# GENETICS OF SARCOSINE AND PHOSPHATE TRANSPORT IN HUMAN KIDNEY

Francis H. Glorieux

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## ABSTRACT

This thesis examines membrane transport phenomena in mammalian kidney. The first part of the thesis describes studies in sarcosinemia, an inborn error of amino acid metabolism. Genotypes and phenotypes were delineated in vivo by exposure to sarcosine excess. A defect in sarcosine oxidation was revealed in the homozygote and the heterozygote. Renal tubular transport of sarcosine remains intact in the blocked catabolic mutant phenotype. In vitro studies revealed that sarcosine is transported by several systems normally utilized by the imino acids and glycine.

The remainder examines a classic genetic disease, X-linked hypophosphatemia. Hyperphosphaturia and hypophosphatemia, the principle discriminants of the mutant genotype, are the result of a primary defect in tubular transport of phosphate. The defect is not parathyroid hormone dependent. The mutant phenotype reveals at least two components of phosphate transport in human kidney. One is PTH sensitive and under the control of an X-linked gene; the other is insensitive to PTH, but can be modulated by calcium ion. Nephrogenous 3',5' cyclic-AMP production is normal in X-linked hypophosphatemia. Long-term supplementation with large amounts of phosphate in the diet and modest supplements of vitamin D effectively neutralize the secondary phenotypic manifestations of the X-linked mutation.

BIOLOGY (Genetics) Ph.D.

# GENETIQUE BIOCHIMIQUE DU TRANSPORT RENAL DE LA SARCOSINE ET DU PHOSPHORE

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## RESUME

Cette thèse traite de certains aspects du transport à travers la membrane cellulaire dans le rein des mammifères. La première partie concerne la sarcosinémie, une erreur innée du métabolisme des acides aminés. Les génotype et phénotype ont été déterminés par des tests de surcharge en sarcosine. Une déficience de l'oxydation de la sarcosine a été mise en évidence chez les sujets homozygotes et hétérozygotes. Le transport rénal de la sarcosine n'est pas affecté par le bloc catabolique chez le sujet homozygote. Les études in vitro ont démontré que la sarcosine est transportée par les différents systèmes normalement utilisés par les acides iminés et la glycine.

La deuxième partie traite d'une maladie génétique classique, l'hypophosphatémie liée au chromosome X. L'hyperphosphaturie avec hypophosphatémie, qui est le caractère discriminant du génotype mutant, est le résultat d'un défaut primaire du transport tubulaire du phosphore. Cette déficience est indépendante de la parathormone. Le phénotype mutant a permis de séparer au moins deux composantes du transport du phosphore dans le rein de l'homme. L'une est sensible à la parathormone et est contrôlée par un gène lié à l'X; l'autre est insensible à la parathormone mais est influencée par l'ion calcium. L'AMP cyclique néphrogénique est normalement produite dans la mutation étudiée. Une importante surcharge en phosphore combinée à un modeste apport en vitamine D neutralise efficacement les manifestations phénotypiques secondaires de la mutation liée au chromosome X.

# BIOCHEMICAL GENETICS OF SARCOSINE AND PHOSPHATE TRANSPORT IN HUMAN KIDNEY

by

Francis H. Glorieux

Presented to the Dean and Faculty of Graduate Studies in partial fulfilment of the requirements for the Degree of Doctor of Phylosophy

Department of Biology (Human Genetics)
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" Que scais-je? "

Michel de Montaigne
Motto on his seal.

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I am deeply grateful to Dr. Charles R. Scriver for introducing me to the biochemical genetics of transport and for his constant guidance and support during my training in his laboratory. I express my gratitude to Dr. Edgard Delvin and Dr. Fazl Mohyuddin who guided my early footsteps in the in vitro work and use of animal models; also to Dr. Claude Arnaud and his team who performed the PTH immuno assays and to Dr. Alan Tenenhouse who performed the c-AMP determinations. I would also like to thank Mrs. Terry Reade for her magnificent and constant support to the patients studied in this thesis.

The many others whose help has been indispensable for this work are acknowledged in the individual papers included in this thesis.

Finally, I wish to thank Mrs. Huguette Ishmael for her loving care in the typing of the manuscript.

# PREFACE

This thesis is submitted according to the newly-accepted regulations for thesis style which have been authorized by the Graduate Training Committee of the Biology Department at McGill. The main body of the thesis is written in a form suitable for publication. The section dealing with the metabolism and transport of sarcosine, the autosomal recessive trait in man known as "sarcosinemia",is published in "The Journal of Clinical Investigation". Two sections on phosphate transport by human kidney in X-linked hypophosphatemia appear in "Science". The last section of the thesis considers the application of knowledge about phosphate transport to the neutralization of the mutant allele in X-linked hypophosphatemic rickets, and has been submitted to "The New England Journal of Medicine" (March 1, 1972).

Appendices have been placed in the text to give more details on procedures used and to present complementary data not published elsewhere.

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The mother tongue of the candidate is French. However, since the published and submitted papers included in this thesis are written in English, the latter has been used for the whole presentation.

## INTRODUCTION

The concept of the "inborn error of metabolism" was introduced by Garrod early in this century in his now famous Croonian Lectures[1]. From his observations on alcaptonuria, albinism, cystinuria and pentosuria, Garrod developed the concept that certain diseases of lifelong duration arise because an enzyme governing a single metabolic step is reduced in activity or missing altogether, the enzyme deficiency resulting from the phenotypic effect of a particular mutant gene. An important implication of the idea is that the normal allele of this gene must in some way be necessary for the formation of the enzyme in the normal organism. This was the first clue to the now well established generalization that genes exert their effects in the organism by directing the synthesis of enzymes and other proteins. The number of conditions which can be explained in these general terms is steadily increasing year after year and constitutes an important chapter of the human biochemical genetics[2].

Two main groups of disorders form the inborn errors of metabolism. In the first one the mutation affects quantitatively or qualitatively a specific enzyme[3] and leads to the accumulation or the lack of a specific metabolite. The metabolic disturbances and clinical abnormalities which result vary widely from effectively lethal in early life (e.g. maple syrup urine disease) to apparently harmless conditions (e.g. sarcosinemia). The other group is constituted by the inborn errors of transport[4,5]. Since the first demonstration by Nägeli in 1844[6] that a semi-

permeable diffusion barrier divides the interior of the cell from the exterior, the investigation of the transfer process of solutes through that barrier, the plasma membrane, was investigated more and more extensively. Several membrane transport systems have been defined with variable properties[7-8]. They involve specific transport or carrier systems to carry certain types of molecules across the membrane. Some systems function merely as permeases (or "here to there" ases) which allow solutes to cross the membrane in either direction but always in the direction of decreasing concentration; this process is called mediated diffusion. However other important membrane transport systems can bring about active transport that is, in the direction of increasing concentration.

The transport process seems to involve particular "carrier" proteins[9] which are essentially analogous to the enzymes implicated in the more classical forms of inborn errors of metabolism. The nature of this active transport and the characteristics of the "carriers" have been investigated[10] by means of chemical, kinetic and genetic probes. Evidence has been established for the ability of the transport site to recognize chemical and molecular specificity. The specificity is also expressed in relation to the concentration of the solute. This is particularly clear for amino acid transport for whom five "common" or group specific sites have been described that operate at substrate concentrations which exceed the usual physiological range[4]. There is also a mode of amino acid transport utilized at physiological concen-

trations of the substrates and characterized by its high affinity and low capacity. Both types of transport are saturable, energy dependent but they exhibit different kinetic characteristics. Furthermore a number of genetically determined and quite specific defects of some of these transport systems have been identified.

The present thesis has been undertaken to investigate different aspects of the biological transport across cell membranes. The first part deals with a classical example of inborn error of metabolism: hypersarcosinemia. The nature of the defect and its phenotypic expression have been identified in the proband and his parents. Moreover the block in the catabolic mutant tends to isolate the transport event and gives the same conditions as if a metabolically inert compound like α-aminoisobutyric acid was used to study transport. To our knowledge, this type of approach has received only limited attention in man, apparently having been studied only in the phenylketonuric[12] and hyperprolinemic subject[11]. The particular interaction of sarcosine with the system for transport of imino acids and glycine[13] in vivo was further investigated in vitro using the rat kidney as a model. Another group of experiments was designed to look at the distribution of the sarcosine dehydrogenase activity (the supposedly deficient enzyme in sarcosinemia). The absence of activity in cultured human fibroblasts and fresh leucocytes eliminated these tissues for further investigation of mutant phenotypes.

In the second part of this thesis, attention has been focussed on the X-linked hypophosphatemic trait. This classical condition first described more than thirty years ago is still in search of a pathogenetic explanation. There is a defect of phosphate transport in the kidney present in all mutant individuals at a variable degree, the male patient being more affected than the female. There has been a long controversy on the nature of the phosphate leak(14). Is it a primary event or is it secondary to hyperparathyroidism caused by a disturbance of calcium and vitamin D metabolism? The question has been answered in the first part of our work where it is demonstrated that the phosphate transport defect is independent of the level of parathyroid hormone activity. The characteristics of the tubular phosphate reabsorption were then studied in hemizygotes and heterozygotes for the X-linked trait by means of infusion techniques. The results of these studies apparently allow one to classify X-linked hypophosphatemia as an inborn error of phosphate transport. The relevance of this work for the treatment of the disease is stressed in the last section of the thesis. The results obtained with aggressive phosphate supplementation to neutralize the mutant phenotype indicate the value of these studies.

## REFERENCES


1. Harris, H. Garrod's Inborn Errors of Metabolism. Oxford University Press, 1963.
2. McKusick, V.A. Mendelian Inheritance in Man. 3rd edition, The Johns Hopkins Press, 1971.
3. Childs, B. and Der Kaloustian, V.H. Genetic Heterogeneity. The New Eng. J. of Med. 279, 1205-1273, 1968.
4. Scriver, C.R. and Hechtman, P. Human Genetics of Membrane Transport with Emphasis on Amino Acids. In H. Harris, K. Hirschhorn Eds. Advances in Human Genetics Vol. 1, Plenum Press, 1970 pp. 211-274.
5. Rosenberg, L.E. Hereditary Diseases with Membrane Defects. In R.M. Dowben, Ed. Biological Membranes, Little Brown and Co. Boston, 1969, pp. 255-295.
6. Scott, D.H. Carl Wilheim von Nägeli. Nature 44, 580-583, 1891.
7. Christensen, H.N. Biological Transport. W.A. Benjamin Inc., New York, 1962.
8. Schultz, S.G. Mechanisms of Absorption. In R.M. Dowben, Ed. Biological Membranes, Little Brown and Co., Boston, 1969, pp. 59-108.
9. Pardee, A.B. Membrane Transport Proteins. Science 162, 632-637, 1968.
10. Berlin, R.D. Specificities of Transport Systems and Enzymes. Science 168, 1539-1545, 1970.
11. Scriver, C.R. The Use of Human Genetic Variations to Study Membrane Transport of Amino Acids in Kidney. Amer. J. Dis. Child. 117, 4-12, 1969.

12. Brodehl, J., Gellissen, K. and Kaas, W.P. The Renal Transport of Amino Acids in Untreated Infants with Phenylketonuria. Acta Paediat. Scand. 59, 241-248, 1970.

13. Mohyuddin, F. and Scriver, C.R. Amino Acid Transport in Mammalian Kidney: Multiple Systems for Imino Acids and Glycine in Rat Kidney. Amer. J. Phys. 219, 1-8, 1970.

14. See references in Section V.

## MATERIALS AND METHODS

1. Metabolism and transport of sarcosine.

Material and methods for the in vivo and in vitro studies are described in the reprint presented in Section IV and are not repeated here.

2. Transport of phosphate in human kidney.

A. The estimation of the tubular maximal reabsorption rate for phosphate (TmP).

The infusion procedure published by Anderson and Parsons (Clin. Sci. 25, 431, 1963) was modified and the following protocol was used:

Conditions:

i. When the patient was on a high phosphate regimen the phosphate supplementation was stopped for at least 12 hours before starting the infusion. This interval was sufficient to allow the serum phosphorus concentration to fall to the usual endogenous level in plasma in X-linked hypophosphatemia.

ii. There is a diurnal rhythm of phosphate excretion. The tests were all performed at the same time of the day between 8:00 A.M. and noon when spontaneous phosphate excretion is lowest.

iii. Patients were fasting. Clear fluids by mouth were offered in order to obtain a urine flow rate above 5 ml/min.

Technique:

i. Solutions:

a. The phosphate solution comprises $Na_2HPO_4$

(10.07 gm) and $NaH_2PO_4.H_2O$ (2.66 gm) diluted in one liter of distilled water. The solution was then filtered under vacuum through a VF sintered glass filter and autoclaved or filtered through a Millipore filter (0.22 micron pore size). The solution for infusion contains 2.8 mg of Pi/ml, at pH 7.35 with osmolarity 280 mOsm.

b. Calcium gluconate (10% W/V in water) from Sterilab Corp. Ltd was used for infusion.

c. Inulin 10% in 0.5% sodium chloride was obtained from Warner-Chilcott laboratories.

ii. Calculations of the infusion rates:

a. Phosphate solution:

The prime infusion ("F") is given at the rate of 0.5 mg Pi/min/Kg of body weight. Plasma Pi is raised by ~3 mg% in 20 mins. by this method. The sustain infusion ("S") is administered at 0.15 - 0.2 mg Pi/min/Kg (about 1/3 of the "prime" rate) in order to maintain the plasma Pi concentration achieved with the prime infusion.

b. Inulin infusion:

The primary dose (10% solution) given at time zero is 0.4 ml/Kg. The sustaining dose (10% solution) is 0.33 ml/min/$m^2$ of body surface area.

c. Calcium infusion:

The prime dose of 4 mg $Ca^{++}$/Kg is given over a 10 - 15 min. period. A very slow sustained infusion is maintained during the last part of the test. The rate

is about 0.05 to 0.9 mg $Ca^{++}$/min/Kg of the 10% Ca gluconate solution, depending of the level of blood calcium before the start of the calcium infusion.

d. Mixture of phosphate and inulin solutions:

The rate of phosphate infusion must be modified 3 times, but the rate of the inulin infusion must be constant. Therefore the concentration of inulin must be adapted to the infusion rate required for "prime" and "sustain" phosphate solutions.

Volumes of solution are prepared about 20% in excess of the calculated requirement. This allows filling of the connection tubes and unpredicted modification of the time schedule.

Example: For a subject 48 Kgs and 1.43 $m^2$ SA

Solution "F" (priming solution) comprises:

a) phosphate: 24 mg/min

8.6 ml/min (of a solution containing 2.8 mg Pi/ml).

For the two "F" periods, the total volume is:

8.6 x 40 mins = 344 ml.

b) inulin required: 0.3 x 1.43 = 0.43 ml/min and for 40 mins: 17.2 ml of 10% inulin. The total volume (a + b) is therefore 361.2 ml which for 40 mins requires an infusion rate of 9 ml/min.

Solution "S" (sustaining infusion) comprises:

a) phosphate: to deliver 8 mg Pi/min, the rate is: 2.8 ml/min and the volume for 105 mins (Periods "S" on the protocol sheet) is 294 ml.

b) inulin: 105 x 0.43 = 45.15 ml. The total volume is 339.15 ml and the infusion rate: 3.23 ml/min.

e. Pumps:

Two Harvard pumps Model 906 were used. This single-barreled model has a speed control device which allows each gearbox speed to be adjusted to 5 - 110% of the preset rate. One pump is used for the phosphate-inulin mixture, the other for the calcium infusion. 50 cc glass syringes are filled under sterile conditions from the stock F and S solutions and exchanged on the pump, by means of Y connections.

iii. Infusion protocol:

The subject awoke around 6:00 A.M., emptied the bladder and started a 2-3 hour urine collection until the beginning of the infusion. A blood sample is drawn prior to the injection of the prime inulin dose for estimating fasting phosphate and calcium concentrations, and inulin blanks.

Fig. III-1 depicts the protocol used during the test. The patient was studied supine but without a bladder catheter when urine flow rates exceeded 5 ml/min. Voiding was done standing. Infusions were performed in arm vein (phosphate) and leg vein (calcium). Blood was withdrawn from the second arm without tourniquet via an indwelling # 21 "butterfly" scalp vein needle. Blood samples are obtained at the end of the two periods of fast infusion and in the middle of the urine collection periods to determine the calcium and phosphorus

concentrations in plasma. The samples were analyzed within 15 minutes with a Technicon Auto-Analyzer and it was possible to adjust the infusion rates if necessary, and avoid prolonged hypo or hyper-calcemic episodes.

Blood samples for PTH determination were drawn before and after both the phosphate and the calcium infusions (see protocol). Fig. III-2 shows the phosphate,calcium and inulin concentrations achieved during a typical experiment.

B. The response to parathyroid hormone (PTH) intravenous infusion.

Purified bovine PTH (urea-TCA extract) was obtained in powder form, from Dr. Claude Arnaud. The specific activity of the extract is about 350 units/mg. Adults received 400 units and children 200 units. The powder is diluted in 5 ml of acetic acid 0.01 M and filtered immediately through a 0.22 micron Millipore filter. It is then diluted in an equal volume of 0.9% sodium chloride and injected intravenously over a 6-7 minute period within one hour of preparation.

The test is performed in the morning in the fasted state and after ingestion of sufficient clear fluids to ensure urine flow rates exceeding 5 ml/min. Three consecutive 30-minute urine collections were obtained for the control periods. At the beginning of the 4th period, the PTH was infused into an arm vein and three 30-minute urine collections were performed subsequently.

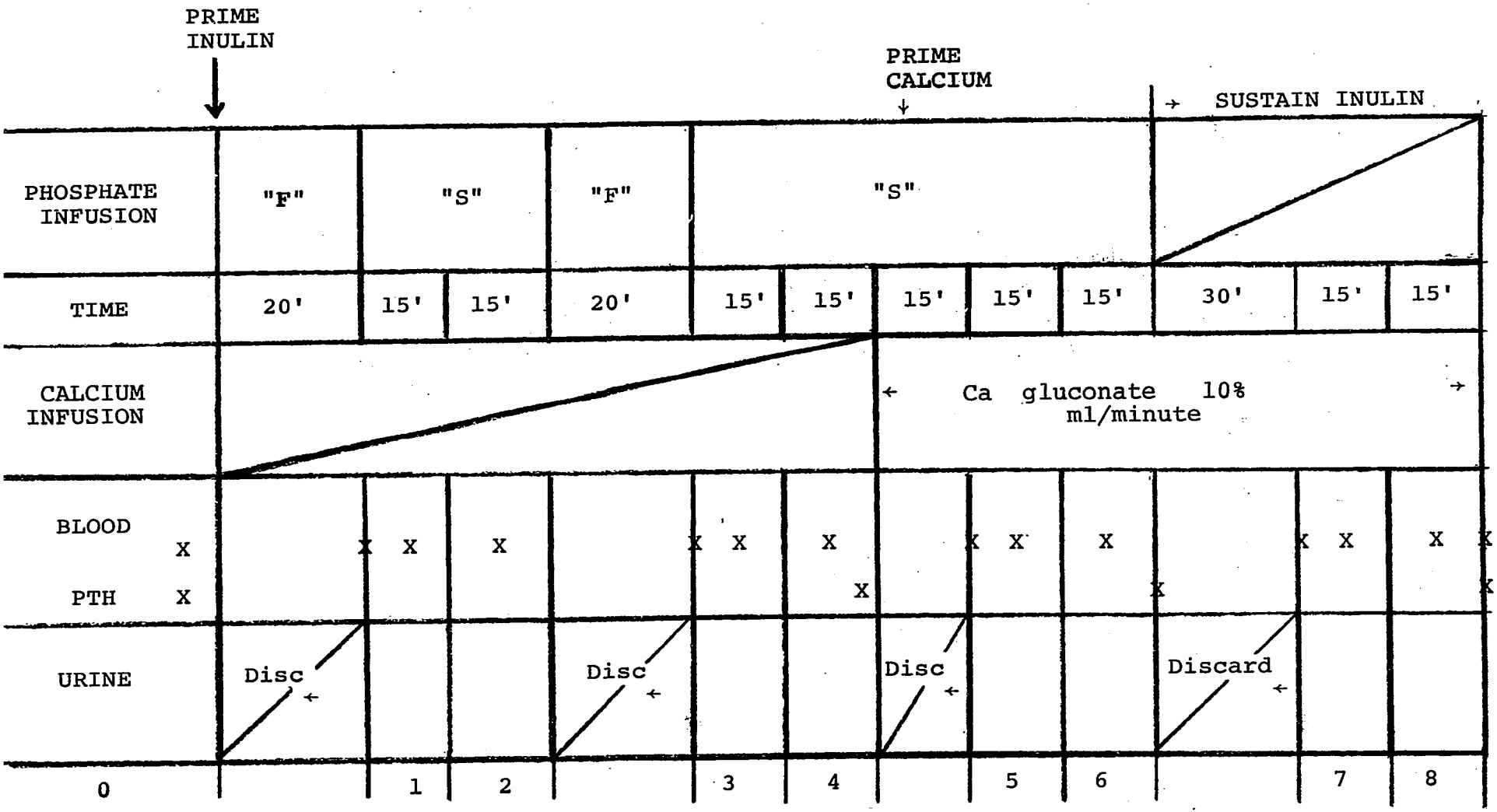
Blood samples are drawn at midpoint during each period for estimating calcium, phosphorus and creatinine concentrations. An indwelling needle was employed to avoid the effects of venipuncture on glomerular filtration rate. The subject was supine during the study except when voiding.

