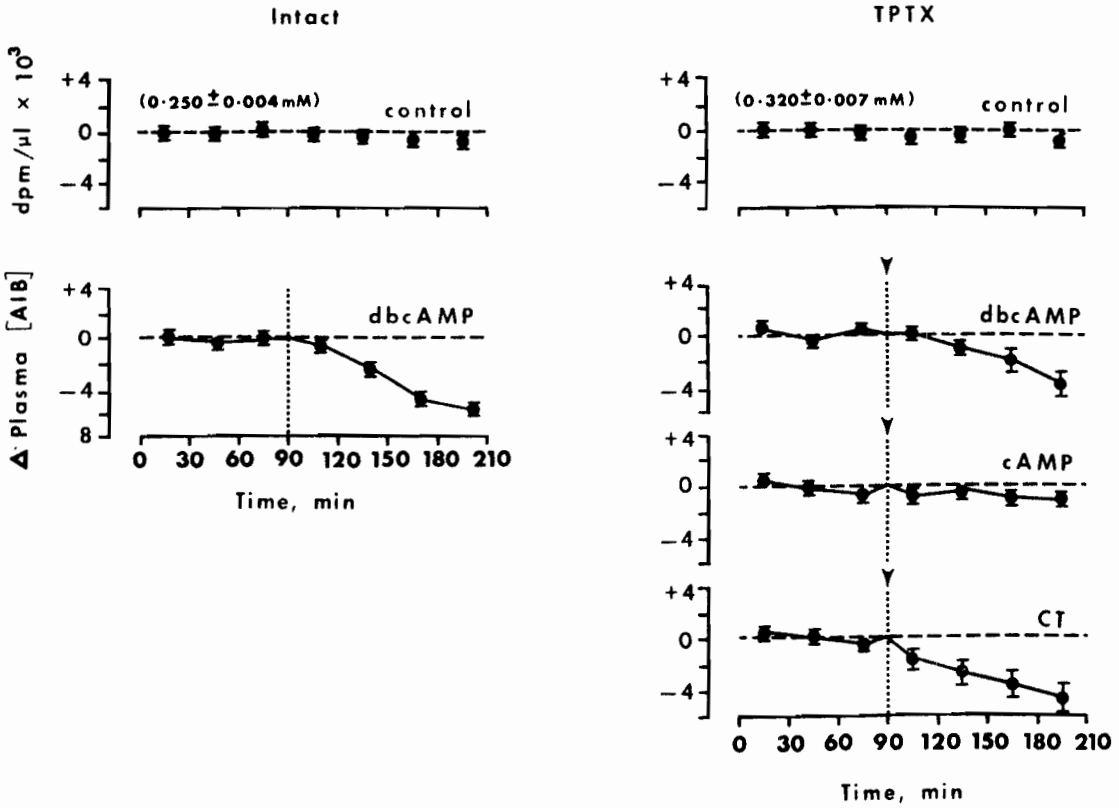
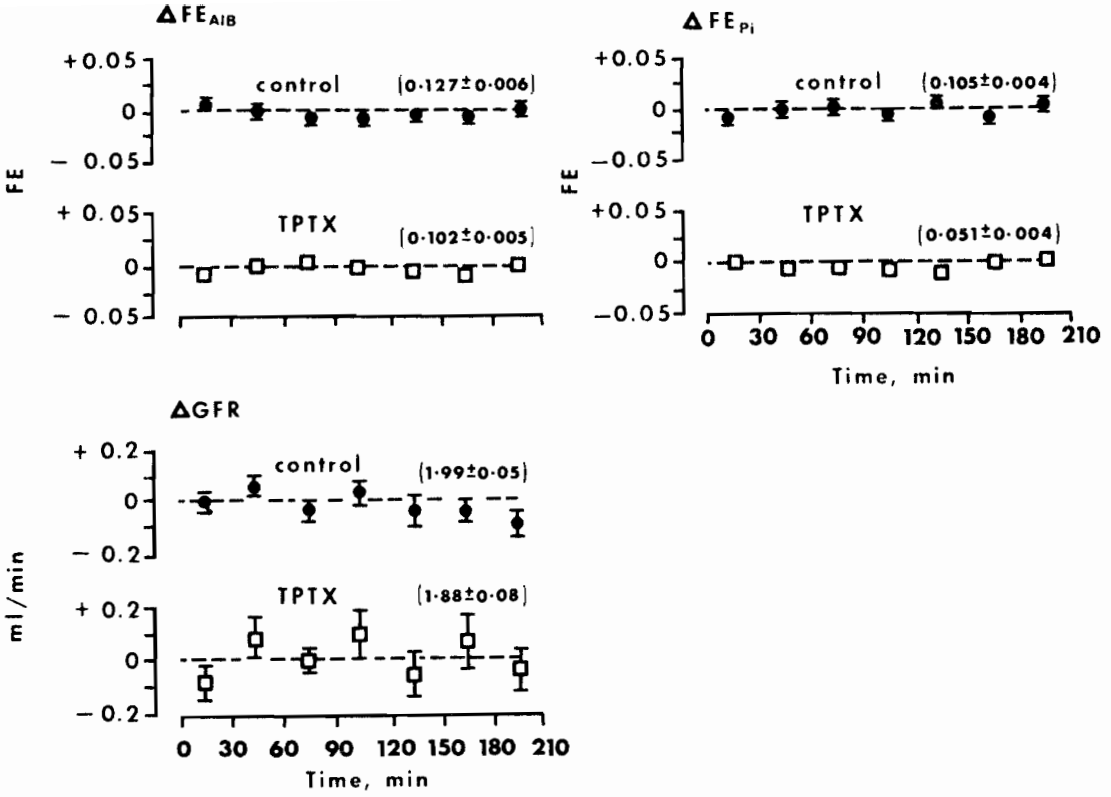