Two aliquots were kept after measuring the total volume of urine in each collection period. One was rapidly acidified and frozen until determination of phosphate, calcium and creatinine concentrations. The other was rapidly frozen without acidification until the determination of adenosine 3',5'-monophosphate content.

C. Analytical methods:

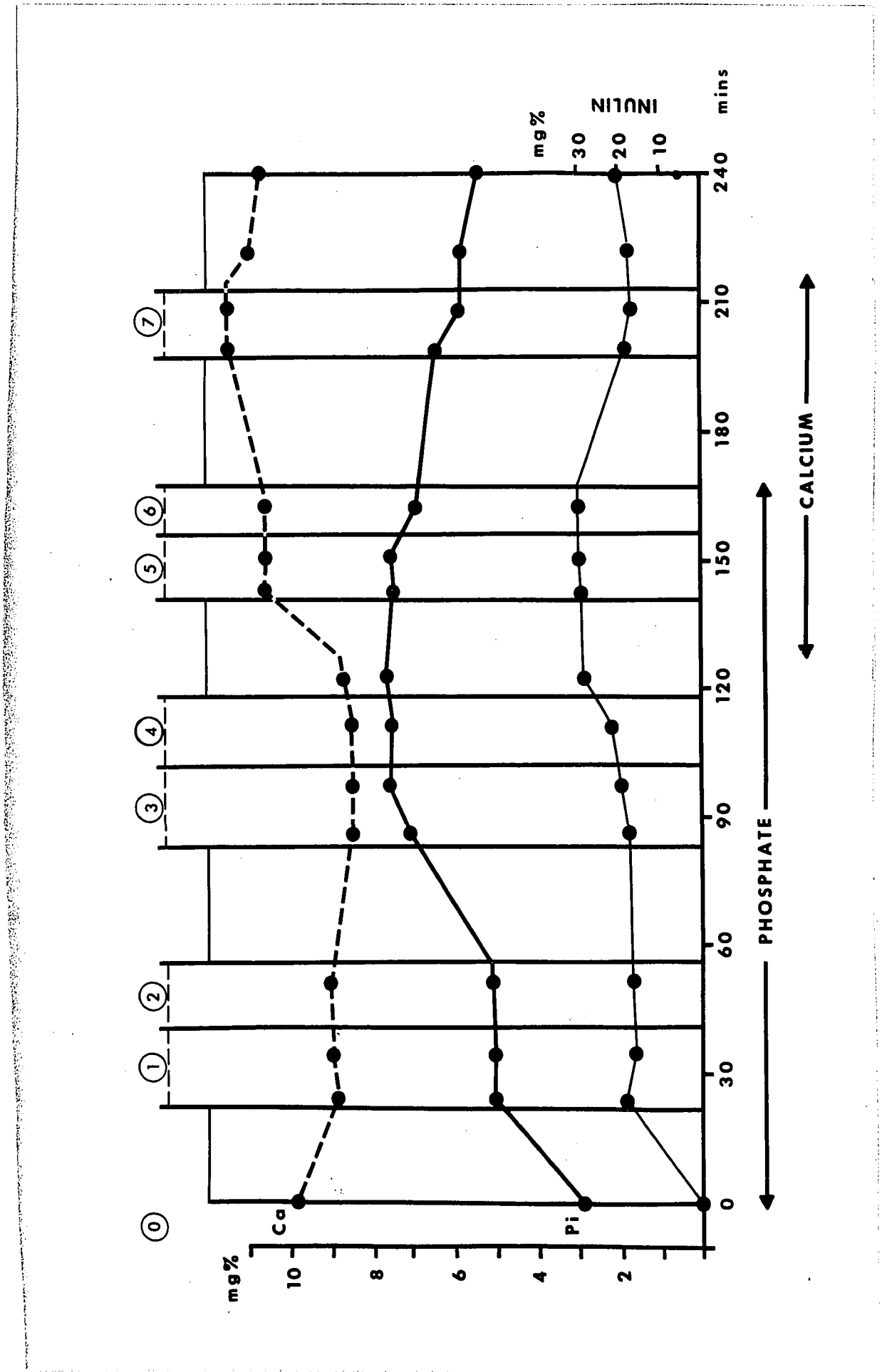
The methods are cited in the relevant publication.

FIGURE III - 1




vol.:
time:
flow rate:

FIGURE III - 2


INULIN
mg%
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90
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CALCIUM
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6
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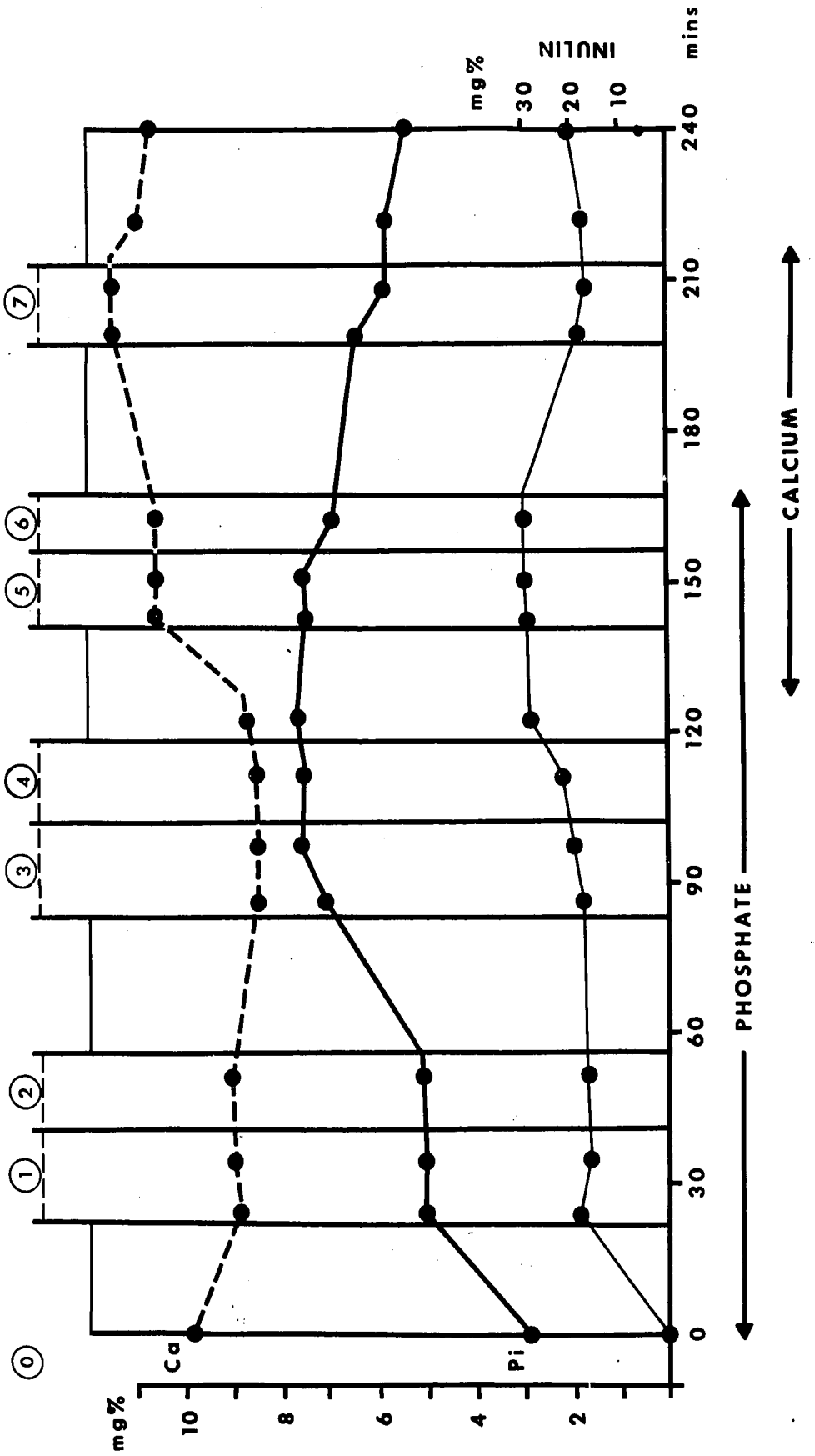
FIGURE III - 2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# TRANSPORT AND METABOLISM OF SARCOSINE IN HYPERSARCOSINEMIC AND NORMAL PHENOTYPES

This study was undertaken as a classical exercise in human biochemical genetics. The clinical part of the work was designed to investigate the pedigree in which the blocked catabolic mutant had been discovered incidentally. The delineation of the phenotype in the homozygote and the presumed obligate heterozygotes for the hypersarcosinemic trait was studied by means of loading tests. The changes in blood concentration of the metabolites involved in the "one carbon cycle" allowed good discrimination of mutant homozygote, heterozygote and homozygous normal phenotypes.

The mutant phenotype also provided an opportunity to study the renal tubular transport of sarcosine in the presence of a block in sarcosine catabolism. The particular response of glycine and proline excretion after sarcosine loading tests led to the hypothesis that interaction of sarcosine occurred with the well studied transport systems for imino acids and glycine. The hypothesis has been tested in vitro and the characteristics of sarcosine transport in kidney were delineated. The presumed mode of sarcosine transport across kidney cellular membranes is summarized in Fig. IV-1.

This study gave the opportunity to investigate a specific problem in biochemical genetics in depth, using a wide variety of approaches. The hypersarcosinemic trait can now be classified with reasonable confidence as a classical inborn error

of metabolism. The study of sarcosine transport provided a useful introduction for the formal investigation of inborn error of transports (viz. Part V).

The results of the sarcosine studies are given in the accompanying publication (J. Clin. Invest. 50, 2313, 1971).

FIGURE IV - 1

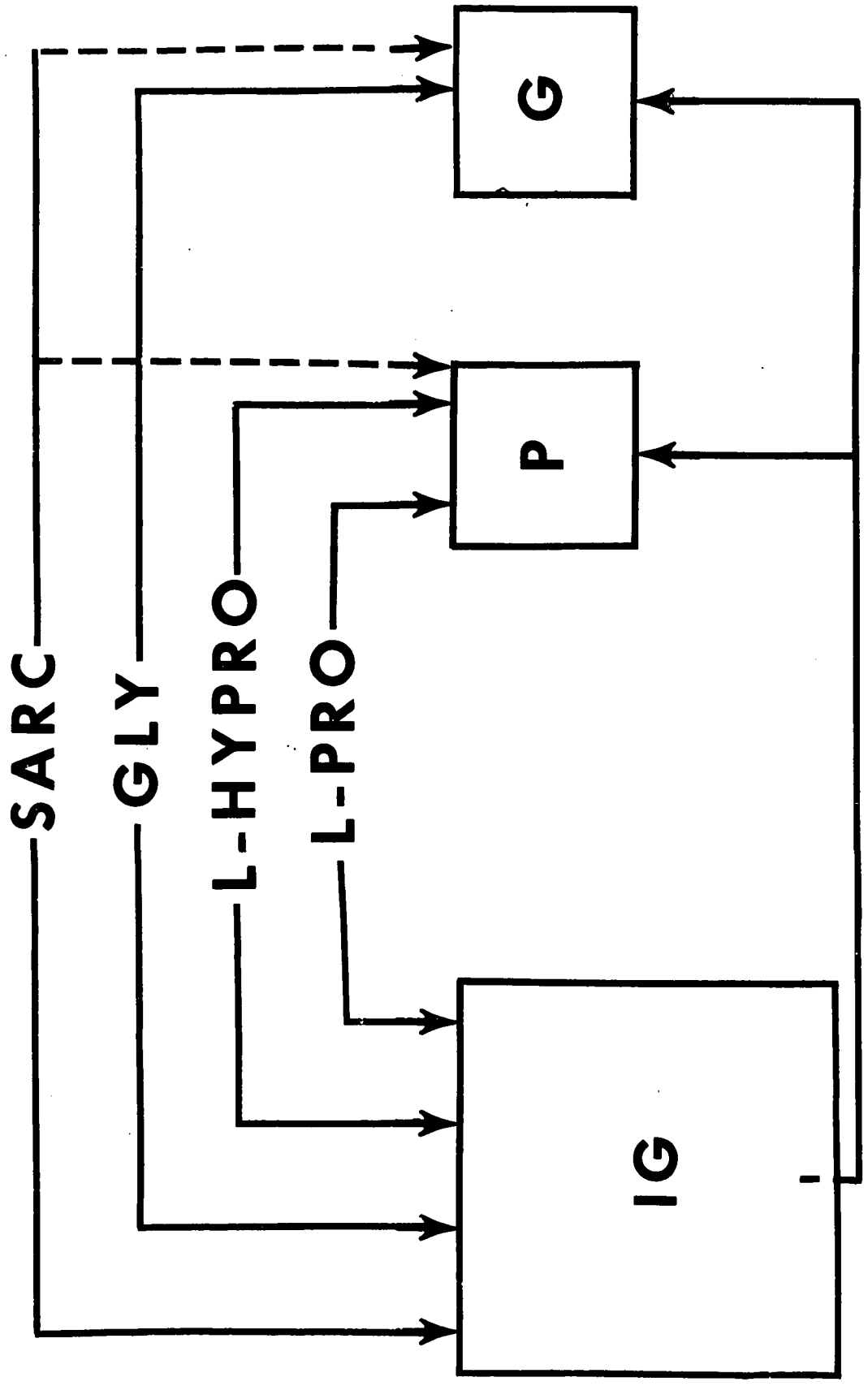
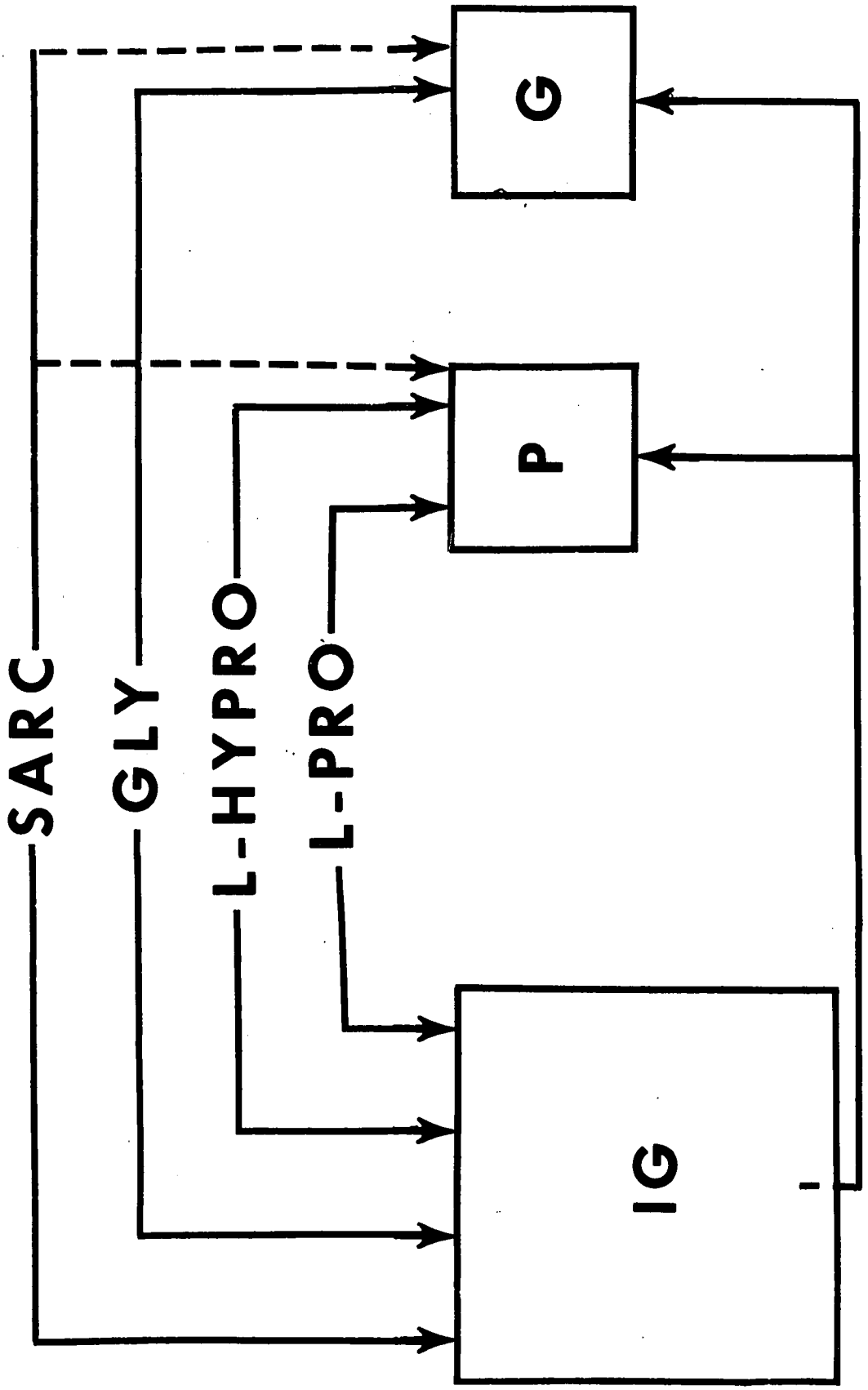


FIGURE IV - 1



## FIGURE IV - 1

The transport sites identified (see ref. in the JCI paper) for the renal tubular transport of iminoacids and glycine are represented. The largest square (IG) corresponds to the high capacity-low affinity site common to the group. The two smaller squares identify the low capacity high affinity sites for iminoacids (P) and glycine (G) respectively. Sarcosine seems to parasitize mainly the low affinity system (IG). The arrows in the lower part of the graph indicate the exchange process proposed to account for the improvement of glycine reabsorption observed during loading studies in the proband.

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# Transport and Metabolism of Sarcosine in Hypersarcosinemic and Normal Phenotypes

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ABSTRACT An adolescent male proband with hypersarcosinemia was discovered incidentally in a French-Canadian family; no specific disease was associated with the trait. The hypersarcosinemia is not diminished by dietary folic acid even in pharmacologic doses (30 mg/day). The normal absence of sarcosine dehydrogenase in cultured human skin fibroblasts and in leukocytes was confirmed, thus eliminating these tissues as useful sources for further investigation of mutant sarcosinemic phenotypes and genotypes.

The response in plasma of sarcosine and glycine, after sarcosine loading, distinguished the normal subject from the subjects who were presumably homozygous and heterozygous for the hypersarcosinemia allele. Sarcosine clearance from plasma was delayed greatly ($t_{\frac{1}{2}}$, 6.1 hr) in the presumed homozygote and slightly ($t_{\frac{1}{2}}$, 2.2 hr) in the presumed heterozygote, while plasma glycine remained constant in the former and rose in the latter. Normal subjects clear sarcosine from plasma rapidly ($t_{\frac{1}{2}}$, 1.6 hr) while their plasma glycine trend is downward. The phenotypic responses suggest that hypersarcosinemia is an autosomal recessive trait in this pedigree.

Renal tubular transport of sarcosine was normal in the proband even though he presumably lacked the sarcosine oxidation which should normally occur in kidney. Sarcosine catabolism is thus not important for its own renal uptake.

Sarcosine interacts with proline and glycine during its absorption in vivo. Studies in vitro in rat kidney showed that sarcosine transport is mediated, saturable, and energy dependent. Sarcosine has no apparent transport system of its own; it uses the low $K_m$ transport systems for L-proline and glycine to a minor extent and a high $K_m$ system shared by these substances for the major uptake at concentrations encountered in hypersarcosinemia. Intracellular sarcosine at high concentration will exchange with glycine on one of these systems, which may explain a paradoxical improvement in renal transport of glycine after sarcosine loading in the hypersarcosinemic proband.

## INTRODUCTION

Hypersarcosinemia with sarcosinuria was first reported in 1965 by Gerritsen and Waisman (2), and subsequently by Hagge, Brodehl, and Gellissen (3) and Scott, Clark, Teng, and Swedberg (4). Only four patients are represented by these reports, but many additional patients have since been reported to Gerritsen.[1] We wish to describe the results of our investigation of another subject with sarcosinemia (1), in whom there is no apparent disease associated with the biochemical disorder, thus affirming the now prevailing impression[1] that sarcosinemia is a "nondisease." We found no expression of the presumed block at sarcosine dehydrogenase (sarcosine:$O_2$ oxidoreductase EC 1.5.3.1) in fibroblasts or leukocytes from our patient, since this enzyme is not active in these tissues normally. Since tetrahydrofolate is apparently involved in the transfer of the formaldehyde group from sarcosine (5), we also evaluated the patient's response to treatment with folic acid in vivo; the trait was not folate responsive in this pedigree. A partial impairment of sarcosine clearance from plasma was demonstrated in the presumed heterozygotes for this trait, indicating that the inheritance of sarcosinemia in man is probably autosomal recessive.

Hereditary hypersarcosinemia provides a valuable opportunity to study the renal transport of sarcosine, since

This work was presented in part at the Society for Pediatric Research, Atlantic City, N. J., 2 May 1970 (1).

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[1] Gerritsen, T. 1970. Personal communication.

oxidation of this amino acid, which occurs normally in mammalian kidney will not contribute to measurements of its renal uptake in vivo in the patient with hypersarcosinemia. We were able to evaluate the characteristics of sarcosine transport both in vivo in the proband and in vitro by using rat kidney cortex slices. Although sarcosine appears to have no membrane transport system of its own, it readily enters renal tissue on the membrane systems used by imino acids and glycine (6).

## METHODS

*Studies in vivo.* Endogenous renal clearance rates and net tubular reabsorption rates of sarcosine and other amino acids were evaluated by the methods described previously (7, 8).