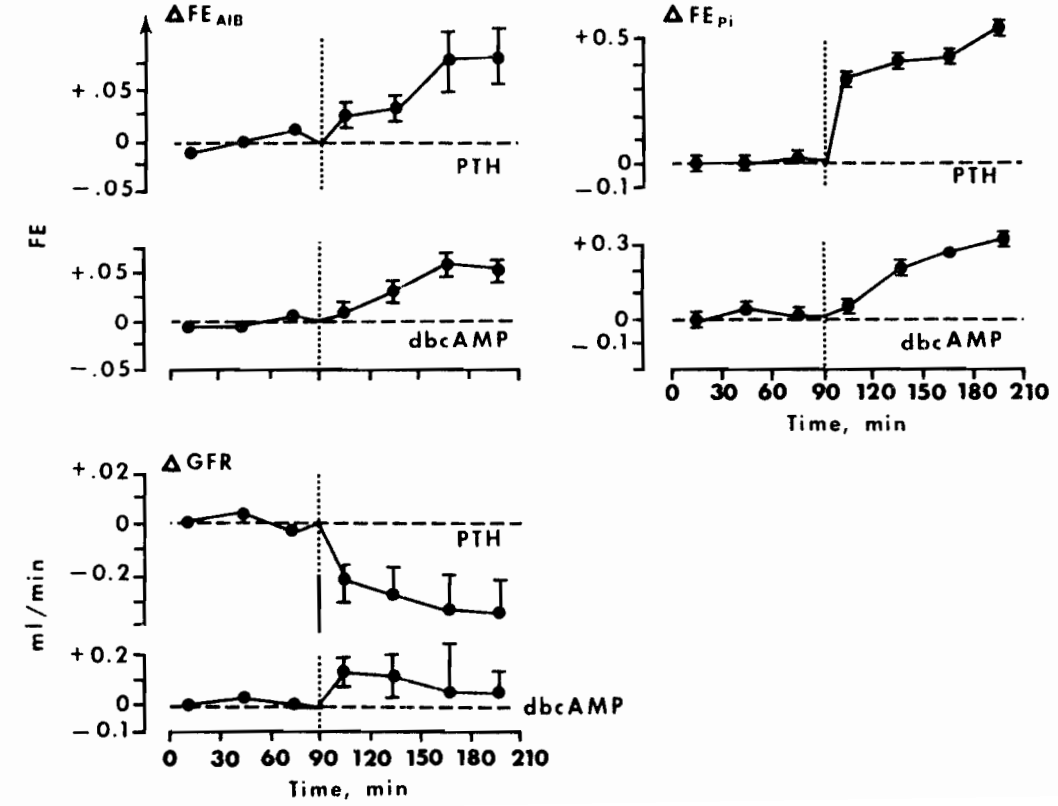

Intact
TPTX
dpm/µl × 10³
Δ Plasma [AIB]
(0·250±0·004 mM)
(0·320±0·007 mM)
control
dbcAMP
cAMP
CT
Time, min

Figure 3: $\Delta FE_{AIB}$, $\Delta FE_{Pi}$ and $\Delta GFR$ as a function of time in the TPTX rat group. Parathyroid extract (PTE), dibutyryl cyclic AMP (dbcAMP), cyclic AMP (cAMP), $CaCl_2$ and calcitonin (CT) were administered. Format is similar to Fig. 2.

Figure 4: Change in plasma AIB concentration ($\Delta$plasma [AIB] as a function of time in intact and TPTX rats under control conditions and after infusion of dibutyryl cyclic AMP (dbcAMP), cyclic AMP (cAMP) and calcitonin (CT). The format of the graph is similar to Fig. 2.


ΔFE_AIB
+0.05
0
− 0.05
control
(0·127±0·006)
+ 0.05
0
− 0.05
TPTX
(0·102±0·005)
FE
ΔFE_Pi
+0.05
0
−0.05
control
(0·105±0·004)
+0.05
0
−0.05
TPTX
(0·051±0·004)
0 30 60 90 120 150 180 210
Time, min
ΔGFR
+ 0.2
0
− 0.2
control
(1·99±0·05)
+ 0.2
0
− 0.2
TPTX
(1·88±0·08)
ml/min
0 30 60 90 120 150 180 210
Time, min


ΔFE_AIB
+ .05
0
−.05
PTH
+.05
0
−.05
dbcAMP
FE
ΔFE_Pi
+0.5
0
−0.1
PTH
+0.3
0
− 0.1
dbcAMP
0 30 60 90 120 150 180 210
Time, min
ΔGFR
+.02
0
−0.2
PTH
+0.2
0
− 0.1
dbcAMP
ml/min
0 30 60 90 120 150 180 210
Time, min

## COMMENTARY

1. Detailed discussions are to be found in the published and pending manuscripts which constitute this thesis (section III - VIII). The following brief commentary provides an overall perspective of the work. An asterisk (*) in this section indicates that the data being discussed were obtained, not by the candidate, but by his coauthors in sections IV and V.

2. The review of the mammalian inborn errors of renal transport (Section III) attempts to determine the precise cellular locus of the transport defect in each condition. It is apparent that assignments made can only be tentative since, with the exception of X-linked hypophosphatemia (section IV), direct evidence is unavailable. Such evidence will only be acquired from animal models, and, as technology improves, perhaps from the study of epithelial cells cultured from the urinary tract of affected subjects. Another possibility, though more removed, is that cells from non-epithelial tissues (e.g. fibroblasts) may be made to express such differentiated functions. Up to now, several investigations have not upheld this latter hope.

3. Four major themes are evident throughout the review (Sections II and III).

3.1 *Transepithelial transport of a solute and cellular accumulation of that solute by any particular part of the nephron are essentially different processes.* In addition to the examples quoted, this thesis presents new

physiological and genetic evidence in support of this generalization. Thus, in Hyp/Y mice, phosphate transport at the basolateral membrane of renal cortical cells is normal, on the basis of kinetic studies using cortex slices (*). Net luminal uptake, on the other hand, is shown to be deficient, indicating that phosphate transport at these two renal cell surfaces is under separate genetic control.

The interactions between AIB and proline described in Section VI also support this theme. While net reabsorption of AIB is stimulated by proline infusion at low rates, renal cortical concentration of AIB is decreased by this procedure. This finding can best be explained by proline-induced enhancement of AIB efflux from cell to peritubular space in association with an increase in net reabsorption. The latter could result from a decreased cell to lumen flux of AIB secondary to lower cell AIB levels. In any case, luminal and basolateral inter actions between these two amino acids appear to be different.

3.2 *Cell-to-lumen flux of a solute can be a significant component of net tubular reabsorption in normal kidney.*

Accordingly it could achieve special importance in situations of abnormally increased solute excretion (decreased net reabsorption). Three mechanisms by which an increased cell-to-lumen flux may arise seem possible:

3.2.1 As revealed in the study of hyperprolinuria in PRO/Re mice (section V) the intracellular pool of the solute can increase (*), secondary to decreased oxidative metabolism

of solute or decreased delivery from cell to peritubular space. The cell-to-lumen flux can increase in proportion to the rise in intracellular solute concentration (*). Cell-to-lumen flux proposed for renal handling of AIB (section VII) may also reflect an analogous event. The greater accumulation of AIB in medulla at equilibrium, compared to cortex (19), is consistent with medulla being the more important source of the cell-to-lumen flux of AIB.