Loading tests were performed with sarcosine (free base, 100 mg/kg body wt) taken as a solution in apple juice at 9 a.m. after an overnight fast. Heparinized venous blood was drawn from the antecubital vein just before the load (0 hr) and at 1, 2, 3, 4, and 5 hr after the load. Plasma was deproteinized immediately with sulfosalycylic acid (3% w/v; plasma : acid, 1 : 5). Timed urine collections were made before and during the sarcosine load.

Amino acids were measured by elution chromatography on ion exchange resins according to the methods of Spackman, Stein, and Moore (9) using a Beckman-Spinco model 120 analyzer, modified for rapid multiple analyses (10).

*Studies in vitro: transport.* Female Long-Evans rats, weighing about 160 g, were killed by decapitation. For studies of sarcosine transport, the kidneys were removed immediately, and cortex slices were prepared and incubated in Tris-electrolyte-glucose buffer (6). The measurements of amino acid uptake and determinations of sarcosine metabolism and oxidation were carried out by methods described previously (6).

*Tissue culture studies.* Skin fibroblasts from normal subjects were subcultured according to the method of Hayflick (11). About $5 \times 10^6$ cells were incubated in a Warburg flask in isotonic medium, pH 7.3, containing cold and $^{14}$C-labeled sarcosine final concentration 0.5 mM. Induction of sarcosine dehydrogenase was tested by culturing cells for 18 hr in the presence of sarcosine (1 mM and 10 mM). Intact and sonicated cells were used in all experiments to determine whether uptake limited their capacity for sarcosine oxidation.

*Leukocyte studies.* Mixed leukocytes were isolated from venous blood by the method of Zucker and Cassen (12). The recovery of mixed leukocytes is 60–70% by this method. Cells were sonicated (22 kc/sec for 3–5 sec at 0°C) and incubated for 45 min as described for fibroblasts.

*Homogenates.* Rat tissues were homogenized in 0.25 M sucrose with a Potter-Elvejhem homogenizer. The centrifuged homogenate (0.1 ml) was then resuspended and incubated for 60 min in Warburg flasks containing Tris buffer and substrate.

*Sarcosine dehydrogenase activity and sarcosine oxidation.* Oxidation was measured with unlabeled sarcosine (1 mM) and sarcosine-1-$^{14}$C (0.1 mM); $^{14}CO_2$ was collected on filter paper (2 cm square soaked with saturated KOH (50 $\mu$l). The papers were dried under vacuum and counted in scintillation fluid. The efficiency of $CO_2$ collecting by this method was 57.7 ±4.9%. Sarcosine dehydrogenase was assayed specifically using methyl-$^{14}$C–labeled sarcosine as substrate, by the isotope method of Rehberg and Gerritsen (13). Pyruvate dehydrogenase was used as a control assay (14) for cell viability in these studies.

*Chemicals.* Sarcosine-1-$^{14}$C (SA 2 mCi/mmole) and sarcosine-$^{14}CH_3$ (SA 3.5 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Their radiochemical purity was confirmed by partition chromatography and high voltage electrophoresis in several systems. Unlabeled amino acids were obtained from Mann Research Labs. Inc., New York. The scintillation mixture for isotope counting contained 4.0 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis[2-(5-phenyloxazolyl)]benzene(POPOP) in 1 liter of toluene; both scintillants were obtained from the Packard Instrument Co., Inc., Downers Grove, Ill.

Counting was performed in a Unilux II Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) operating at 40% efficiency.

*Calculations.* The half-life for sarcosine disappearance from plasma was calculated by plotting the plasma response on log scale against time (arithmetic scale) and fitting the best straight line to the points by the minimum root mean square method; half-time for disappearance was obtained directly from the slope.

Data for distribution ratios in transport studies indicate an isotope distribution ratio (counts per minute per unit time per milliliter intracellular fluid : counts per minute per milliliter of initial incubation medium) unless stated otherwise. Net uptake velocity was corrected for the nonsaturable component by the method of Akedo and Christensen (15). The steady-state conditions used in our studies allow satisfactory estimates of the Michaelis constant for uptake of an amino acid (16), and under these conditions biphasic uptake kinetics were observed when the uptake velocity of sarcosine was examined in relation to its concentration in the medium. Assuming that the observed sarcosine uptake was the sum of one or more components, then:

$$u \text{ observed} = u_1 + u_2 \ldots + u_n. \quad (1)$$

$V_{max}$ and $K_m$ for each component can then be determined by solving:

$$u = \frac{V_{max_1}[S]}{K_{m_1} + S} + \frac{V_{max_2}[S]}{K_{m_2} + S} + \frac{V_{max_n}[S]}{K_{m_n} + S} \quad (2)$$

A revised computer method (17), adapted from our earlier work (6, 18), was used to solve this equation.

## RESULTS

### The proband

L. V. was born 8 August 1959, the fifth son of nonconsanguinous French-Canadian parents. He developed normally to the age of 13 months, when an episode, diagnosed as poliomyelitis led to a mild spastic paraplegia. Investigation performed in 1969 during an orthopedic admission to another hospital included chromatographic examination of amino acids in urine. This showed the presence of a ninhydrin-positive substance subsequently identified as sarcosine. Sarcosine was also present in blood in concentration varying from 0.18 to 0.76 $\mu$moles/ml.

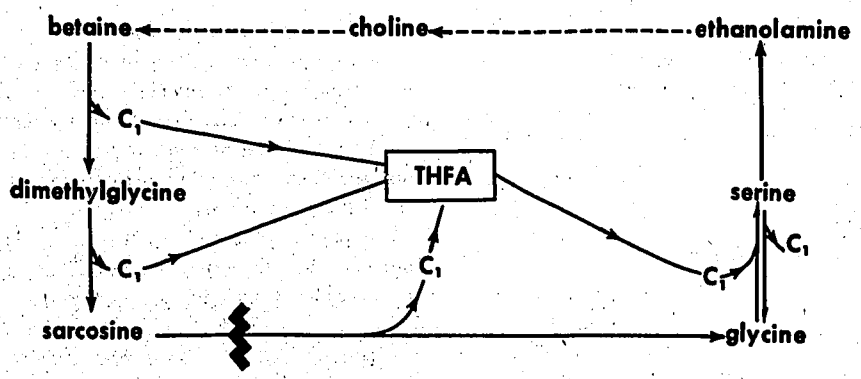


FIGURE 1 Scheme depicting sarcosine metabolism. THFA indicates the presumed $N^5$, $N^{10}$-methylenetetrahydrofolate derivative which serves transfer of the 1-carbon formaldehyde group formed during oxidation of sarcosine to glycine. The presumed block in sarcosine metabolism is indicated.

## Identity of sarcosine

The identification of sarcosine in urine and plasma was performed by partition chromatography on Whatman filter paper No. 4 in three different solvent systems (water-saturated phenol; lutidine: water, 2.2:1; and butanol: acetic acid: water, 12:3:5); by high voltage electrophoresis on Whatman filter paper No. 3 MM soaked in formic, acetic buffer pH 2, and by column chromatography on ion exchange resins. Sarcosine (free base) was run in all systems to identify its position. The unknown spot and the standard cochromatographed in the five systems and yielded identical color reactions with ninhydrin in the partition and electrophoretic systems; the 570 mμ to 440 mμ OD ratio after elution chromatography was identical for unknown and standard. There was no other ninhydrin-positive material in equivalent amounts in the patient's urine.

## Sarcosine metabolism

*Response to folic acid in vivo.* The proband was given an excess of folic acid (30 mg/day; normal requirement about 1 mg/day) for 3 consecutive days. The rationale for this trial is indicated in Fig. 1. The methyl group is transferred as formate from sarcosine, presumably as an $N^5$, $N^{10}$-methylenetetrahydrofolate derivative of folic acid. Fasting concentrations of sarcosine in plasma were 0.18 and 0.34 μmoles/ml before and after folate supplementation, respectively. The latter value falls within the range for plasma sarcosine in this patient when no folate was administered. The values for Δ-sarcosine in plasma after a sarcosine load by mouth (the sum of the difference between values at 1, 2, 3, 4, and 5 hr after the load, and the preload value) were +1.02 μmoles/ml·5 hr before and +2.18 μmoles/ml·5 hr after folate, indicating that folate did not influence sarcosine catabolism in this patient.

*Response to sarcosine loading.* Sarcosine loading by mouth was performed on the proband, his parents, and four normal adult subjects. The half-time for plasma sarcosine disappearance rates in each individual in the three groups and the simultaneous change in glycine, the product of sarcosine conversion, are shown in Fig. 2.

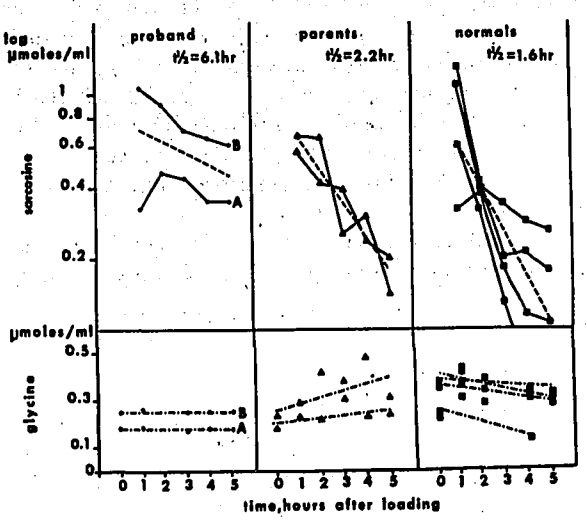


FIGURE 2 Response of sarcosine and glycine in plasma to sarcosine loading (100 mg/kg body wt given by mouth at 9 a.m.). Upper portion: A and B indicate studies in proband before and after folic acid supplement, respectively. Half-life for sarcosine clearance from plasma ($t_{\frac{1}{2}}$) was calculated as described under Methods; the interrupted line is the average decay slope for each group studied. The initial (preload) concentration of sarcosine in plasma was: zero in both parents and all normal subjects, and 0.18 μM and 0.34 μM in studies A and B, respectively, in the proband. Lower portion: Change in plasma glycine with time after sarcosine loading. Dotted lines define the average trend for each subject in the group. Changes in serine are not shown since they paralleled glycine.

TABLE I

*Sarcosine Oxidation and Dehydrogenase Activity in Tissues of Man and Rat*

| Tissue source | Oxidation to $CO_2$* |
|---|---|
| | *dpm/hr per g tissue* |
| Human | |
| Skin fibroblast | 0 |
| Mixed leukocytes from blood | 0 |
| Rat | |
| Liver‡ | 15,750 |
| Kidney‡ | 6,375 |
| Muscle | 0 |
| Brain | 0 |
| Spleen | 0 |

* Sarcosine dehydrogenase activity was also examined by a specific assay (13). Oxidation and specific dehydrogenase activities complemented each other in all tissues examined.

‡ Organ weights (average from 10 rats): liver, 6.5 g; both kidneys, 1.6 g.

Normal subjects showed rapid disappearance of sarcosine from plasma ($t_{\frac{1}{2}}$, 1.6 hr). The concentration of glycine in plasma actually declines modestly during this period. In the proband, sarcosine clearance from plasma was greatly delayed ($t_{\frac{1}{2}}$, 61 hr), and there was no change in plasma glycine. Both parents had a slightly delayed disappearance of sarcosine ($t_{\frac{1}{2}}$, 2.2 hr), but the most striking finding in them was a modest but steady rise in the concentration of glycine in plasma after sarcosine loading. Changes in the concentration of serine in plasma in these studies paralleled those of glycine.

*Sarcosine metabolism in vitro.* No oxidation of sarcosine or evidence for specific dehydrogenase activity was detected in skin fibroblasts cultured for at least 14 passages from explants grown from the proband and two normal subjects (Table I). No activity was induced by exposure of cells for 18 hr to sarcosine (1.0 mM and 10 mM in the growth medium). Neither intact nor sonicated cells exhibited dehydrogenase activity under any of these conditions.

Leukocytes obtained from venous blood of normal subjects and the patient contained no specific sarcosine dehydrogenase activity. Measurement of sarcosine oxidation to $CO_2$ yielded similar negative results.

*Tissue specificity of sarcosine oxidation (in the rat).* The tissue specificity of sarcosine oxidation was examined in the Long-Evans rat. Significant oxidation of sarcosine was found only in liver and kidney (Table I). When the relative weights of liver and kidney were taken into account, we found that kidney accounts for 10% of total body oxidation of sarcosine in the rat, a value close to that reported for a different strain of rat by Rehberg and Gerritsen (13).

If it is assumed that man resembles the rat in his ability to oxidize sarcosine, we should expect sarcosine accumulation to occur initially in liver and kidney in the "blocked catabolic mutant" we know as hypersarcosinemia. Therefore, we examined the transport of sarcosine in kidney to determine whether it is impaired in this metabolic derangement.

## Sarcosine transport in human kidney in vivo

*Tubular absorption.* The filtered renal load of sarcosine in the proband varied between 54.22 and 119.77 μmoles/min per 1.73 $m^2$ under fasting conditions; the corresponding value is almost zero in normal subjects. The equivalent endogenous renal clearance rates for sarcosine in the proband were about 8 ml/min per 1.73 $m^2$, indicating that the majority of filtered sarcosine experiences net tubular absorption in the sarcosinemic phenotype. Tubular reabsorption of sarcosine in our patient is probably a saturable phenomenon, although true saturation was not demonstrated. The $T_m$ for sarcosine was estimated to be at least 160 μmoles/min per 1.73 $m^2$ (Fig. 3). Data on tubular absorption of sarcosine in another sarcosinemic patient (3) were obtained from Dr. Johannes Brodehl. Sarcosine transport at equivalent filtered loads was similar in both sarcosinemic probands and in our control subjects (Fig. 3).

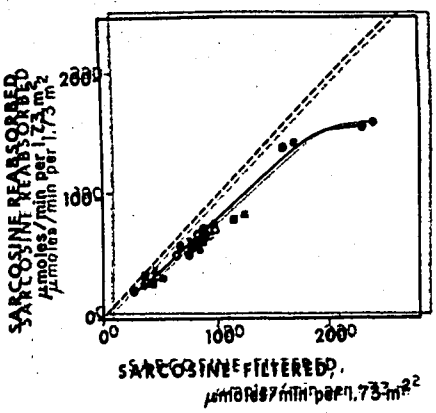


FIGURE 3. Renal tubular reabsorption of sarcosine in man, plotted in relation to its filtered load: in proband (●); in the patient of Brodehl and associates (3)* (○); and in a normal subject (■). Plasma sarcosine was raised by venous infusion method, and glomeral filtration was monitored with inulin (8).

*Interaction with other amino acids.* Interaction between sarcosine, proline, and glycine was observed during renal transport in vivo. Intravenous infusion of sarcosine in the proband and in the normal subject altered the urinary excretion of proline and glycine. This phenomenon could not be explained by changes in the plasma concentration of the latter substances, and it was clearly related to the rising concentration of

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| Tissue source | Oxidation to $CO_2$* |
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## Sarcosine transport in human kidney in vivo

*Tubular absorption.* The filtered renal load of sarcosine in the proband varied between 54.2 and 119.7 μmoles/min per 1.73 $m^2$ under fasting conditions; the corresponding value is almost zero in normal subjects. The equivalent endogenous renal clearance rates for sarcosine in the proband were about 8 ml/min per 1.73 $m^2$, indicating that the majority of filtered sarcosine experiences net tubular absorption in the sarcosinemic phenotype. Tubular reabsorption of sarcosine in our patient is probably a saturable phenomenon, although true saturation was not demonstrated. The Tm for sarcosine was estimated to be at least 160 μmoles/min per 1.73 $m^2$ (Fig. 3). Data on tubular absorption of sarcosine in another sarcosinemic patient (3) were obtained from Dr. Johannes Brodehl. Sarcosine transport at equivalent filtered loads was similar in both sarcosinemic probands and in our control subject (Fig. 3).

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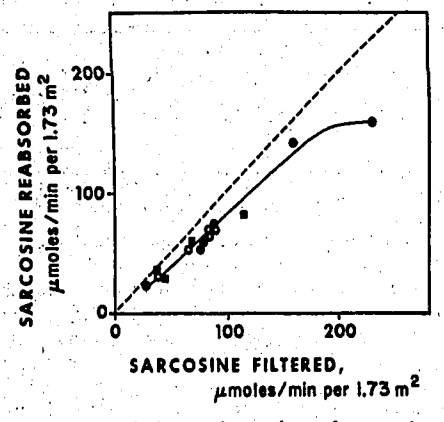


FIGURE 3 Renal tubular reabsorption of sarcosine in man, plotted in relation to its filtered load: in proband (●); in the patient of Brodehl and associates (3)* (○); and in a normal subject (■). Plasma sarcosine was raised by venous infusion method, and glomeral filtration was monitored with inulin (8).

TABLE II

*Renal Response to Sarcosine Loading: Excretion and Absorption of Sarcosine, Glycine, and Proline*

| Subject and procedure | Sarcosine | | Glycine | | Proline | |
|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After |
| Sarcosine infusion* | | | | | | |
| Proband | | | | | | |
| Urinary excretion, *μmoles/min per 1.73 m²* | 6.8 | 128 | 3.94 | 3.68 | 0 | 1.03 |
| Net tubular absorption, *% of filtered load* | 94 | 68 | 89 | 94 | 100 | 97 |
| Control | | | | | | |
| Urinary excretion, *μmoles/min per 1.73 m²* | 0 | 38 | 1.0 | 8.05 | 0 | 0 |
| Net tubular absorption, *% of filtered load* | 0 | 65 | 96.7 | 80.7 | 100 | 100 |
| Sarcosine load by mouth,‡ urinary excretion, *μmoles/min per 1.73 m²* | | | | | | |
| Proband: on folate | 6.35 | 10.3 | 2.75 | 2.27 | 0 | 0 |
| off folate | 2.83 | 9.15 | 2.76 | 1.81 | 0 | Tr.§ |
| Father | 0 | 9.70 | 1.73 | 3.75 | 0 | Tr. |
| Mother | 0 | 9.42 | 1.10 | 4.10 | 0 | Tr. |
| Controls, adult | | | | | | |
| R. V. | 0 | 3.62 | 0.17 | 1.52 | 0 | 0 |
| F. G. | 0 | 50.6 | 1.02 | 4.61 | 0 | 0 |
| J. N. | 0 | 7.25 | 0.27 | 3.30 | 0 | Tr. |
| D. W. | 0 | 18.4 | 1.54 | 7.91 | 0 | 0 |

* Data taken from periods of control and highest filtered load of sarcosine as shown in Fig. 3.
‡ Load, 100 mg/kg at 9 a.m. after overnight fast.
§ Indicates a detectable change but less than 0.1 μmoles/min per 1.73 m².

sarcosine in plasma and urine. Tubular reabsorption of glycine in the proband increased from 89 to 94% of its filtered load (Table II), but decreased from normal to 81% in the control subject. Net tubular absorption of proline clearly decreased during sarcosine infusion in the patient, but was not changed in the control subject.

When sarcosine was administered by mouth to the proband, his parents, and four normal adults, its concentration increased in plasma and urine of all subjects (Table II). At this time in the proband, there was a minimal increase in proline excretion whereas glycine excretion actually diminished. On the other hand, the urinary excretion of glycine clearly increased in all normal subjects and in the parents, while there was no comparable effect on proline excretion.

Rapid elevation of plasma and urinary sarcosine thus produced consistent, but divergent, effects on the urinary excretion of glycine, in particular, in the sarcosinemic proband when compared with subjects who do not have impaired sarcosine metabolism. An explanation for this was sought by the study of sarcosine transport in kidney in vitro. Rat kidney cortex slices were used for this work, since it has been shown that there are many homologies in the transport of *N*-substituted amino acids and glycine in the kidney of rat and man (6).

## Sarcosine transport in rat kidney in vitro

*Time course of uptake.* The time course of sarcosine uptake was evaluated at 0.3 and 2.1 mM (Fig. 4). Sarcosine at both concentrations accumulated against an isotope gradient at the steady state; the latter is achieved within 40 min of incubation.

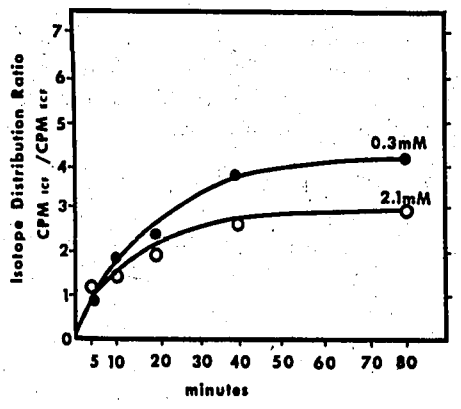


FIGURE 4 Time course of sarcosine uptake into rat kidney cortex slices at two initial concentrations in the medium. Uptake is expressed as an isotope distribution ratio. Additional studies (see text) show that the sarcosine (chemical) distribution ratio is only slightly less than isotope distribution ratio and unequivocally greater than 1.0 at steady state.

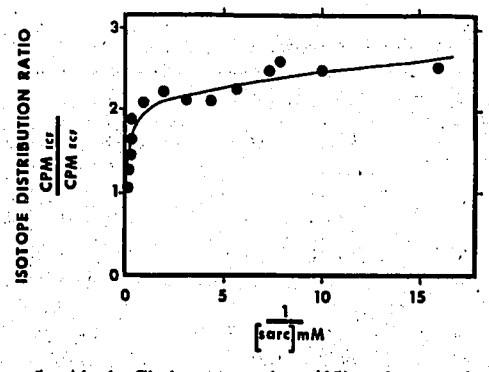


FIGURE 5 Akedo-Christensen plot (15) of sarcosine uptake by rat kidney cortex slices, at various external concentrations of substrate, under steady-state conditions. Uptake of sarcosine is saturable over the whole concentration range employed.