3.2.2 Abnormal cell-to-lumen flux may occur via an abnormal carrier at the brush-border membrane that allows excessive backflux. The abnormal efflux of phosphate in Hyp/Y mice may represent such a situation. That efflux occurs is indisputable, since net tubular secretion of phosphate is noted during phosphate loading (*). That a brush-border component of phosphate transport is defective has also been directly demonstrated (*). It is unknown, however, whether the net phosphate efflux results from unmasking of a residual, normally active phosphate efflux system, or from abnormal phosphate efflux on a mutant carrier, as might result from a decrease in the normally high (relative to exterior) internal Km. The latter is a prerequisite for asymmetry of solute flux at the luminal membrane in normal conditions (section III-3). In situations with increased cell-to-lumen flux of this type, the intracellular concentration of the solute, in contrast to type I, is low or normal, although examples only of the latter type are currently known. Thus, in Hyp/Y mice, the cellular phosphate pools in

cortex are normal (*), despite the net decrease in uptake from lumen.

3.2.3 A general abnormality in cell membrane permeability prevents retention of an adequate cellular pool during net reabsorption. The maleic acid model of the renal Fanconi syndrome is an example (1). In this situation the intracellular concentration of affected solutes may be normal or decreased, with no defect in luminal uptake or in the membrane carriers per se, although cell-to-lumen flux (and thus renal excretion) is increased for a broad spectrum of solutes. The most probable basic abnormalities are either a decrease in cellular energy production (as from maleic acid (8)), or a generalized alteration in cell membrane permeability. The latter mechanism may account for the generalized hyperaminoaciduria of vitamin-D deficiency, as suggested by Lorentz (9,10) and by the work described in section VIII of this thesis. I propose that cytoplasmic calcium ion depletion changes membrane permeability and results in a generalized increase in solute excretion, Thus, in stage-I vitamin-D deficiency ricket (5), no abnormality of amino acid excretion is present. The studies of Borle (2) indicate that the cytoplasmic $[Ca^{++}]$ is elevated under the conditions of early vitamin-D deficiency. As vitamin-D and calcium depletion progress, cytoplasmic $[Ca^{++}]$ falls. Decreased net reabsorption of amino acids is observed in stages II and III of vitamin-D deficiency. A similar series of events would be expected in calcium deficiency states, with similar consequences. The fact that the renal dysfunction in the

rare cases of isolated calcium deficiency in man mimics the findings in vitamin-D deficiency (7), suggests that vitamin D per se is not a factor in the hyperaminoaciduria. Two other pathological associations of vitamin-D deficiency, hypophosphatemia with phosphate depletion and hyperparathyroidism, do not seem to be responsible for the increased solute excretion. The former does not produce hyperaminoaciduria when it occurs as an isolated condition (13) while the importance of the latter is questioned by the data presented in section VIII. Direct proof of the low $Ca^{++}$-permeability hypothesis must be sought in studies of amino acid transport in vitamin-D deficient animals using micropuncture methods in vivo, and by examining amino acid uptake and efflux in vitro, using epithelial cells from vitamin-D deficient animals. In addition, the presence of an increased cell-to-lumen flux of amino acid in vitamin-D deficient animals could be sought using the methods described in section VII of this thesis.

3.2.4 The ability to demonstrate a cell-to-lumen flux of amino acid in vivo using non-invasive methods would be of significant value in studies of human subjects with the renal Fanconi syndrome. Any non-metabolizable solute with significant urinary excretion may be adequate, since the method depends on kinetic analysis of the delay in equilibrium of the solute with body water; thus no endogenous pools of the solute can be present initially. Urinary tract dead-space could be measured using any good marker of glomerular

filtration (14). Patients with increased cell-to-lumen flux of AIB, but with normal luminal uptake, would be expected to have normal AIB reabsorption in the clearance periods immediately following AIB injection. As AIB equilibrates with renal tissues, however, an enhanced cell to lumen flux would result in an abnormally low net tubular reabsorption. This method could initially be evaluated in animal studies, for example in maleic acid-treated rats, since micropuncture studies have already identified normal luminal uptake and increased capillary to lumen flux of amino acid in this model (1).

A significant deficiency of this method is that the cells responsible for the cell to lumen flux of AIB remain unidentified. Medulla is a potential source (section VII) as suggested by the longer time required for AIB equilibration with it, and the higher (than cortex) distribution ratio attained by it at equilibration. The fact that the natural amino acids are also accumulated more by medulla than by cortex (11,12) corresponds with the other similarities between AIB and natural amino acid behaviour in kidney noted in section VI.