The effect of metabolic conversion of sarcosine (0.1 mM) on its uptake ratio was examined. $^{14}C$ label from sarcosine had appeared in other water-soluble metabolites after 40 min incubation; 14% of the soluble label was present in glycine, 7% in serine, and the remainder was predominantly sarcosine. The sarcosine (chemical) distribution ratio during uptake under steady-state conditions was then calculated from the isotope distribution ratio after correcting for the conversion of sarcosine to other labeled forms. The corrected ratio is greater than 3.0 for 0.3 mM sarcosine, indicating that sarcosine is transported in kidney against a true chemical gradient.

When the isotope distribution ratio is also corrected for loss of label by oxidation to $CO_2$, the net uptake ratio obtained by adding soluble counts to counts in $CO_2$ is greater than depicted in Fig. 4. The adjusted "uptake ratios" are 6.8 and 3.4 at 0.3 mM and 2.1 mM sarcosine, respectively, after 40 min incubation.

Exposure of slices to cyanide ($10^{-3}$ M NaCN) or anaerobic conditions, completely abolished concentrative uptake indicating that the mechanisms for sarcosine uptake are apparently coupled to energy metabolism.

*Concentration-dependent uptake.* When sarcosine is present at 0.3 mM in the initial medium, the steady-state isotope distribution ratio is greater than when the starting concentration is 2.1 mM (Fig. 4). As the external substrate concentration is increased, the steady-state distribution ratio approaches 1.0 (Fig. 5); this behavior indicates that sarcosine uptake in kidney at concentrations equivalent to those encountered in the proband occurs on a saturable mediation.

The kinetics of sarcosine uptake on the saturable component under steady-state conditions were examined by the Eadie and Augustinsson transformation (u vs. u/S) of the Michaelis equation (Fig. 6). Biphasic uptake kinetics were revealed by this method, indicating that more than one component probably exists for sarcosine uptake. This behavior was also observed when the Lineweaver and Burk transformation (1/u vs. 1/S) was used to examine the same uptake data. At low external substrate concentrations, the apparent $K_m$ for sarcosine uptake under steady-state conditions is about 0.1 mM, while at high substrate concentrations it is about 3 mM. These values are comparable with those which describe the uptake of glycine and proline by rat kidney (6).

If it is assumed that more than one type of membrane system accommodates sarcosine transport in kidney, the theoretical contribution of each component to the observed (total) uptake can be calculated. This was accomplished substituting the values for uptake, at 12 different sarcosine concentrations between 0.1 mM and 12.1 mM into equation 2. When the results were drawn as a Michaelis plot (Fig. 7), it became evident that at extracellular concentrations of sarcosine above 0.1 mM, the major fraction of its uptake takes place on a high capacity system. The revised $K_m$ values for sarcosine transport were 0.1 mM on the low capacity system and 3.1 mM on the high capacity system.

*Specificity of sarcosine uptake.* Glycine and L-proline inhibit sarcosine uptake in vitro under steady-state conditions (Table III). The effect of these inhibitors was shown to be competitive. Glycine and L-proline did not have any additional effect on sarcosine oxidation in kidney, and their effect on net sarcosine uptake was restricted to interaction at the transport site.

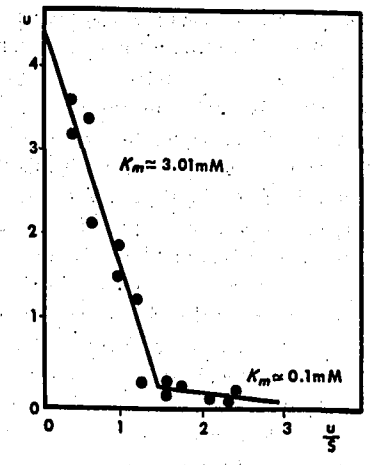


FIGURE 6 Eadie-Augustinsson plot of saturable sarcosine uptake under steady-state conditions in rat kidney cortex slices. Uptake occurs on more than one system for which first approximation $K_m$ values are shown. When corrections were made for simultaneous uptake of sarcosine on multiple systems (viz. equation 2), the corrected $K_m$ values were not significantly different, since uptake on the low $K_m$ system is extremely small.

TABLE III

*Comparative Interactions between Sarcosine, Glycine, and L-Proline during Uptake by Rat Kidney Cortex Slices*

| Substrate | Inhibitor | Inhibition of uptake at 5 min | Inhibition of uptake at 40 min |
|---|---|---|---|
| *mM* | *20 mM* | % | |
| Sarcosine 0.3 | L-Proline | 74 | 76 |
| | Glycine | 44 | 79 |
| Sarcosine 2.0 | L-Proline | 88 | 92 |
| | Glycine | 71 | 85 |
| Glycine 0.01 | Sarcosine | | 80 |
| Glycine 2.0 | Sarcosine | | (−27)* |
| L-Proline 0.01 | Sarcosine | | 55 |
| L-Proline 2.0 | Sarcosine | | 82 |

Slices were incubated at 37°C in pH 7.4 Tris-electrolyte-glucose buffer for specified times. The effect of the second amino acid (at 20 mM) upon uptake of the first is expressed as per cent inhibition in relation to uptake of substrate in paired slices incubated without the second amino acid in the medium. All values are mean of triplicate observations. Inhibition was significant ($P < 0.01$ by Student's $t$ test) in all cases. Per cent inhibition was comparable when measurement of uptake included or excluded label lost as $^{14}CO_2$.

* Indicates significant *stimulation* of uptake.

Sarcosine competitively inhibits the uptake of glycine and L-proline (Table III) when the latter are present in the medium at concentrations low enough to assign most of their transport to the respective substrate-specific, low $K_m$, low capacity systems which accommodate their uptake in kidney (6). On the other hand, the uptake of 2 mM glycine was actually stimulated by external sarcosine, whereas the uptake of 2 mM L-proline was still inhibited. At this concentration the renal uptakes of glycine and L-proline occur predominantly on a high capacity system shared by proline and glycine (6).

TABLE IV

*Effect of Preloading with Sarcosine upon Uptake of Amino Acids in Rat Kidney Cortex Slices*

| Substrate uptake medium | Concn of sarcosine in preload medium* | Isotope distribution ratio at 10 min‡ Control | Isotope distribution ratio at 10 min‡ Preloaded |
|---|---|---|---|
| *mM* | *mM* | | |
| Sarcosine 0.1 | 1 | 1.80 | 1.52 |
| Sarcosine 1.1 | 10 | 1.22 | *1.34*§ |
| Glycine 0.012 | 20 | 1.75 | 1.17 |
| Glycine 2.0 | 20 | 1.30 | *1.95*§ |
| L-Proline 0.011 | 20 | 0.36 | *0.42*§ |
| L-Proline 2.0 | 20 | 0.38 | 0.28 |

* Slices were preloaded during 40 min incubation in buffer containing unlabeled sarcosine, followed by removal, rinsing, and blotting. The slices were then transferred to fresh medium containing $^{14}C$-labeled substrate.

‡ Uptake of substrate was measured at 10 min and compared with uptake by control slices carried through preincubation in the absence of sarcosine. Values are the mean of at least triplicate observations.

§ Indicates significant stimulation ($P < 0.02$).

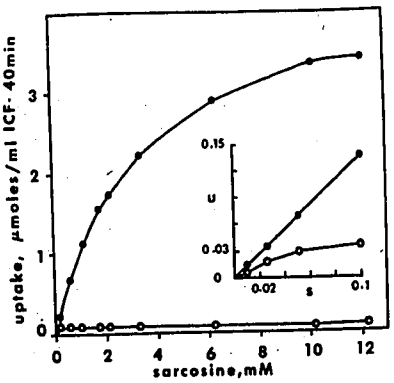


FIGURE 7 Michaelis plots of sarcosine uptake assigned to low $K_m$ (○) and high $K_m$ (●) systems in rat kidney; the $K_m$ values are 0.1 mM and 3 mM, respectively. It is evident that the major fraction of sarcosine uptake is achieved on a high $K_m$ system, at all external concentrations of the substrate.

*Sarcosine uptake and exchange with other amino acids.* The likelihood that sarcosine uptake occurs on more than one system and thus might enter by one system and exchange on another, was investigated further by methods described previously (6). In slices preloaded with sarcosine, the uptake of 1.1 mM sarcosine, 2 mM glycine, and 0.01 mM L-proline was enhanced (Table IV). Uptake at other concentrations of these external substances was not stimulated by high concentrations of internal sarcosine.

*Renal metabolism of sarcosine; effect on transport kinetics in vitro.* We examined whether renal oxidation of sarcosine or lack thereof as in the blocked catabolic mutant, influenced its transport kinetics in that tissue. $^{14}CO_2$ from sarcosine was collected during incubations by the technique described earlier (6); counts appearing in $CO_2$ were then either included or omitted when calculating the net uptake rate. The $K_m$ for sarcosine binding by the transport system(s), with or without oxidation, was the same (Fig. 8).

## DISCUSSION

*Sarcosine metabolism.* The conversion of sarcosine to glycine is catalyzed by a mitochondrial oxidase system which has two components (19, 20). One is a soluble sarcosine dehydrogenase; the other is a particu-

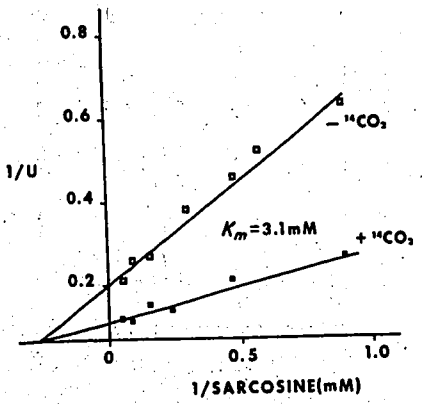


FIGURE 8 Effect of oxidation on uptake kinetics for sarcosine in rat kidney cortex slices. $^{14}C$ counts lost from slice in $CO_2$ were included (■) or excluded (□) from calculation of uptake (u) at various substrate concentrations (S). Results presented as Lineweaver-Burk plot. Oxidation of sarcosine has no effect on its affinity for the uptake system, since the $K_m$ value is not changed when the effect of oxidation on observed uptake is considered or neglected.

late electron transfer system. Sarcosine-specific dehydrogenase can be further fractionated to yield the dehydrogenase and a flavoprotein. Mitochondrial oxidation of the *N*-methyl group of sarcosine thus requires an electron transfer flavoprotein that accepts electrons from the substrate-specific flavoprotein dehydrogenase (20). A formaldehyde group is formed during sarcosine conversion to glycine, and by reaction with tetrahydrofolate, the intermediate $N^5$,$N^{10}$-methylenetetrahydrofolate is presumably formed (21). The 1-carbon fragment can be reutilized from the activated folic acid intermediate, to form the carbon of serine by condensation with glycine (22, 23).

The nature of the enzyme defect in human hypersarcosinemia is still unknown, but the weight of available evidence indicates a block in the conversion of sarcosine to glycine, presumably involving the substrate-specific dehydrogenase. Because this enzyme is neither active nor inducible in normal human fibroblasts or leukocytes as shown in the present and in previous studies (4, 13), it will not be possible to evaluate the enzymatic basis of the hypersarcosinemic trait further without recourse to organ biopsy. Sarcosine dehydrogenase activity in mammalian tissues is largely restricted to liver and kidney, as shown in the present work and in that of others (13).

The failure to ameliorate the hypersarcosinemic phenotype in the proband with pharmacologic doses of folic acid, suggests that the trait in this pedigree at least does not involve a reversible defect in the formation of "activated" formaldehyde from the specific dehydrogenase reaction. The potential responsiveness of mutant biochemical phenotypes to vitamin supplements is an important consideration in those hereditary amino-acidopathies where a vitamin is the precursor of one of the reactants in the enzymatic conversion of metabolites (24). The proband appeared to become more hypersarcosinemic after sarcosine loading while receiving folate, when compared with the response in the absence of folate. We believe this variation is not significant since the patient's plasma sarcosine concentration was quite variable anyway. However the possibility that folate in some way enhanced intestinal absorption of sarcosine in this patient should be considered.

*Loading studies: interpretation of phenotype.* Sarcosine disappearance from plasma should be delayed and plasma glycine should not change after a sarcosine load given to subjects with deficient conversion of sarcosine to glycine; this response was obtained in our proband. Brodehl and coworkers (3)[2] observed a similar response after comparable sarcosine loading in their proband. Sarcosine clearance from plasma was delayed in the patients studied by Gerritsen and Waisman (2) and by Scott et al. (4). In the latter study (4) glycine rose in the proband's plasma but not until the 4th hr after loading; this could have reflected conversion of sarcosine to glycine by bacteria in the intestinal lumen, rather than an endogenous response. The plasma glycine response after sarcosine loading was not reported by Gerritsen and Waisman (2).

The heterozygote for hypersarcosinemia should have only modest impairment of sarcosine clearance from plasma after loading, and one anticipates the initial plasma concentration of sarcosine to be normal under fasting conditions. We found this to be the case in both parents of our proband. The rise in their glycine after sarcosine loading was a response opposite to that obtained in normal subjects. The renal response to sarcosine loading did not account for observed changes in plasma glycine of parents and control subjects.

Sarcosine clearance from plasma and the concomitant glycine response provide a useful distinction between normal subjects, parents, and proband in the present sarcosinemic pedigree. Moreover, they appear to be more reliable indices of the presumed genotype than sarcosine excretion data, which, as shown in Table III, are poor indices, contrary to earlier conclusions (2). On the basis of the plasma amino acid response to sarcosine loading, we tentatively suggest that the sarcosine trait is autosomal recessive in this French-Canadian pedigree.

*Clinical significance of the sarcosinemic trait.* Hypersarcosinemia appears to be a benign condition. Our pro-

[2] Brodehl, J. 1970. Personal communication.

band had no disease which could be directly attributed to his hypersarcosinemia. The same can be said for the hypersarcosinemic sibling of the first reported proband (2) and for another reported proband (3). A larger series[1] apparently also indicates that no consistent disease state accompanies hypersarcosinemia, and thus, as with other aminoacidopathies such as hydroxyprolinemia, hyperprolinemia, and cystathioninemia, hypersarcosinemia appears to be a "nondisease" in medical terms.

The likelihood is great that the sarcosinemic trait in man will exhibit genetic heterogeneity. For this reason we must be cautious in assuming that sarcosine dehydrogenase activity is necessarily deficient in our proband or that he is exactly similar to others described in the literature. However, we believe our assumption about deficient renal sarcosine dehydrogenase activity to be reasonable, in view of the particular nature of amino acid reabsorption in the proband and considering how these observations may be related to what we have learned about sarcosine transport in kidney.

*Sarcosine transport.* Sarcosine is oxidized in mammalian kidney, but hypersarcosinemia, due to sarcosine dehydrogenase deficiency, offers an opportunity to study its renal transport in man independent of its metabolism. Renal transport of proline and of phenylalanine has been investigated to some extent in man under the equivalent conditions of the blocked catabolic phenotypes found in hyperprolinemia and phenylketonuria, respectively. In neither case did the hereditary impairment in metabolism of the amino acid appear to influence its uptake by kidney in vivo (8, 25, 26). In fact, should intracellular amino acid at high concentration exchange with intraluminal amino acid, it is possible that renal transport could be enhanced as may be the case in phenylketonuria (25). We have now shown that renal transport of sarcosine is similar in the subject with hereditary hypersarcosinemia and in normal subjects. We were able to confirm in vitro that metabolism of transported sarcosine plays no role in determining its binding kinetics to membrane carrier in kidney.

Sarcosine apparently does not have its own transport system. It is a nonessential amino acid concerned in only a limited repertoire of intracellular metabolism being synthesized only from an intracellular precursor (dimethylglycine). Although bound sarcosine has been identified in human glycopeptides (27), it is believed to achieve this form by N-methylation of peptide-linked glycine. Our studies indicate that sarcosine is transported in vivo primarily on the tubular transport system(s) used by proline, hydroxyproline, and glycine in human kidney (6, 8, 28). The kinetics of sarcosine uptake in vitro and the inability of proline or glycine transport to resist sarcosine inhibition at low or high concentrations, clearly reveal that more than one transport site serves its uptake in mammalian kidney. We have tentatively identified the systems used in sarcosine transport as the low $K_m$ system for proline, the low $K_m$ system for glycine, and the high $K_m$ system shared by both; this constellation of sites has been characterized in detail (6, 8, 28–30), using the same methods employed in the present investigation. The in vitro studies suggest that the principle carrier available for sarcosine entry into kidney is probably the high $K_m$, high capacity system shared by proline, hydroxyproline, and glycine. This corroborates earlier studies of sarcosine transport in hamster intestine (31).

The data which describe sarcosine transport in rat kidney in vitro are likely to be informative about the nature of sarcosine transport by human kidney in vivo, since it has already been shown that the principle system for sarcosine transport has many homologies in human and rat kidney (6). The different responses in urinary amino acid excretion obtained in proband and control subjects after sarcosine loading can be explained by applying these insights.

The sarcosine concentration in kidney should be unusually high after sarcosine loading in the sarcosinemic proband; under such conditions, sarcosine could exchange with glycine in vivo as it will in vitro. The ability of sarcosine to stimulate glycine uptake under certain conditions presumably arises because the first amino acid is allowed to enter by one system and exchange with the second amino acid on another (32). Glycine reabsorption could improve in the proband when the intracellular sarcosine pool is further expanded by loading. On the other hand, renal oxidation of sarcosine can occur in normal subjects thus preventing a sustained high concentration in kidney after loading. In this circumstance sarcosine can exert its inhibitory effect on glycine entry on the low $K_m$ system to an extent which would outweigh the opportunity for exchange on the high $K_m$ system.

*Significance of studies.* The foregoing information can be used to counsel future sarcosinemic probands in the likelihood that this autosomal recessive trait is harmless. The mechanism by which glycine alters its steady state in response to sarcosine loading awaits clarification. The further demonstration of parasitic transport wherein membrane systems serving "primary" substrates are used under certain circumstances by "secondary" susbstrates (in this case sarcosine) illustrates the value to be gained from designing pharmacologically active compounds which can be accommodated on directed transport systems in tissues.

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## REFERENCES

1. Glorieux, F. H., F. Mohyuddin, D. T. Whelan, and C. R. Scriver. 1970. Hypersarcosinemia: new observations. *Pediat. Res.* **4**: 450. (Abstr.)
2. Gerritsen, T., and H. Waisman. 1966. Hypersarcosinemia. An inborn error of metabolism. *N. Engl. J. Med.* **275**: 66.
3. Hagge, W., J. Brodehl, and K. Gellissen. 1967. Hypersarcosinemia. *Pediat. Res.* **1**: 409. (Abstr).
4. Scott, C. R., S. H. Clark, C. C. Teng, and K. R. Swedberg. 1970. Clinical and cellular studies of sarcosinemia. *J. Pediat.* **77**: 805.
5. Meister, A. 1965. Biochemistry of the amino acids. Academic Press, Inc., New York. 2nd edition. **2**: 658.
6. Mohyuddin, F., and C. R. Scriver. 1970. Amino acid transport in mammalian kidney: multiple systems for imino acids and glycine in rat kidney. *Amer. J. Physiol.* **219**: 1.
7. Scriver, C. R., and E. Davies. 1965. Endogenous renal clearance of free amino acids in pre-pubertal children. *Pediatrics.* **36**: 592.
8. Scriver, C. R., M. L. Efron, and I. A. Schafer. 1964. Renal tubular transport of proline, hydroxyproline and glycine in health and in familial hyperprolinemia. *J. Clin. Invest.* **43**: 374.
9. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30**: 1190.
10. Scriver, C. R., E. Davies, and P. Lamm. 1968. Accelerated selective short column chromatography of neutral and acidic amino acids on a Beckman-Spinco analyzer, modified for simultaneous analysis of two samples. *Clin. Biochem.* **1**: 179.
11. Hayflick, L. 1965. The limited "in vitro" lifetime of human diploid cell strains. *Exp. Cell Res.* **37**: 614.
12. Zucker, R. M., and B. Cassen. 1969. The separation of normal human leukocytes by density and classification by size. *Blood.* **34**: 591.
13. Rehberg, M. L., and T. Gerritsen. 1968. Sarcosine metabolism in the rat. *Arch. Biochem. Biophys.* **127**: 661.
14. Blass, J. P., J. Avigan, and B. W. Uhlendorf. 1970. A defect in pyruvate decarboxylase in a child with an intermittent movement disorder. *J. Clin. Invest.* **49**: 423.
15. Akedo, H., and H. N. Christensen. 1962. Nature of insulin action on amino acid uptake by the isolated diaphragm. *J. Biol. Chem.* **237**: 118.
16. Tenenhouse, A., and J. M. Quastel. 1960. Amino acid accumulation in Ehrlich ascites carcinoma cells. *Can. J. Biochem.* **38**: 1311.
17. Neal, J. 1971. Analysis of non-linear Lineweaver-Burk plots. *J. Theor. Biol.* In press.
18. Scriver, C. R., and F. Mohyuddin. 1968. Amino acid transport in kidney: heterogeneity of alpha aminoisobutyric uptake. *J. Biol. Chem.* **243**: 3207.
19. Frisell, W. R., and C. G. Mackenzie. 1962. Separation and purification of sarcosine dehydrogenase and dimethyl-glycine dehydrogenase. *J. Biol. Chem.* **237**: 94.
20. Frisell, W. R., J. R. Cronin, and C. G. Mackenzie. 1962. Coupled flavoenzymes in mitochondrial oxidation of N-methyl groups. Purification of the electron transfer flavoprotein. *J. Biol. Chem.* **237**: 2975.
21. Mackenzie, C. G. 1955. Conversion of N-methyl glycines to active formaldehyde and serine. *In* A Symposium on Amino Acid Metabolism, Sponsored by the McCallum-Pratt Institute of the Johns Hopkins University. W. D. McElroy and B. Glass, editors. The Johns Hopkins Press, Baltimore. 684.
22. Blakley, R. L. 1957. The interconversion of serine and glycine: some further properties of the enzyme system. *Biochem. J.* **65**: 342.
23. Mackenzie, C. G., and W. R. Frisell. 1958. The metabolism of dimethylglycine by liver mitochondria. *J. Biol. Chem.* **232**: 417.
24. Rosenberg, L. E. 1969. Inherited amino acidopathies demonstrating vitamin dependency. *N. Engl. J. Med.* **281**: 145.
25. Brodehl, J., K. Gellisen, and W. P. Kass. 1970. The renal transport of amino acids in untreated infants with phenylketonuria. *Acta Paediat. Scand.* **59**: 241.
26. Scriver, C. R. 1969. The use of human genetic variation to study membrane transport of amino acids in kidney. *Amer. J. Dis. Child.* **117**: 4.
27. Cherian, M. G., and A. N. Radhakrishnan. 1965. A new glycopeptide containing hydroxyproline and sarcosine in human urine. *Biochim. Biophys. Acta.* **101**: 241.
28. Scriver, C. R. 1968. Renal tubular transport of proline, hydroxyproline, and glycine. III. Genetic basis for more than one mode of transport in human kidney. *J. Clin. Invest.* **47**: 823.
29. Wilson, O. H., and C. R. Scriver. 1967. Specificity of transport of neutral and basic amino acids in rat kidney. *Amer. J. Physiol.* **213**: 185.
30. Baerlocher, K., C. R. Scriver, and F. Mohyuddin. 1970. Ontogeny of iminoglycine transport in mammalian kidney. *Proc. Nat. Acad. Sci. U. S. A.* **65**: 1009.
31. Hagihira, H., T. H. Wilson, and E. C. C. Lin. 1962. Intestinal transport of certain N-substituted amino acids. *Amer. J. Physiol.* **203**: 637.
32. Jacquez, J. A. 1967. Competitive stimulation: further evidence for two carriers in the transport of neutral amino acids. *Biochim. Biophys. Acta.* **135**: 751.

# X-LINKED HYPOPHSOPHATEMIA

## A. The nature of the disease.

### i) Clinical phenotype:

- Hypophosphatemia

The only constant abnormality in individuals with X-linked hypophosphatemia is a low fasting serum concentration of inorganic phosphate. This is the marker for the condition and it reveals the mode of inheritance of the mutation. In designating a person as hypophosphatemic, allowance must be made for the normal changes in serum phosphorus with age and for difference between the sexes. Since there is also a diurnal variation and a rapid influence of the oral intake by the diet, phosphate plasma concentration has always been determined in our patients on a morning blood sample after an overnight fast. The result was then compared to the normal values published by Greenberg et al[1] and declared abnormal when falling lower than 2.5 S.D below the normal mean for age and sexe.

The postnatal age at which hypophosphatemia occurs has been variably reported. Harrison et al[2] observed normal levels up to the age of 6 months in children who then developed the complete phenotype of X-linked hypophosphatemic rickets. The authors related this finding to the relatively low glomerular filtration rate observed during the first few months of life. Stickler[3], on the other hand, found consistently low serum phosphorus values from birth. In our series, only one patient (E.M. see

Section VI) was diagnosed relatively early in life, at 8 months of age; his plasma phosphorus,at that time, was 3.3 mg% which is well below the normal range.

The hypophosphatemia is accompanied by decreased net tubular reabsorption of phosphate[4] which seems to be the main cause of the low plasma phosphate steady-state. The phosphate transport defect, however, may not be confined to renal tubular epithelium. Intestinal absorption of phosphate may also be impaired, but much additional work will be required to evaluate intestinal transport of phosphate in the trait and to assesswhether other tissues have similar mechanisms for phosphate transport that could be affected by the hypophosphatemic trait. Condon et al[5] have suggested that there is an intestinal transport defect for phosphorus in hypophosphatemic subjects. However, their study is made on an heterogeneous group of patients and it seems erroneous to assess that a low plasma phosphorus,60 minutes after an oral load, reflects solely a defective intestinal absorption. The role of the unmineralized bone and of the renal transport defect in modulating the plasma phosphate steady-state cannot be ignored.

Clinical findings

Bone disease is present in all male patients. The female individuals are affected at a variable degree, their phenotype being anywhere between the normal and the male one. Usually the disease is recognized first by the presence of leg deformities starting at the age of weight-

bearing. Muscular weakness and atony, frequent in vitamin-D deficient rickets, are not usually observed. Frontal and occipital bossing and a rachitic rosary are often present. Abnormalities of the spinal column or pelvis usually found in vitamin D deficiency, are frequently absent.

The radiologic findings are the same as those seen in rickets from other causes. Bowing of the lower extremities is the most evident change.

Growth Failure

While it is within normal limits at birth, in most cases the height is below the third percentile after two years of age. In the subsequent years of childhood, the growth retardation becomes more important, particularly in the male. McNair and Stickler[6]. noted that the shortness of stature is predominantly segmentary and primarily limited to the lower extremities, during childhood. This is apparent because of the changes in body proportions during that period of life. From infancy to pre-adolescent period, the lower segment increases from 30% to 50% of total height. It is therefore not surprising that the effects of a general growth failure will be more striking in the lower limbs. The defect is not related to a growth hormone deficiency[7]. A tentative explanation for growth failure, involving a defect in oxygen transport secondary to the low phosphate concentration in serum is discussed in Section VI.

ii) Inheritance:

It has been clearly demonstrated that in familial hypophosphatemia the degree of manifestation of the inherited trait could vary from individuals who have severe to mild bone disease and hypophosphatemia to those in whom only hypophosphatemia can be demonstrated, when the latter is taken as a discriminant for identifying the genotype[4,8]. Transmission can best be explained by the presence of a single mutant gene on the X-chromosome. It is dominant since it is transmitted from generation to generation without interruption and there is absence of male-to-male transmission. Therefore, an affected female is heterozygous, whereas an affected male is hemizygous. The fact that the male patients show full expression of the disease, while females are variably affected, can best be explained by the inactivation of one of the X-chromosome in the latter[9]. This will result in phenotypic mosaicism and account for the variation in degree of manifestation encountered in the heterozygote females.

Not all cases of familial hypophosphatemic rickets are clearly inherited. Sporadic cases are reported that could be due to phenocopy, a recessive trait or new mutations.

No estimate of genetic fitness has been reported. The frequency of the trait is broadly estimated at $0.5 \times 10^{-4}$[8].

iii) Possible mechanisms of pathogenesis:

The controversy over the pathogenesis of familial

hypophosphatemic rickets has centered about the primary or secondary nature of the reduced tubular maximum capacity for phosphate reabsorption and the resultant hypophosphatemia, the one (and sometimes only) abnormality present in all genetically affected persons. The first tentative explanation was proposed by Albright et al[10] who suggested that the primary defect is in intestinal calcium absorption. The defect leads to a decreased concentration of calcium in the extracellular fluid which results in secondary hyperparathyroidism. This factor is known to impair tubular reabsorption of phosphorus. Hypophosphatemia is the final step and the cause of the bone disease.

As an alternative, Dent[11] and Fanconi and Girardet[12] suggested that the primary abnormality is a specific, genetically determined defect in the renal tubular transport of phosphorus.

More recently, an abnormality of vitamin D metabolism has been proposed[13] on the basis of a quantitative decrease in the conversion of vitamin $D_3$ to 25-hydroxycholecalciferol[4]. However clinical trials with 25-HCC[14] have failed to correct the abnormal phenotype.

The possibility that 25-HCC or one of its derivatives acts directly upon the kidney tubule has been lately suggested[15,16]. However, the incriminated factor has still to be isolated and made available for clinical use.

B. The absence of secondary hyperparathyroidism in X-linked hypophosphatemia.

i) Any pathogenic hypothesis that involves a defect in vitamin D dependent calcium metabolism has as an obligate corollary, secondary hyperparathyroidism to explain the renal loss of phosphorus. Therefore clear evidence for the presence or absence of hyperparathyroidism was an important initial step in our attempt to clarify the mechanism of the X-linked hypophosphatemic rickets.

Histologic examinations of the parathyroid glands have given variable results[4]. Hyperplastic and normal glands have both been described in the trait.

The decrease in tubular reabsorption of phosphorus was in the range observed in normal individuals injected with parathyroid extract[17]. Furthermore the well known beneficial effect of calcium infusion on tubular transport of phosphorus in vitamin D resistant rickets has been interpreted as evidence for suppression of the parathyroid hormone hyperactivity[18]. This latter point will be discussed in a further section.

The development by Arnaud and colleagues of a new radioimmuno assay for the measurement of human parathyroid hormone in serum provided the desired opportunity to study our group of patients.

Data on the status of parathyroid gland activity in X-linked hypophosphatemia are presented in the following manuscript (Science: 173, 845-847, 1971)

Note 1: Since the publication of this work, a new patient (a 10 year old girl) with X-linked hypophosphatemic rickets, never treated, has been included in the group. Her IPTH value before any treatment was 28 µl eq/ml (normal: <40 µl eq/ml).

2: The authorship in the published paper is alphabetical in accordance with our laboratory policy for collaborative work with another group. The candidate is in fact the senior author of the paper.

# Serum Parathyroid Hormone in X-Linked Hypophosphatemia

Abstract. *Serum immunoreactive parathyroid hormone (IPTH) is normal in patients with X-linked hypophosphatemic rickets who are not treated with phosphate salts. Phosphate raises IPTH in these patients. Endogenous IPTH does not influence the existing defect in tubular reabsorption of phosphate in male patients.*

X-linked hypophosphatemic rickets is a dominant disease in which a low concentration of orthophosphate in plasma is the most constant index of the phenotype (*1*). In males, the mutant allele also causes bone disease, while in carrier females it is variably expressed, some showing only hypophosphatemia, while others have bone disease as well. Impairment of renal tubular reabsorption of phosphate has long been recognized as another important phenotypic feature of this trait.

Two opposing views have evolved concerning the pathogenesis of X-linked hypophosphatemia. One opinion favors a primary disorder of intestinal calcium absorption due to impaired endogenous conversion of vitamin D (*2*) to normal biologically active polar derivatives (*3*). This would result in secondary hyperparathyroidism, renal loss of phosphate, hypophosphatemia, and bone disease as an ultimate consequence.

The well-known suppressive effect of intravenous calcium infusion upon the hyperphosphaturia (*4*) appeared to support this hypothesis. The failure of X-linked hypophosphatemia to respond unambiguously to treatment with 25-hydroxycholecalciferol (*5*) is against defective hepatic biosynthesis of vitamin D metabolites; a failure to synthesize the derivative active in the intestine (*3*) has not yet been examined.

The other opinion favors a primary disorder of phosphate transport in the renal tubule (*6*), and perhaps also at the general cellular level (*7*). The ability to heal the bone disease more effectively with phosphate administration alone (*8*), or with phosphate and modest supplements of vitamin D (*9*), than with very large amounts of vitamin D alone in either its precursor or hydroxylated forms (*5*) emphasizes the apparent primacy of the phosphate leak in the pathogenesis of the complete phenotype. If the calcium-transport hypothesis, including its essential codicil about secondary hyperparathyroidism, could be eliminated by direct measurements of circulating parathyroid hormone, investigative efforts could be focused on the phosphate transport hypothesis. The development of a new radioimmunoassay for the measurement of human parathyroid hormone in serum (*10*) provided an opportunity to study this problem.

Serum was obtained from patients with familial hypophosphatemic rickets meeting the criteria for the X-linked trait (*11*). Untreated patients had serum immunoreactive parathyroid hormone concentrations (IPTH) within or close to the normal range (Fig. 1). Patients who had been receiving vitamin D but who still retained their hypophosphatemia, hyperphosphaturia, and active bone disease, also had normal serum IPTH. Only those patients receiving large quantities of therapeutic phosphate salts by mouth had increased serum IPTH. Three patients on the phosphate regimen developed roentgenographic and biochemical signs of hyperparathyroidism; reduction of the phosphate intake and supplementation of their diet with calcium, as calcium gluconate (1 to 3 g/day), and vitamin $D_2$ (100,000 unit/day) for at least 4 weeks suppressed serum IPTH to normal or near normal in these patients (Table 1).

Tubular reabsorption of phosphate [$\mu$mole/100 ml glomerular filtration rate (GFR)] was measured (*12*) on many occasions in four male probands. The results were plotted in relation to the serum IPTH at the same time (Fig. 2). Tubular reabsorption of phosphate was greatly impaired [mean for group = 36.8 $\mu$mole/100 ml GFR, normal, > 90 $\mu$mole/100 ml GFR (*1*)]. The serum phosphorus concentration

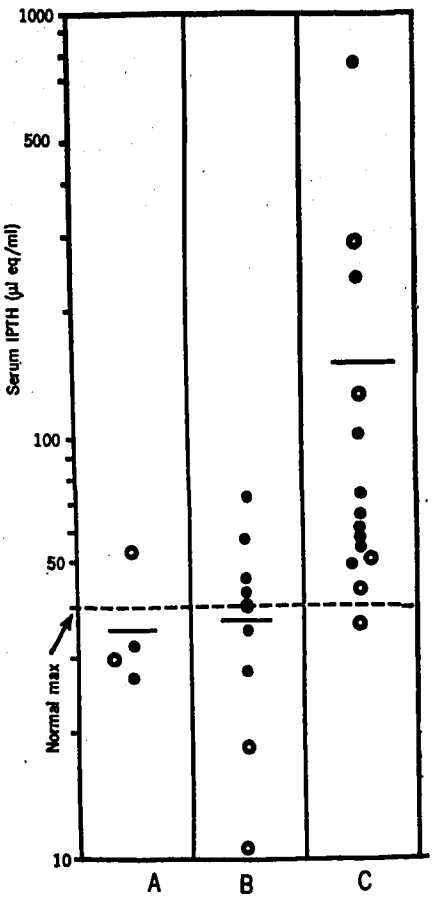


Fig. 1. Serum IPTH in patients with X-linked hypophosphatemic rickets. Column A, no treatment; column B, treatment with large doses of vitamin $D_2$ alone (about 100,000 unit/day); column C, treatment with a phosphate supplement by mouth (3 g/day, as orthophosphate) and vitamin $D_2$ (up to 100,000 unit/day). Open circles; female patients; closed circles; male patients. All patients have bone disease.

Table 1. Response of serum IPTH to change in dietary phosphate in patients with X-linked hypophosphatemic rickets and acquired secondary hyperparathyroidism.

| Subject | Sex | Dietary regimen: Phosphate (3 g/day); vitamin $D_2$ (< 25,000 unit/day) | | | Dietary regimen: Reduced phosphate (1 g/day); vitamin $D_2$ (100,000 unit/day) | | |
|---|---|---|---|---|---|---|---|
| | | Serum values | | IPTH ($\mu$l eq/ml) | Serum values | | IPTH ($\mu$l eq/ml) |
| | | P* | Ca* | | P* | Ca* | |
| M.C. | F | 5.4 | 8.9 | 290 | 3.9 | 10.4 | 40 |
| L.A. | M | 5.0 | 9.3 | 100 | 3.8 | 11.4 | 67 |
| E.M. | M | 4.4 | 7.4 | 750 | 3.9 | 10.6 | 43 |
| | Mean | 4.9 | 8.5 | 380 | 3.9 | 10.8 | 50 |

* Values in milligrams per 100 milliliters.

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was $3.3 \pm 1.4$ mg/100 ml (mean and S.D.) for the group at the time these determinations were done and when the patients were taking varying amounts of phosphate by mouth. There was no relationship between tubular reabsorption of phosphate and serum IPTH, indicating that the defect in phosphate transport in male patients is not influenced by endogenous serum IPTH.

We believe that this is the first unambiguous evidence of normal serum IPTH activity in patients with untreated X-linked hypophosphatemia. Serum IPTH is increased only when these patients are treated with phosphate supplements. The mechanism involved in producing this increase in serum IPTH is probably the negative feedback control of parathyroid hormone secretion by the concentration of calcium in serum (*13*). Serum calcium decreased in the patients whose serum phosphorus had been increased by daily oral phosphate administration (Table 1).

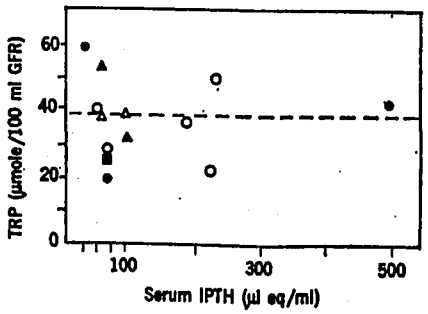


Fig. 2. Relationship of TRP [tubular reabsorption of phosphate, μmole/100 ml glomerular filtration rate (GFR)] to endogenous serum IPTH in four male probands with X-linked hypophosphatemic rickets, at serum phosphate values for group of $3.3 \pm 1.4$ mg/100 ml (mean $\pm$ S.D.). The dotted line indicates the regression of TRP on serum IPTH; the latter does not influence TRP in these patients. Maximum normal serum IPTH, 40 μl eq/ml.

A phosphate transport defect is present in male patients with X-linked hypophosphatemia even when their serum IPTH is normal (Fig. 2). The insensitivity of residual phosphate transport to endogenous IPTH suggests to us that the renal tubule of patients with X-linked hypophosphatemia contains a second parathyroid hormone–insensitive phosphate-transport system. We also suggest that this component of phosphate transport in kidney is responsive directly to calcium, perhaps in a manner analogous to that documented in canine kidney by Lavender and Pullman (*14*). This would account for the well-known effect of hypercalcemia on tubular reabsorption of phosphate in X-linked hypophosphatemia (*4*).

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### References and Notes

1. R. Winters, J. Graham, T. F. Williams, V. McFalls, C. Burnett, *Medicine* **37**, 97 (1958); T. F. Williams, R. W. Winters, C. H. Burnett, in *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, D. S. Fredericksen, Eds. (McGraw Hill, New York, ed. 2, 1966), p. 1179.
2. L. V. Avioli, T. F. Williams, J. Lund, H. F. DeLuca, *J. Clin. Invest.* **46**, 1907 (1967).
3. E. B. Olson and H. F. DeLuca, *Science* **165**, 405 (1969); D. R. Fraser and E. Kodicek, *Nature* **228**, 764 (1970); M. F. Holick, H. K. Schnoes, H. F. DeLuca, *Proc. Nat. Acad. Sci. U.S.* **68**, 803 (1971).
4. D. Fraser, J. Leeming, E. Cerwenka, *Helv. Paediatr. Acta* **14**, 497 (1959); W. Falls, N. Carter, R. Rector, D. Seldin, *Ann. Intern. Med.* **68**, 553 (1968).
5. H. Earp, R. Ney, M. Gittelman, R. Richman, H. DeLuca, *N. Engl. J. Med.* **283**, 627 (1970).
6. B. R. Robertsen, R. C. Harris, D. J. McClure, *Am. J. Dis. Child.* **64**, 948 (1942); C. E. Dent, *J. Bone Jt. Surg.* **34B**, 266 (1952); G. Fanconi and R. Girardot, *Helv. Paediatr. Acta* **7**, 14 (1952); B. Frame and R. W. Smith, Jr., *Am. J. Med.* **25**, 771 (1958); R. W. Winters and J. B. Graham, *Pediatrics* **25**, 932 (1960).
7. J. Rey and J. Frezal, *Rev. Fr. Etud. Clin. Biol.* **11**, 771 (1966).
8. D. Fraser, D. Geiger, J. Munn, P. Slater, R. Jahn, E. Liu, *Am. J. Dis. Child.* **96**, 460 (1958); F. Glorieux, C. Arnaud, C. Clow, H. Goldman, T. Reade, C. R. Scriver, *Clin. Res.* **18**, 726 (1970).
9. C. West, J. Blanton, F. Silverman, N. Holland, *J. Pediatr.* **64**, 469 (1964); H. Harrison, F. Lifshitz, A. D. Johnson, *Am. J. Dis. Child.* **112**, 290 (1960); C. Nagant de Deuxchaisnes and S. Krane, *Am. J. Med.* **43**, 508 (1967).
10. C. Arnaud, H. Tsao, T. Littledyke, *J. Clin. Invest.* **50**, 21 (1971).
11. The criteria for diagnosis of X-linked hypophosphatemia were: proven hypophosphatemia for age on a morning blood sample after overnight fast; absence of hypocalcemia and hyperaminoaciduria in the untreated state; no male-to-male transmission; female clinical phenotype not more severe than male in same pedigree.
12. Tubular reabsorption of phosphate (TRP) was measured after an overnight fast. A timed urine collection lasting about 180 minutes was obtained, and venous blood was obtained at the end of the period. Phosphorus and creatinine determinations were made and the TRP value was calculated. TRP was also determined with inulin clearance rates to monitor GFR on several occasions. Inulin and creatinine clearances were comparable in the same subject.
13. E. Reiss, J. Canterbury, M. Bercovitz, E. Kaplan, *J. Clin. Invest.* **49**, 2146 (1970).
14. A. R. Lavender and T. N. Pullman, *Am. J. Physiol.* **205**, 1025 (1963).
15. Supported by grants from the Medical Research Council of Canada (MT-1085), the Department of National Health and Welfare (DPG 647-7-643), and NIH (NIH-AM-12302). F.G. is a national Genetics Foundation trainee in genetics. We thank Mrs. Terry Reade, Mr. Peter Lamm, and Dr. H. Goldman of the deBelle Laboratory, and Miss H. S. Tsao, Miss Judith Hess, Miss Karen Laakso, and Miss Julianna Bishoff of the Mayo Clinic for their excellent technical assistance. This is publication No. 231 from the McGill University–Montreal Children's Hospital Research Institute. Send reprint requests to C.S.