3.3 *Genetic heterogeneity for inborn errors of transport exists as it does for the Mendelian mutations of man in general.* This theme was not further developed in the experimental portion of the thesis. Note however that there is little phenotypic variation in human subjects with X-linked hypophosphatemic rickets. Resolution of the molecular

defect using the Hyp/Y model may suggest a biochemical basis for heterogeneity.

3.4 *The renal transport of a given solute may be served by more than one transport system.* Multiple transport systems for single solutes have been identified using either genetic or kinetic methods. By necessity, these methods are complimentary. Thus, the fact that Hyp/Y mice are still able to reabsorb as much as 65% of filtered phosphate suggests the presence of alternative phosphate carriers in the luminal membrane ($Na^+$-independent, for example). Nevertheless, the mere demonstration of residual reabsorption is insufficient evidence for proving the existence of other transport systems. It leaves unanswered the concern that the residual transport may occur on partially functioning (mutant) carriers, rather than on fully functionally carriers of another type. In the mutant individual, however, abnormal reabsorption kinetics may disclose the presence of a partially functioning mutant carrier, as described for the "Km" mutant with iminoglycinuria in section II. Other physiological evidence indicating the existence of two or more transport systems also lends credibility to the suggestion that only one is deleted by mutation, as in the case of phosphate transport (4) and X-linked hypophosphatemia (6).

A novel exploitation of the fact that multiple carriers for glycine and the imino acids are present in several mammalian species (16) is described in section V. Integrity of the shared system at the luminal membrane for glycine and

the imino acids is indicated by the expected degree of inhibition of glycine reclamation by proline infusion in PRO/Re mice. On the other hand, well-characterized disorders of transport might be better utilized to clarify some of the complexity arising from the overlapping specifities of multiple transport systems in the normal kidney.

For example, the extent to which AIB transport is shared between the major transport systems of mammalian kidney could be ascertained by comparing its net reabsorption in individuals with cystinuria, Hartnup disease, dicarboxylic aminoaciduria, and iminoglycinuria, to its net reabsorption in normal individuals. From the data assembled in section VI, together with the demonstration of active $Na^+$-dependent luminal uptake of AIB (18), it is apparent that the behaviour of AIB in mammalian kidney is representative of natural amino acids to a significant extent, regardless of the disposition of its transport amongst the major transport systems. Indeed, it would be difficult to argue, at present, that the transport of any one of the natural amino acids is more representative of the others than is the analogue AIB. Only further studies in a variety of normal and pathophysiological situations will resolve this issue.

The relevance of studies of renal transport using AIB is of no greater concern elsewhere than in the examination of the relationship between calcium metabolism and AIB excretion described in section VIII. The generality of the

observation that neither parathyroid hormone nor its intracellular messenger, cyclic AMP, affect AIB excretion in the thyroparathyroidectomized rat must now be evaluated for the natural amino acids. It is not improbable that previous research on this problem (described in section VIII) was merely describing a secondary effect of parathyroid hormone infusion namely stimulation of calcitonin secretion.

The research in section VIII underscores one of hazards involved in attempting to draw meaningful conclusions from studies in which cyclic nucleotides are infused into whole animals. One cannot neglect the fact that the specificity of tissue responses to these intracellular messengers resides at the cell surface, in the hormonal receptor (15). Consequently, cyclic nucleotide infusion in vivo corresponds to the simultaneous stimulation, by numerous polypeptide hormones, of their various target organs - hardly an appropriate context for physiological research.

We did not anticipate finding increased AIB excretion (with hypocalcemia and hypophosphatemia) following infusion of calcitonin at dose levels that are not phosphaturic. This important finding suggests that the phosphaturia reported in the literature is a pharmacological action of calcitonin. It also suggests that calcitonin may modify cellular ion levels in a manner that can be reflected sensitively through measurement of amino acid transport. The localization of calcitonin receptors solely to the ascending limb (medulla) and with distal convoluted tubule (3), sites at which amino

acid reabsorption is minimal (17), may indicate that calcitonin acts primarily by increasing the cell-to-lumen flux of AIB in these parts of the nephron. Even if calcitonin is not found to increase natural amino acid excretion, a significant insight into its cellular responses will be gained by understanding how it increases AIB excretion. The identification of such a clear-cut discrepancy between the renal transport characteristics of the natural amino acids and AIB would be equally fascinating, in view of their otherwise remarkable similarities.

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# SECTION X

## Claims to Original Work

In sections of this thesis in which the candidate was not the senior author, claims to original work are made only for those facets of the research done by the candidate.

Section IV: Phosphate transport in Hyp/Y mice:

1. Fractional excretion of phosphate in Hyp/Y is abnormally increased, despite the fact that the serum phosphate concentration is low compared to controls.
2. Inulin clearance is not different in Hyp/Y and control mice, indicating that the increased phosphate excretion is not secondary to an unusually high filtered load of phosphate.

Section V: Hyperprolinuria in PRO/Re mice:

1. Renal proline clearance in PRO/Re mice is about fifty-fold higher than in controls over a wide range of plasma proline concentrations. Net tubular reabsorption of proline is therefore very diminished in the PRO/Re nephron.
2. Proline and glycine share a reabsorptive system for tubular reabsorption in mice, as indicated by competitition studies.
3. Glycine reclamation is impaired to the same degree in the PRO/Re and control mouse at various concentrations of L-proline. This finding indicates that the shared luminal membrane system serving reclamation is retained in PRO/Re kidney.

4. Integrity of the luminal and peritubular proline transport systems in PRO/Re mice demonstrates that renal proline metabolism and transport are independent functions in mammalian kidney.

Section VI: Net Reabsorption of α-aminoiosbutyrate (AIB) in rats:

1. Renal loss of AIB in rats determines its endogenous pool-size at steady state, consistent with its non-metabolizable nature.

2. The fractional excretion of AIB varies widely between individual rats of one inbred species, but is stable within individual animals.

3. Net reabsorption of AIB in rats resembles the reabsorptive process for the natural amino acids in at least five ways:

(a) The fractional excretion of AIB ($FE_{AIB}$) is not influenced by spontaneous fluctuations in GFR or urine flow rate.

(b) The $FE_{AIB}$ and renal cortex AIB accumulation are not directly related.

(c) AIB and phosphate do not interact during absorption.

(d) AIB impairs the reabsorption of natural short-chain aliphatic amino acids which also have high endogenous excretion rates.

(e) Various natural amino acids impair AIB reabsorption.

4. Certain AIB : natural amino acid interactions in vivo are novel:

(a) Stimulation of AIB reabsorption by proline, and of glutamic acid reabsorption by AIB suggests that AIB and proline, and AIB and glutamate may share luminal membrane carriers.

(b) The preferential accumulation of AIB by medulla in vivo is abolished by proline infusion but not by amino acids from other major transport groups.

Section VII: Evidence for a cell to lumen flux of AIB:

1. Following rapid intravenous injection in the rat, the fractional excretion of AIB increases over a 60-90 minute period despite a concomitant fall in plasma [AIB]. This paradoxical finding is best explained by the presence of a cell to lumen flux of AIB. This represents the first demonstration of such a flux using only clearance techniques.

2. Extrapolation of the decay of AIB reabsorption to zero time allows quantitation of the contribution of the cell to lumen flux of AIB to total AIB excretion..

3. AIB equilibrates with renal medulla more slowly than with cortex.

4. AIB reabsorption by rat kidney is saturable when plasma [AIB] exceeds ∿6mM, indicating that renal AIB transport is mediated.

5. When filtered AIB rises from ∿1 to ∿3 micromoles $.min^{-1}$. 100 $cm^{-2}$, AIB reabsorption falls sharply.

Section VIII: Calcium metabolism and AIB excretion:

1. Parathyroid extract (PTE) and dibutyryl cyclic AMP each increase the fractional excretion of AIB, but only in rats with the thyroid and parathyroid glands intact.

2. Dibutyryl cyclic AMP infusion (15mg/Kg.hr) increases the glomerular filtration rate of both intact and thyroparathyroidectomized (TPTX) rats.

3. Dibutyryl cyclic AMP decreases the plasma [AIB] after 30 min. of infusion in both intact and TPTX rats, consistent with its well-known stimulation of hepatic AIB accumulation.

4. Cyclic AMP and $CaCl_2$ both reduce $FE_{AIB}$ in TPTX rats. This effect is probably secondary to altered renal hemodynamics.

5. Calcitonin infusion in TPTX rats increases the $FE_{AIB}$. An effect of calcitonin on free amino acid excretion has not been previously demonstrated.

6. Calcitonin infusion at a rate of 25mU/Kg.hr in TPTX rats does not alter the $FE_{Pi}$, although at this infusion rate other physiological effects are evident. Phosphaturia is not a physiological action of calcitonin.