25 March 1971

ii) One of our patients (D.R.), because of the particular course of his disease, has been extensively studied. In particular, attention was given to the relationship between tubular reabsorption of phosphorus and the activity of the parathyroid glands. After several months of an aggressive phosphate supplementation, he developed a secondary hyperparathyroidism with bone lesions. Ionized calcium rose to 75% of total calcium (normal: <55%). The persistence of secondary hyperparathyroidism led to a state of autonomous (tertiary) hyperparathyroidism. Therefore a 7/8 parathyroidectomy was performed and 400 mg of hyperplastic glands were removed. Tubular transport of phosphorus was assessed at several occasions. The findings are summarized on Figure V-1. In the upper part of the graph, the changing status of the parathyroid activity and the corresponding IPTH values are represented (IPTH expressed in nanograms/ml, normal <4). The middle square depicts severe hyperparathyroidism. In the middle horizontal series, data for tubular reabsorption of phosphate are presented (in percent of the filtered load). A defect in phosphate reabsorption is clearly present at all stages of hyperparathyroid hormone activity which was not corrected by surgical removal of the hyperplastic glands. The lower part of the graph relates the tubular reabsorption of phosphorus to its plasma concentration. Appropriate symbols represent the different stages of parathyroid activity. It was noted that reabsorption varied inversly with plasma concentration. This indicates that the

phosphate transport system present in this hemizygote for X-linked hypophosphatemic rickets is saturated at all concentrations of the filtered load. The TRP value was therefore equal to the TmP and calculated to be about 45 μmoles/100 ml GF in every occasion. This observation,in addition to the normal values of IPTH in untreated patients, provided a basis upon which to study more closely the characteristics of phosphate transport in the X-linked hypophosphatemic mutants.

FIGURE V - 1

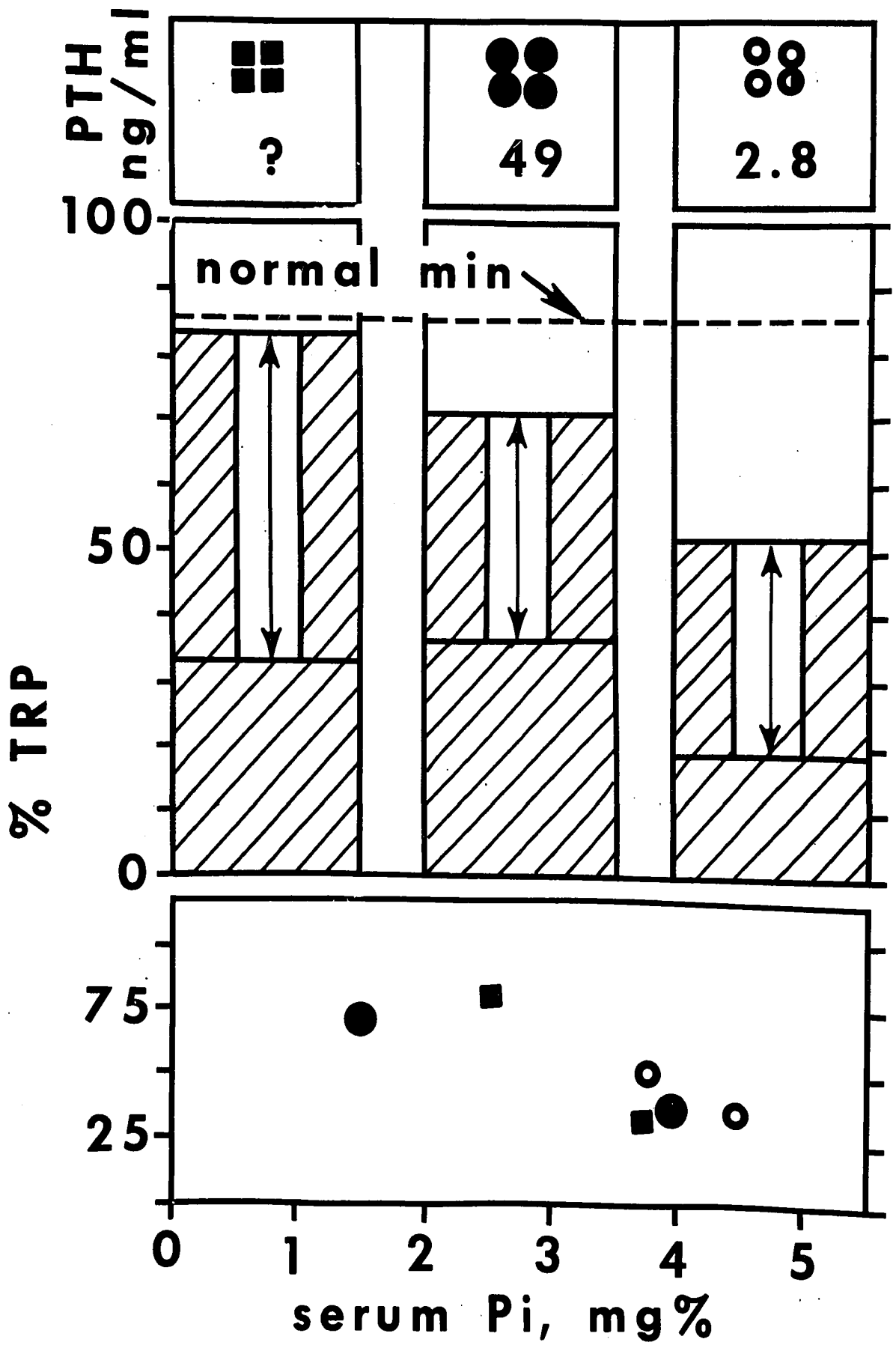
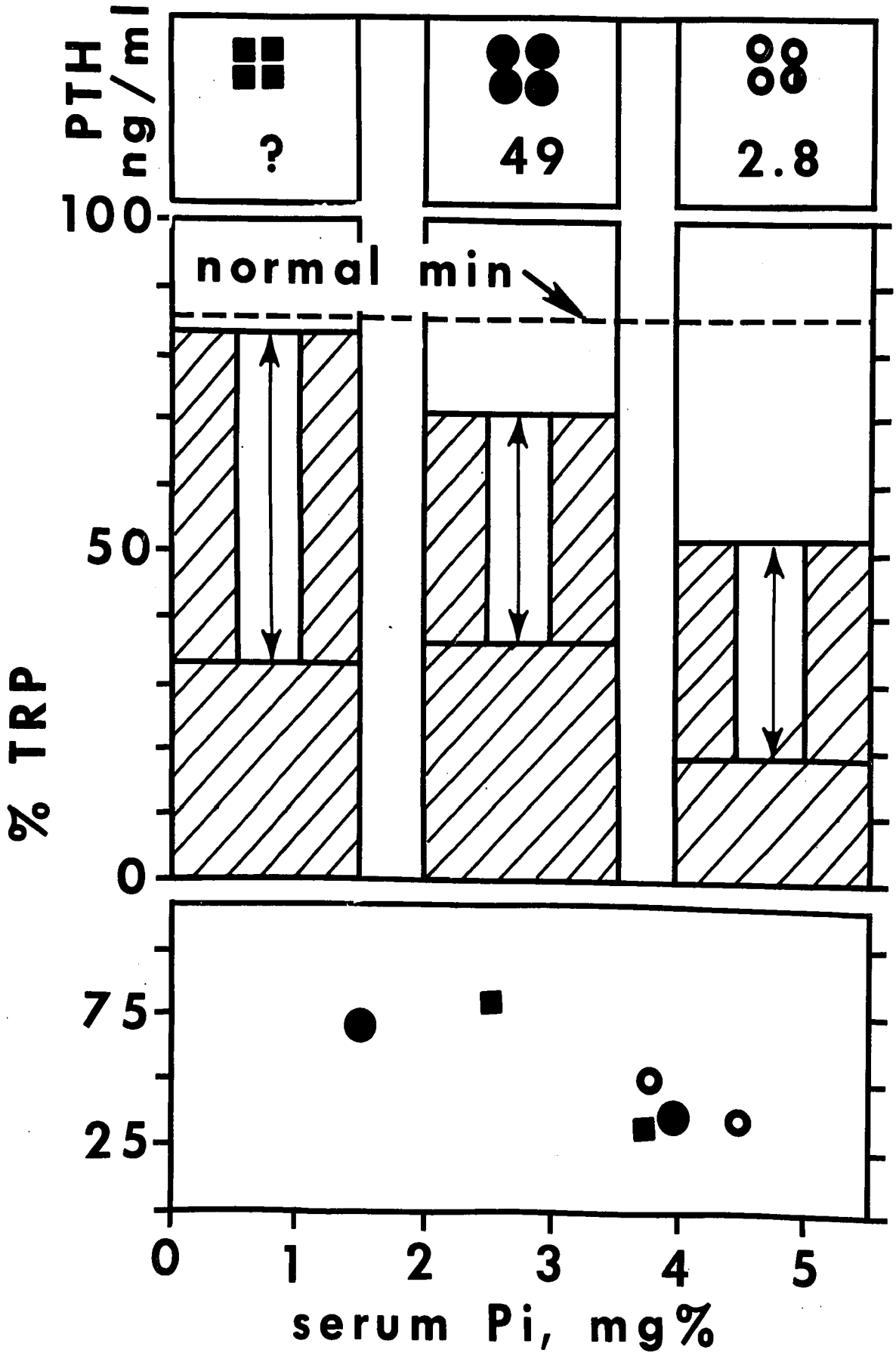


FIGURE V - 1



C. The phosphate transport defect.

The findings published in the first Science paper implicate a specific phosphate transport defect in the pathogenesis of X-linked hypophosphatemic rickets. In subsequent work, the particular inheritance of the trait allowed us to separate the patients into two groups: mutant hemizygotes and heterozygotes.

Evidence is given in the second Science paper that phosphate transport in human kidney normally involves at least two components, one of which is completely inactivated in the mutant hemizygote and variably so in the heterozygote. The type of phosphate transport retained in the hemizygote is not influenced by an increase in endogenous PTH or by infusion of bovine PTH. The role of calcium will be discussed in Section V-D. The interpretation of these data utilizes the Lyon hypothesis[9] which proposes the random inactivation of one X-chromosome early in the development in the female. We are suggesting that an X-linked gene is responsible for regulation of phosphate transport by kidney. The results of these studies are presented in the following paper (Science 175, 997-1000, 1972).

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SCIENCE

# Loss of a Parathyroid Hormone–Sensitive Component of Phosphate Transport in X-Linked Hypophosphatemia

Francis Glorieux and Charles R. Scriver

# Loss of a Parathyroid Hormone–Sensitive Component of Phosphate Transport in X-Linked Hypophosphatemia

Abstract. *Mutant hemizygotes with X-linked hypophosphatemia lack a parathyroid hormone–sensitive component of inorganic phosphate transport in kidney; female heterozygotes retain a variable proportion of this type of transport. The residual mechanism for reabsorption in affected males allows inorganic phosphate efflux from the kidney to urine so that net "secretion" is sometimes observed; the latter is directly proportional to the serum concentration of inorganic phosphate. Calcium acts on the kidney tubule to enhance net reabsorption by this component of inorganic phosphate transport.*

The primary defect of X-linked hypophosphatemia has eluded clarification since the first descriptions of this form of vitamin D–resistant rickets (*1*, *2*). Because of the impaired clinical responsiveness to vitamin D, and because of the discovery that intravenous calcium infusion could suppress the elevated renal clearance of inorganic phosphate ($P_i$) which accompanies hypophosphatemia (*2*, *3*), attention was originally focused on vitamin D–dependent mineral metabolism in this trait. The primary defect was thought to involve impairment of calcium absorption in the intestine, or abnormal vitamin D metabolism, and secondary hyperparathyroidism was invoked as the basis for the renal loss of $P_i$ (*2*, *4*). These hypotheses are improbable because untreated patients with X-linked hypophosphatemia have normal concentrations of parathyroid hormone (PTH) in the blood (*5*).

An alternate hypothesis emphasizes a primary disturbance of $P_i$ transport in kidney, and perhaps also in other tissues (*2*, *6*). Hypophosphatemia is the single constant phenotypic trait in carriers of this X-linked mutant allele (*2*), suggesting that the allele primarily influences $P_i$ metabolism. Replacement of $P_i$ is particularly effective in correcting the mutant clinical phenotype (*7*), as would be expected if $P_i$ metabolism is mainly affected in the disease.

We present evidence that the defect in X-linked hypophosphatemia concerns a PTH-sensitive component of $P_i$ transport that is responsible for about two-thirds of the total net reabsorption of $P_i$ in human kidney; this component is partially absent in female patients and completely absent in male patients. A residual component of $P_i$ transport, capable of being saturated, is retained in male patients. This permits $P_i$ flux from tubular lumen to plasma and also allows a flux in the reverse direction. Although this transport is insensitive to PTH it can be modulated by calcium ion. The net reabsorption of $P_i$ is enhanced immediately upon intravenous infusion of calcium.

Tubular transport of $P_i$ is saturable in normal human subjects (*8*). We examined the tubular reabsorption rate [$TRP_i$, micromoles per 100 ml of glomerular filtrate (GF)] and the maximum reabsorption rate ($TmP_i$, micromoles per 100 ml of GF, at saturation) at various concentrations of serum $P_i$ (*9*) in six males (all children) and nine females (three children, six adults) with X-linked hypophosphatemia. Subjects with bone disease (six male and three female children) were on treatment regimens with $P_i$ (1 to 3 g/day) and vitamin $D_2$ (10,000 to 50,000 unit/day) prior to investigation; each patient had a normal glomerular filtration rate (GFR).

The tubular reabsorption rate is lower in mutant hemizygotes than in female heterozygotes, and below the normal mean in all patients (Fig. 1); this finding confirms many earlier reports (*2*). The $TRP_i$ in female heterozygotes overlaps the normal range, as predicted by the Lyon hypothesis (which implies random inactivation of one X chromosome early in development) if $P_i$ transport in the kidney is closely coupled to the effect of an X-linked mutation. However, we could find no constant relation between the $TRP_i$ and the degree of bone disease in heterozygotes.

A saturable component of $P_i$ reabsorption is present in both hemizygotes and heterozygotes but is much lower in the former (Fig. 1), confirming previous reports (*2*, *10*). There is no unusual "splay" in the graph of $P_i$ reabsorption, indicating no change in binding of $P_i$ by the available transport system. The residual $P_i$ transport, in mutant hemizygotes, is virtually saturated even at endogenous concentrations of plasma phosphate. This suggests the presence of more than one form of $P_i$ transport, the residual form being unaffected by the X-linked mutation.

The renal excretion of $P_i$ in mutant hemizygotes was often greater than its filtered load; this was observed over a threefold range of $P_i$ concentration in serum (lower part of Fig. 1). These observations were monitored by inulin clearance, and the rate of net tubular secretion was found to be directly proportional to the $P_i$ concentration in serum. When net reabsorption of $P_i$ occurred, the serum calcium concentration in these subjects was $9.9 \pm 0.9$ mg/100 ml (mean ± standard deviation, 27 samples) and when there was net secretion of $P_i$, it was $9.9 \pm 0.4$ mg/100 ml (14 determinations).

We examined the effect of bovine PTH (*11*) on the endogenous $P_i$ ex-

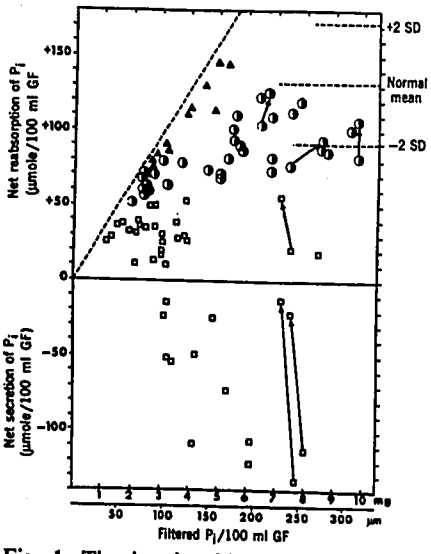


Fig. 1. The in vivo kinetics of net reabsorption of inorganic phosphate ($P_i$) by the kidney of normal subjects and these with X-linked hypophosphatemia. The substrate concentration ($P_i$) is varied in the glomerular filtrate by an intravenous infusion method (*8*). Reabsorption rate is calculated as described (*9*). Mutant hemizygotes are represented by squares, heterozygotes by half-filled circles and normal subjects by triangles. The mean ± 2 standard deviations (S.D.) for the normal maximal rate of tubular reabsorption ($TMP_i$) in man (*2*) is shown. The interrupted diagonal line (upper graph) indicates complete reabsorption of filtered phosphate; points falling below this line indicate incomplete net tubular reabsorption of phosphate. The net reabsorption rate ($TRP_i$), the difference between filtered and excreted $P_i$, is plotted upward as a positive value when the excreted $P_i$ is less than the filtered $P_i$. Negative reabsorption (net secretion), found in several hemizygotes (excreted $P_i >$ filtered $P_i$), is plotted downward. Arrows indicate the change in $P_i$ reabsorption 30 to 60 minutes after intravenous infusion of calcium (4 mg/kg) over a 15-minute period; this dose was sufficient to raise total calcium in serum by 2 mg/100 ml. A single patient is represented by the two studies of the effect of calcium on

cretion in normal, heterozygous, and mutant hemizygous phenotypes. Because of prior phosphate therapy, the endogenous serum immunoreactive PTH (*12*) (IPTH) was above normal in one male and one female patient before bovine PTH infusion (*5*); the concentration was normal in the remaining subjects. Renal clearance of $P_i$ relative to creatinine increased sharply in the four normal subjects after infusion of bovine PTH (Fig. 2). The GFR was not changed significantly by this procedure in any patient. The $P_i$ excretion in three heterozygotes overlapped the normal response. On the other hand, each of the three mutant hemizygotes were less responsive to PTH than were the heterozygotes, and one male actually excreted less $P_i$ after the PTH infusion. One of the hemizygotes (second from right, Fig. 2) was studied when his serum $P_i$ had been raised to 8 mg/100 ml by the infusion method (*9*). His response to PTH was like that of the other two male patients who were studied when their concentrations of serum $P_i$ were low (< 3 mg/100 ml). We reported previously (*5*) that a tenfold increase in endogenous serum IPTH does not influence $TRP_i$ in mutant hemizygotes. We conclude, from the present and the early studies, that the residual $P_i$ transport, unmasked in the hemizygote with X-linked hypophosphatemia, is virtually insensitive to PTH.

Phosphaturia may not be a satisfactory index of the renal tubular response to PTH. Urinary excretion of adenosine 3′,5′-monophosphate (cyclic AMP) is said to be a more reliable index (*13*), and for this reason we measured cyclic AMP in the urine of the patients infused with bovine PTH (*14*). Urinary cyclic AMP was normal in all patients before infusion. The excretion rate of cyclic AMP in female heterozygotes, mutant hemizygotes, and normal subjects increased by 19 to 80 nmole/min in the first 30 minutes after bovine PTH injection; the response for mutant and normal subjects overlapped each other and all were in the normal range (*13*). We also measured the change in calcium excretion relative to creatinine, before and after PTH infusion. Calcium excretion dropped by 50 to 70 percent after PTH infusion in normal, heterozygous, and hemizygous subjects. Therefore, PTH acts in a normal manner on the renal tubule in X-linked hypophosphatemia but the hormone is unable to exert its usual effect on $P_i$ transport.

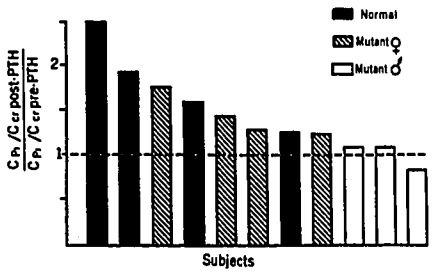


Fig. 2. Effect of an intravenous infusion of purified bovine parathyroid hormone (PTH) on phosphate excretion by mutant hemizygotes (open bars) and heterozygotes (hatched bars) with X-linked hypophosphatemia, and normal subjects (black bars). The ordinate is the ratio $(C_{P_i}/C_{cr})$ after PTH infusion to the ratio $(C_{P_i}/C_{cr})$ before PTH infusion, where C is the endogenous renal clearance rate of inorganic phosphate ($P_i$) and creatinine (cr) averaged for three 30-minute periods before and after PTH infusion.

Intravenous infusion of calcium enhances $P_i$ transport in the mammalian kidney (*2*, *15*). The effect is mediated both through a parathyroid-dependent mechanism and by a direct effect upon the tubule (*15*). The latter can be the only mode of action of calcium in male patients with X-linked hypophosphatemia who respond to calcium infusion with improvement in the net tubular reabsorption of $P_i$ (*2*, *3*), because $P_i$ reabsorption is insensitive to PTH, and serum IPTH is not abnormally elevated in the untreated mutant hemizygote (*5*). Studies of the change in $P_i$ reabsorption after calcium infusion in X-linked hypophosphatemia, often were performed with large amounts of calcium infused over several hours (*3*). We examined five patients (three female, two male) at 30 to 60 minutes after a calcium infusion (4 mg/kg over 15 minutes) that was sufficient to raise serum calcium by 2 mg/100 ml. Tubular transport of $P_i$ was studied at concentrations of serum $P_i$ sufficient to saturate the transport. Tubular reabsorption of $P_i$ improved under these conditions in each subject (Fig. 1), whether net tubular reabsorption or net secretion of $P_i$ was present initially. Although serum immunoreactive PTH fell by about 20 percent in all subjects after the calcium infusion, this response would not account for the change in $TRP_i$ in hemizygotes.

Familial hypophosphatemia is a dominantly expressed X-linked disease (*2*) and, according to the Lyon hypothesis, such a disease will be expressed fully in male hemizygotes and variably in the females. We must conclude, however, that the loss of $P_i$ transport in kidney is not complete even in mutant hemizygotes with X-linked hypophosphatemia. A component of $P_i$ transport, with a saturable capacity for net reabsorption at about one-third the normal value, is still active in the male patient. Transport of $P_i$ in females is between normal and hemizygous values; this is compatible with partial retention of the transport that is absent in males. We propose, therefore, that $P_i$ transport in human kidney involves at least two components (*16*). One component accounts for about two-thirds (about 100 μmole per 100 ml of GF) of the total net reabsorptive capacity; this transport is modulated by endogenous parathyroid hormone. A second component accounts for all or most of the remaining uptake; its capacity is about 50 μmole per 100 ml of GF, and its $P_i$ transport can be directly modulated by the calcium ion. These findings suggest that calcium may regulate $P_i$ efflux (plasma to lumen) relative to net influx (lumen to plasma) by the second type of $P_i$ transport. It is possible that whatever benefit has been achieved by massive vitamin D treatment in X-linked hypophosphatemia was actually derived from the effect of vitamin D on calcium metabolism which in turn modulated $P_i$ transport by the calcium-sensitive system.

It is still not known whether the two (or more) components of $P_i$ transport are separate binding sites (proteins) with different capacities and sensitivities to PTH and calcium, functioning in parallel at the brush border, or whether they function in series, perhaps at opposite poles of the tubular epithelial cell. The latter hypothesis implies a mediated brush border transport with high capacity, which is sensitive to PTH and calcium, and an antiluminal carrier capable of transporting $P_i$ from cell to plasma and also from plasma to cell. The X-linked mutation presumably influences only one mode of transport ni the parallel hypothesis and there is precedence for this type of membrane transport mutation (*17*). In the series hypothesis the mutation concerns transport on the brush border site which allows $P_i$ binding but not a coupled transport to allow accumulation of $P_i$ in the cell; there is also precedence for mutations which "uncouple" substrate-specific transport (*18*). The X-linked mutation may affect the membrane system directly or it may affect another cell function which modulates membrane transport of phosphate.

We do not know whether other cells and organs in man have similar mechanisms for $P_i$ transport. A number of the clinical features of X-linked hypophosphatemia, particularly in the female heterozygote, cannot be explained solely on the basis of an hereditary defect in $P_i$ transport in kidney. Moreover, some nonsaturable but relatively inefficient $P_i$ transport in cell membrane (*19*) is likely to be present since high concentration of $P_i$ improves phosphate retention (*7*). We anticipate that mediated $P_i$ transport may be impaired in other tissues in this disease; there is evidence for this in other inborn errors of membrane transport (*17*). The X-linked hypophosphatemia may be genetically heterogeneous and different types of defective $P_i$ transport may be identified; such genetic heterogeneity has been found in the other inborn errors of membrane transport (*17*).

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### References and Notes

1. F. Albright, A. M. Butler, E. Bloomberg, *Amer. J. Dis. Child.* **54**, 529 (1937); J. F. Christensen, *Acta Paediat. Scand.* **28**, 247 (1940–1941).
2. R. W. Winters, J. B. Graham, T. F. Williams, V. W. McFalls, C. H. Burnett, *Medicine* **37**, 97 (1958); T. F. Williams, R. W. Winters, C. H. Burnett, in *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, Eds. (McGraw-Hill, New York, ed. 2, 1966), pp. 1179–1204.
3. D. Fraser, J. Leeming, E. Cerwenka, *Helv. Paediat. Acta* **14**, 497 (1959); W. Falls, N. Carter, R. Rector, D. Seldin, *Ann. Intern. Med.* **68**, 553 (1968).
4. F. Albright and H. W. Sulkowitch, *J. Clin. Invest.* **17**, 305 (1938); L. V. Alvioli, T. F. Williams, J. Lund, H. F. DeLuca, *ibid.* **46**, 1907 (1967); H. Earp, R. Ney, M. Gittleman, R. Richman, H. DeLuca, *N. Engl. J. Med.* **283**, 627 (1970).
5. C. Arnaud, F. Glorieux, C. R. Scriver, *Science* **173**, 845 (1971).
6. B. R. Robertson, C. R. Harris, D. J. McClure, *Amer. J. Dis. Child.* **64**, 948 (1942); C. E. Dent, *J. Bone Joint Surg. Brit. Vol.* **34**, 266 (1952); G. Fanconi and P. Girardet, *Helv. Paediat. Acta* **7**, 14 (1952); B. Frame and R. W. Smith, Jr., *Amer. J. Med.* **25**, 771 (1958); R. W. Winters and J. B. Graham, *Pediatrics* **25**, 932 (1960); J. Rey and J. Frezal, *Rev. Fr. Etud. Clin. Biol.* **11**, 771 (1966).
7. D. Fraser, D. Geiger, J. Munn, P. Slater, R. Jahn, E. Lin, *Amer. J. Dis. Child.* **96**, 460 (1958); H. Harrison, F. Lifshitz, A. D. Johnson, *ibid.* **112**, 290 (1960); C. West, J. Blanton, F. Silverman, N. Holland, *J. Pediat.* **64**, 469 (1964); C. Nagant de Deuxchaisnes and S. Krane, *Amer. J. Med.* **43**, 508 (1967); F. Glorieux, C. Arnaud, C. Clow, H. Goldman, T. Reade, C. R. Scriver, *Clin. Res.* **18**, 726 (1970).
8. D. Longson, J. N. Mills, S. Thomas, P. A. Yates, *J. Physiol.* **131**, 555 (1956); D. D. Thompson and H. H. Hiatt, *J. Clin. Invest.* **36**, 550 (1957); J. Anderson and V. Parsons, *Clin. Sci.* **25**, 431 (1963).
9. Tubular reabsorption of $P_i$ was determined by calculating the filtered load (serum $P_i$ × GFR), and measuring the excreted $P_i$ in urine ($P_i$ per milliliter times flow rate [milliliters per minute]); the reabsorbed amount was calculated as the difference and expressed as a coefficient of GFR. The latter was estimated from the inulin clearance or creatinine clearance. The $TmP_i$ was determined when the serum $P_i$ was raised by a modification of the infusion method of Anderson (*8*). All determinations were made between 0800 and 1200 hours when the patient was in the fasting state, at rest, and had a water intake sufficient to ensure a free urine flow exceeding 4 ml/min; all urine collection periods were 30 minutes or longer.
10. W. Rupp and W. Swoboda, *Helv. Paediat. Acta* **10**, 135 (1955).
11. Parathyroid hormone, prepared by trichloroacetic acid precipitation from bovine glands and with specific activity of about 350 unit/mg, was supplied by Dr. Claude Arnaud. The PTH powder was diluted in 0.01*M* acetate buffer and filtered through a Millipore filter (0.22-μm pore size) immediately before use. The filtrate containing the hormone was then diluted with an equal volume of sodium chloride. Adults received 400 units, and children 200 units, by intravenous infusion for 10 minutes. Urine collections were performed, with minor variation, according to the method of Chase *et al.* (*13*).
12. C. Arnaud, H. Tsao, T. Littledyke, *J. Clin. Invest.* **50**, 21 (1971).
13. L. R. Chase, G. L. Melson, G. D. Aurbach, *ibid.* **48**, 1832 (1969).
14. The determinations were performed by the kinase method of A. G. Gilman [*Proc. Nat. Acad. Sci. U.S.* **67**, 305 (1970)] in the laboratory of Dr. Alan Tennenhouse.
15. J. E. Howard, T. R. Hopkins, T. B. Connor, *J. Clin. Endocrinol.* **13**, 1 (1953); H. H. Hiatt and D. D. Thompson, *J. Clin. Invest.* **36**, 573 (1957); A. R. Lavender and T. N. Pullman, *Amer. J. Physiol.* **205**, 1025 (1963).
16. R. W. Winters and colleagues (*2*) also offered this suggestion in their earlier attempt to interpret the primary defect in X-linked hypophosphatemia.
17. L. E. Rosenberg, in *Biological Membranes*, R. M. Dowben, Ed. (Little, Brown, Boston, 1969), pp. 255–259; C. R. Scriver and P. Hechtman in *Advances in Human Genetics*, H. Haris and K. Hirschorn, Eds. (Plenum, New York, 1970), pp. 211–274.
18. P. T. S. Wong, E. R. Kashket, T. H. Wilson, *Proc. Nat. Acad. Sci. U.S.* **65**, 63 (1970); P. Hechtman and C. R. Scriver, *Biochim. Biophys. Acta* **219**, 428 (1970).
19. S. L. Schrier, L. D. Moore, A. P. Chiapella, *J. Lab. Clin. Med.* **75**, 422 (1970).
20. Supported by grants from the Medical Research Council of Canada (MT-1085) and the National Genetics Foundation (New York). We are grateful to Mrs. Terry Reade and Peter Lamm for their assistance with these studies and to Dr. Claude Arnaud for many valuable discussions and for a gift of bovine PTH. Publication No. 279 from the McGill University–Montreal Children's Hospital Research Institute.

26 July 1971; revised 28 October 1971

D. Supplementary data.

i) The effect of calcium on phosphate transport

In X-linked hypophosphatemic rickets, intravenous infusion of calcium clearly enhances the tubular reabsorption for phosphorus. This effect has been interpreted as evidence for secondary hyperparathyroidism in the hereditary trait[18]. In view of our own previous observations which were against this hypothesis, it was necessary to reevaluate this effect and particularly in our patient D.R. after parathyroidectomy. The results obtained in this hemizygous patient,using the infusion method described in Section III,are represented in the Figure V-2. The three-dimensional figure inscribed in the graph shows changes in TRP obtained over a range of plasma values for calcium and phosphorus. The white area indicates that the transport system for phosphorus is saturated in this subject even at endogenous concentration of phosphate. However the rapid infusion of calcium enhances the phosphate transport function as represented by the right hatched area.

A similar response to calcium infusion was obtained in the heterozygote mother of D.R. (Fig. V-3). Her response is less striking but obviously present. The interpretation of these data as a direct effect of calcium upon the renal tubule[19] is discussed in Section V-C (Second Science paper).

FIGURE V - 2

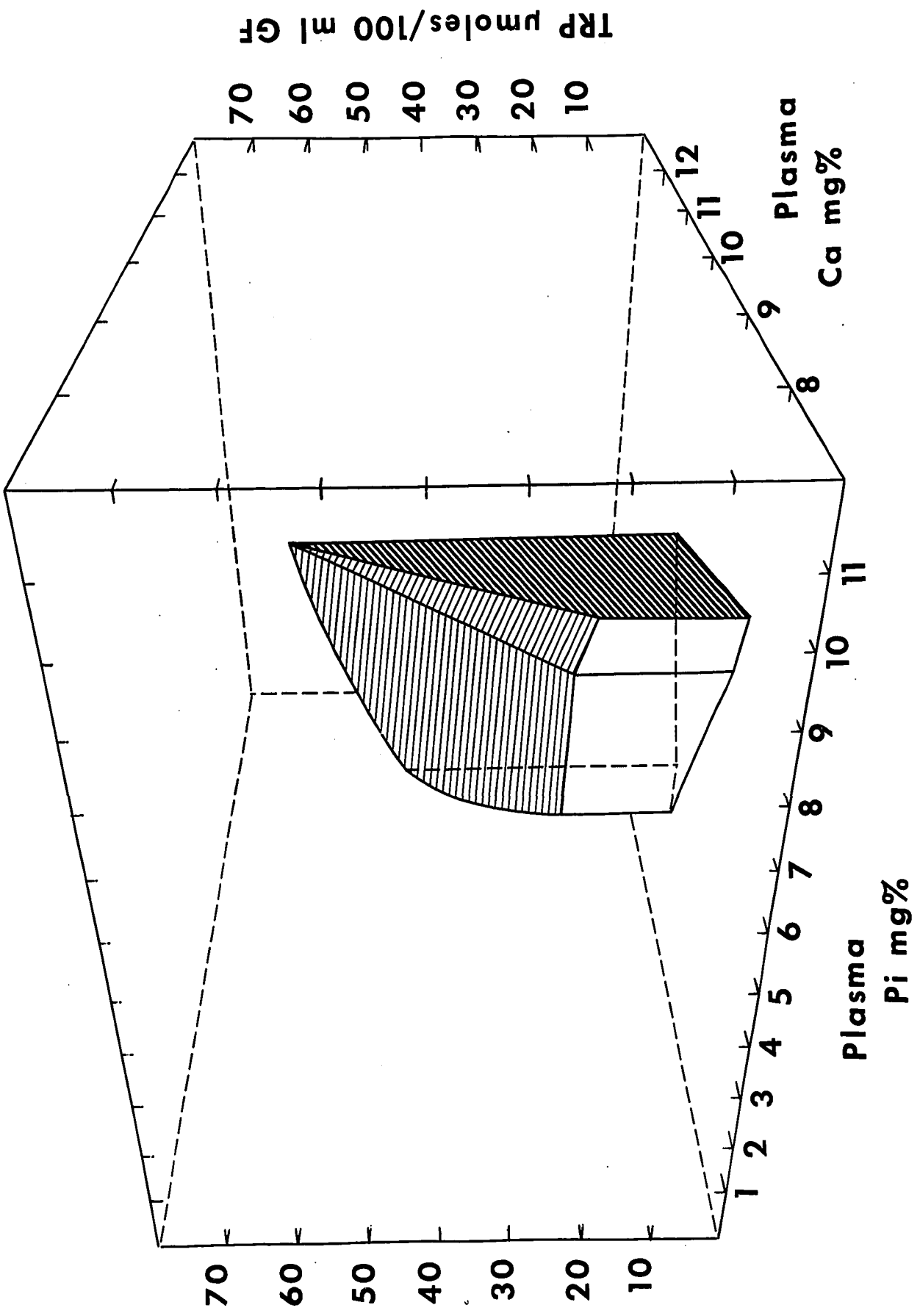

TRP μmoles/100 ml GF
70
60
50
40
30
20
10
Plasma
Ca mg%
8
9
10
11
12
Plasma
Pi mg%
1
2
3
4
5
6
7
8
9
10
11
70
60
50
40
30
20
10

FIGURE V - 2

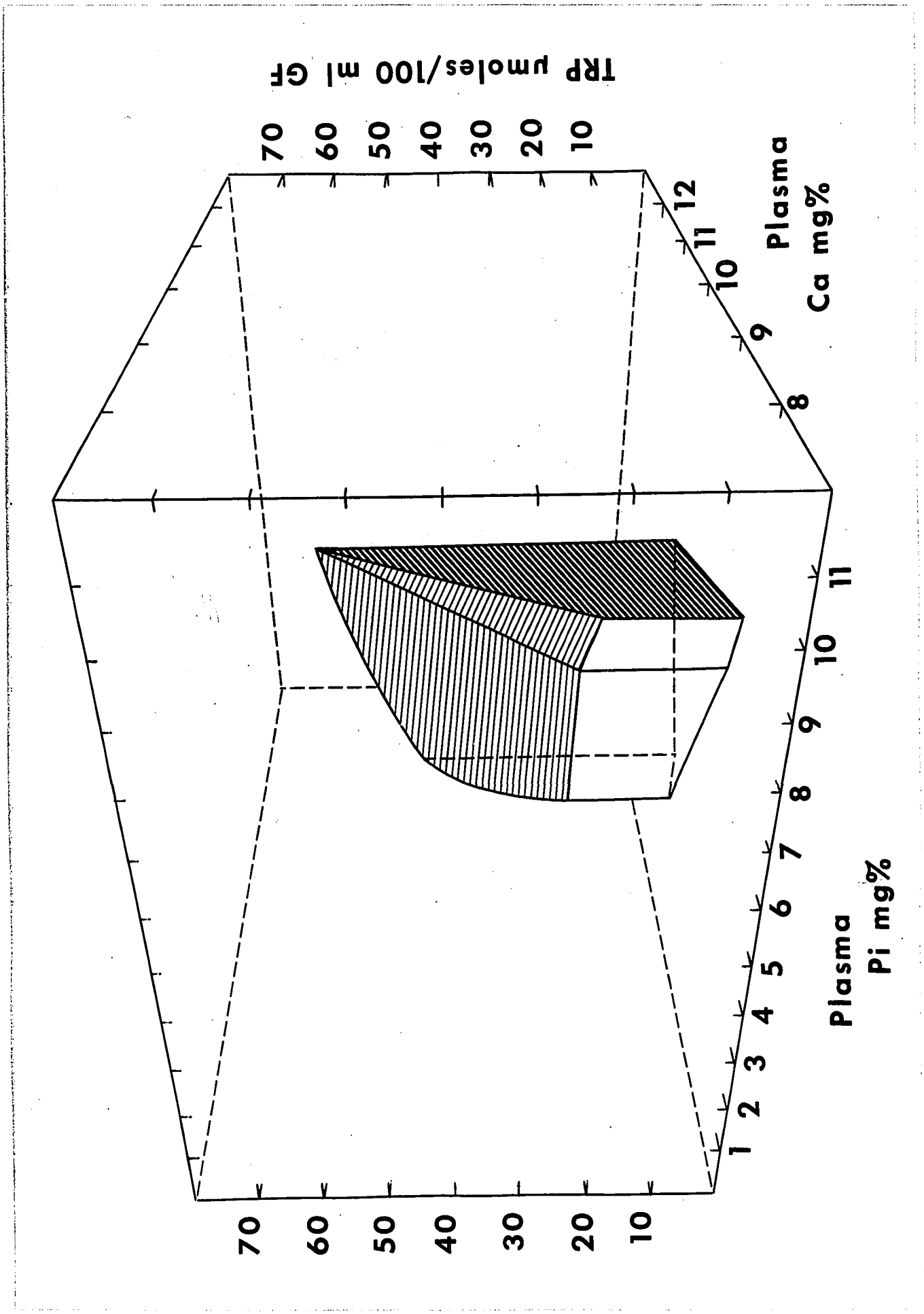

TRP μmoles/100 ml GF
70
60
50
40
30
20
10
Plasma
Ca mg%
8
9
10
11
12
Plasma
Pi mg%
1
2
3
4
5
6
7
8
9
10
11
70
60
50
40
30
20
10

FIGURE V - 3

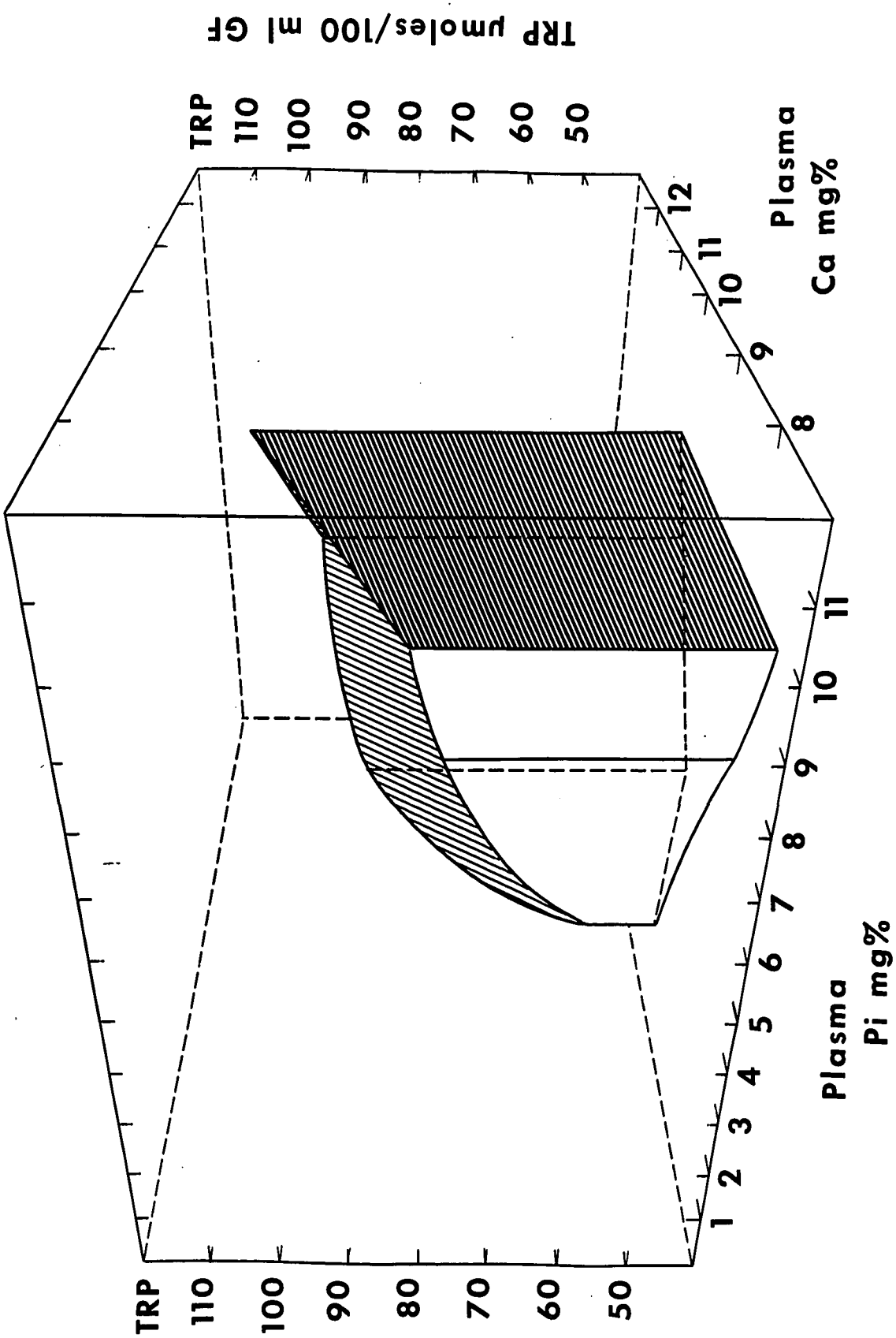
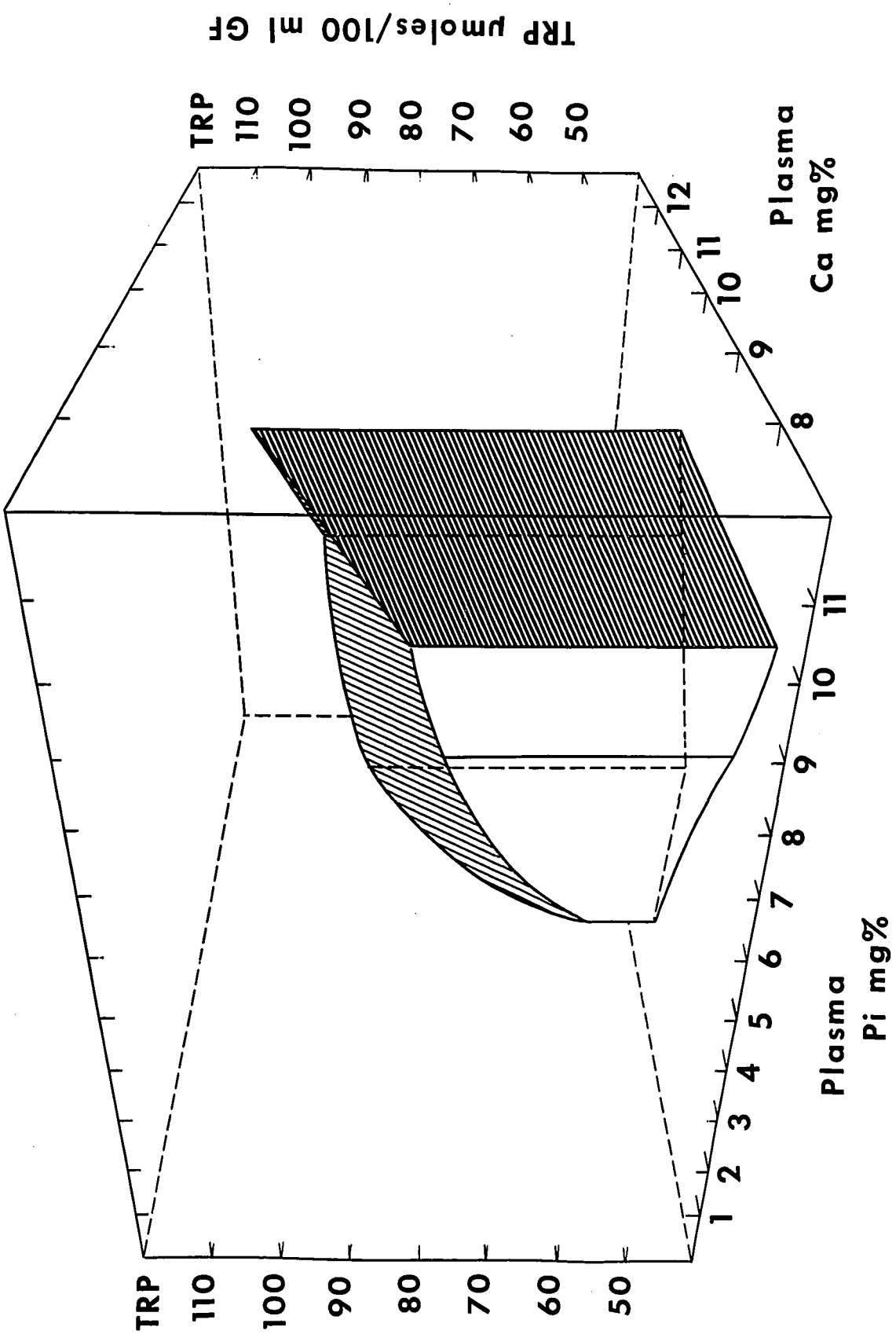

TRP μmoles/100 ml GF
TRP
110
100
90
80
70
60
50
Plasma
Ca mg%
12
11
10
9
8
Plasma
Pi mg%
1
2
3
4
5
6
7
8
9
10
11
TRP
110
100
90
80
70
60
50

FIGURE V - 3


TRP μmoles/100 ml GF
TRP
110
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Pi mg%
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TRP
110
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50

ii) The relation between plasma phosphate and filtered phosphate during calcium infusion

It is generally assumed that at endogenous concentration of phosphate in plasma 100% of that phosphate is filtered through the glomerulus. Direct measurement of the plasma phosphate can therefore be used for the determination of the filtered load of phosphorus. However, when both calcium and phosphorus are raised in blood, there is a possibility that formation of a calcium phosphate colloidal complex may occur with the plasma proteins[20]. Should this occur, the value (measured by the Fiske and Subbarow method) of plasma phosphate used for estimation of the filtered load of phosphorus will be larger than the actual value in vivo. The reabsorbed fraction obtained by subtracting the excreted amount of phosphorus from the filtered load will thus be overestimated. Thus, the observed "calcium effect" reported in the previous paragraph (Section D-i) could be an artefact of overestimation of the filtered load of phosphorus.

To clarify the point, the following experiment has been done:

A sample of heparinized fresh blood was obtained by venipuncture from a normal individual and separated in two plasma fractions:

- Plasma sample A containing 9.6 mg% of calcium and 3.3 mg% of phosphate. This sample was used as control.

- Plasma sample B was artificially enriched so that calcium and phosphorus concentrations were 11.1 mg% and 7.8 mg% respectively.

The samples were applied to separate G-25 Sephadex columns (42.5 cm long) and eluted with normal saline. Eighteen fractions (2 ml) were collected from each column and analyzed for protein, calcium and phosphorus.

Proteins (measured by the Lowry method) appeared in fractions 2 to 9 from both columns.

Phosphorus was not detected in any of the A and B fractions. Calcium was present at the concentration of 0.2 mg% in all fractions.

The limits of the technique used for measuring phosphate concentration in the fractions (on a Technicon Auto-Analyzer) were such that not more than 0.5 mg% of the plasma phosphorus could have been present as a colloidal complex and consequently, not available for filtration through the glomerulus. This amount would not alter significantly the estimation of the filtered load of phosphorus. The use of the formula: (Pi x GFR) for calculating the filtered load of phosphorus is therefore apparently justified for the experiments involving calcium infusion.

iii) The excretion of cyclic adenosine 3',5'-monophosphate (c-AMP)

Parathyroid hormone exerts its action on the renal tubular cell through the activation of the membrane bound enzyme, adenyl cyclase[21]. A subsequent increase in intracellular c-AMP is indicated by an increase in the urinary excretion of the nephrogenous c-AMP. In pseudohypoparathyroidism, the absence of phosphaturic effect after intravenous infusion of PTH is coupled to a non-response in c-AMP excretion and this syndrome is said to be refractory to PTH[22].

Since we had observed a complete or partial lack of phosphaturic response to a PTH infusion in our hemizygous and heterozygous patients respectively, it was important to assess c-AMP excretion after PTH infusion.

The results are presented in Figure V-4. Three normal controls, four heterozygotes and three mutant hemizygotes with X-linked hypophosphatemia were studied. All patients had normal excretion of c-AMP before the infusion. In all subjects the maximum excretion occurred 30 minutes after the PTH injection; this is the normal response[22]. There is complete overlap among the three groups and the results are well within the published normal range.

Therefore there is a normal activation of adenyl cyclase in X-linked hypophosphatemia, but some intermediate step between that response and the increased phosphaturia after PTH infusion may be quantitatively or qualitatively deficient. We suggest that this reflects the complete or

partial inactivation of the PTH-sensitive component of phosphate transport in the hemizygotes and heterozygotes for X-linked hypophosphatemia respectively.

FIGURE V - 4

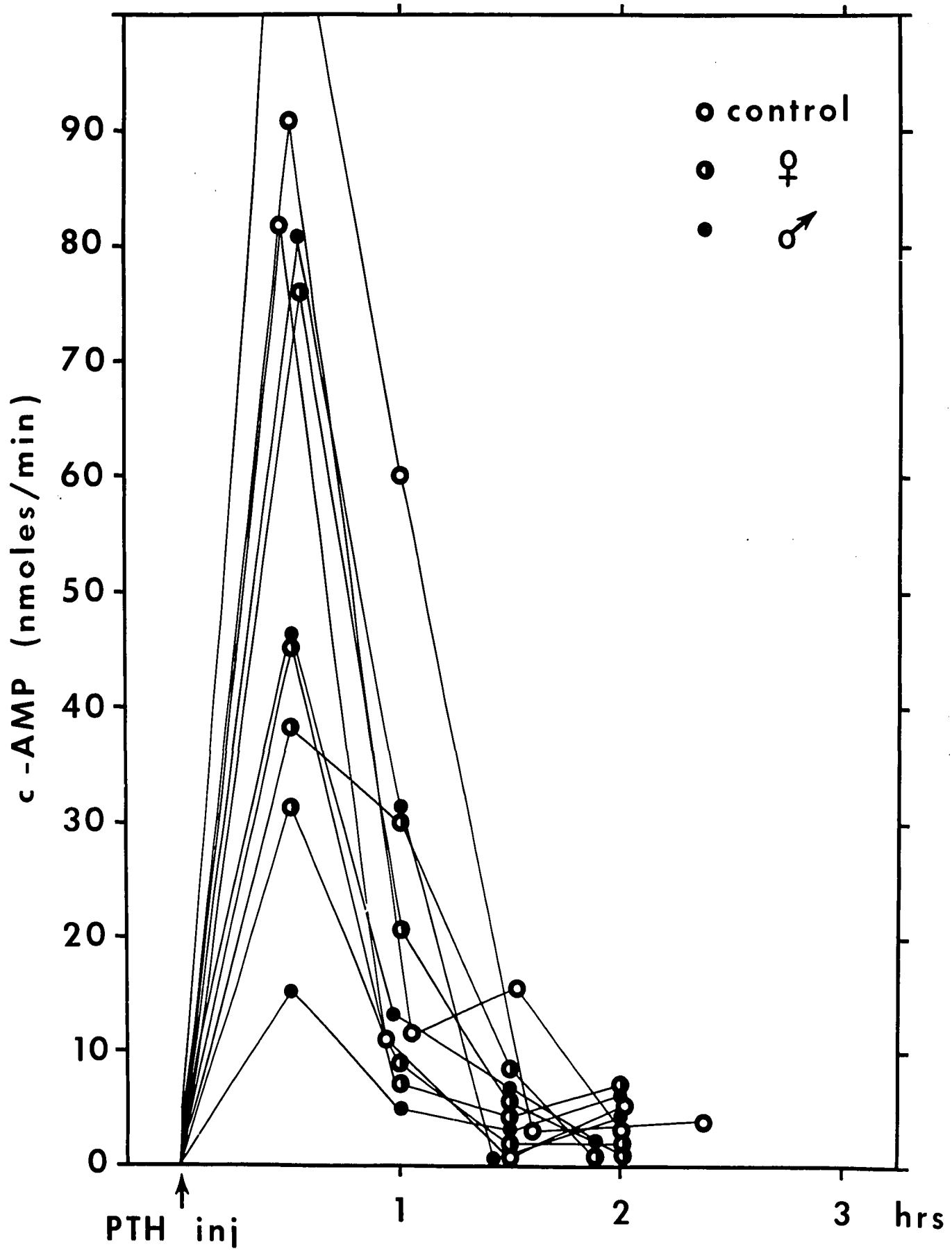
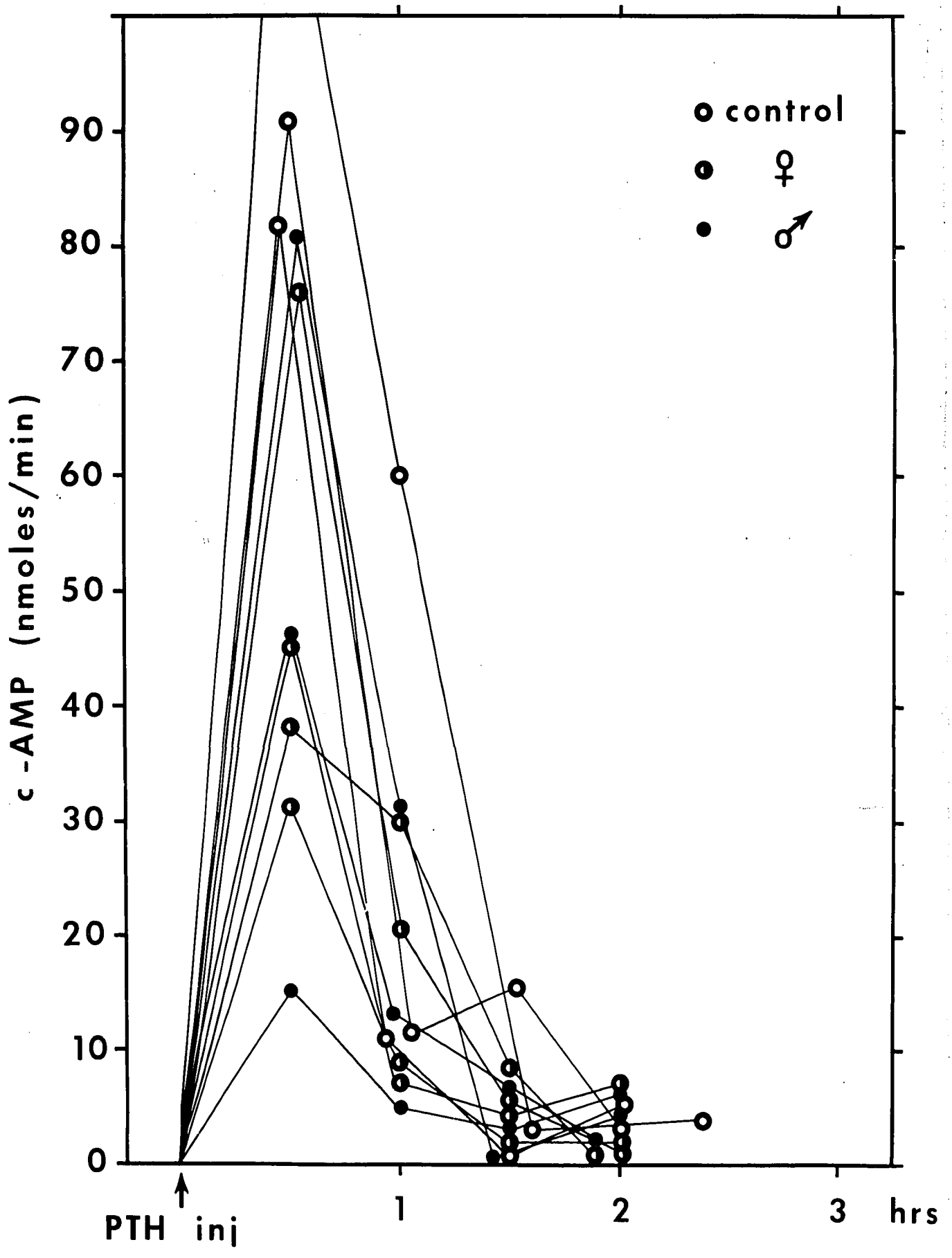

control
♀
♂
c-AMP (nmoles/min)
90
80
70
60
50
40
30
20
10
0
PTH inj
1
2
3
hrs

FIGURE V - 4


control
♀
♂
c-AMP (nmoles/min)
90
80
70
60
50
40
30
20
10
0
PTH inj
1
2
3
hrs

iv) Levels of circulating IPTH during infusion studies.

To clarify whether the "calcium effect" we have observed during our infusion studies is a direct effect on the tubular transport of phosphorus[19] or could be related to changes in circulating IPTH induced by the infusion[18], blood samples were drawn before and after both the phosphate and the calcium infusion and assessed for IPTH levels in Dr. Arnaud's laboratory. The available results are summarized on table V-1: five female heterozygotes (F) and one hemizygote (M) are represented. The latter was hyperparathyroid following long term phosphate therapy, it seems however that the decrease in IPTH caused by the calcium infusion is probably not important enough to explain the neutralization of the observed secretion process. In the heterozygotes, on the other hand, calcium effect on TRP is variable and some phosphaturic effect of PTH is also observed. These results again are in accordance with the conclusion of the bovine PTH infusion tests and support the idea that hemizygotes for X-linked hypophosphatemia completely lack a PTH sensitive transport component for phosphorus while heterozygotes retain a variable percentage of it.

TABLE V-1

| | Time mins. | $Ca^+$ mg% | P mg% | IPTH µl eq/ml | Infusion** protocol | | TRP µmoles/100 ml GF |
|---|---|---|---|---|---|---|---|
| 1. F | 0 | 9.6 | 2.3 | 8 | ↑ | | 77 |
| (BD-)* | 119 | 8.3 | 8.0 | 22 | Pi | ↑ | 88 |
| | 200 | 10.4 | 7.2 | 20 | ↓ | Ca | 117 |
| | 230 | 10.7 | 5.6 | 13 | | ↓ | 96 |
| 2. F | 0 | 9.3 | 2.2 | 23 | ↑ | | 70 |
| (BD-) | 112 | 8.1 | 7.8 | 28 | Pi | ↑ | 121 |
| | 162 | 10.4 | 7.4 | 14 | ↓ | Ca | 110 |
| | 239 | 12.1 | 6.5 | 16 | | ↓ | 128 |
| 3. F | 0 | 9.0 | 1.5 | 54 | ↑ | | 69 |
| (BD+) | 168 | 7.3 | 8.9 | 98 | Pi | ↑ | 83 |
| | 215 | 9.4 | 9.0 | 69 | ↓ | Ca | 106 |
| | | | | | | ↓ | |
| 4. F | 0 | 10.7 | 2.3 | 24 | ↑ | | 72 |
| (BD+) | 111 | 7.2 | 5.5 | 100 | Pi | ↑ | 125 |
| | 162 | 9.6 | 5.5 | 37 | ↓ | Ca | 102 |
| | 231 | 10.7 | 2.6 | 36 | | ↓ | 77 |
| 5. F | 0 | 10.4 | 2.8 | 49 | ↑ | | 66 |
| (BD+) | 114 | 8.7 | 5.1 | 60 | Pi | ↑ | 33 |
| | 166 | 10.2 | 4.5 | 51 | ↓ | Ca | 51 |
| | 230 | 10.4 | 4.0 | 54 | | ↓ | 51 |
| 6. M | 0 | 10.8 | 3.6 | 240 | ↑ | | 28 |
| (BD+) | 123 | 8.6 | 7.4 | 350 | Pi | ↑ | -112 |
| | 186 | 10.4 | 7.2 | 280 | ↓ | Ca | - 22 |
| | 249 | 10.2 | 4.2 | 210 | | ↓ | - 24 |

* BD = Bone Disease

** = The arrows encompass the overall duration of Pi or Ca infusions.

## REFERENCES

1. Greenberg, B.G., Winters, R.W., Graham, J.B. The normal range of serum inorganic phosphorus and its utility as a discriminant in the diagnosis of congenital hypophosphatemia. J. Clin. Endocr. and Metab. 20, 364-379, 1960.

2. Harrison, H.E., Harrison, H.C., Lifshitz, F., Johnson, A.D. Growth disturbance in hereditary hypophosphatemia. Amer. J. Dis. Child. 112, 290-297, 1966.

3. Stickler, G.B. Familial hypophosphatemic vitamin D resistant rickets: The neonatal period and infancy. Acta Paed. Scand. 58, 213-219, 1969.

4. Williams, T.F., Winters, R.W. Familial (hereditary) vitamin D resistant rickets: with hypophosphatemia. In J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, eds. The metabolic basis of inherited disease, 3rd ed., New York, McGraw-Hill, 1972, pp. 1465-1485.

5. Condon, J.R., Nassim, J.R., Rutter, A. Pathogenesis of rickets and osteomalacia in familial hypophosphatemia. Arch. Dis. Child. 46, 269-272, 1971.

6. McNair, S.L., Stickler, G.B. Growth in familial hypophosphatemic vitamin D-resistant rickets. New Engl. J. Med. 281, 511-516, 1969.

7. Gershberg, H., Neumann, L.L., Mari, S. Studies on vitamin-D-resistant rickets; Effects of human growth hormone. Metabolism 13, 636-649, 1969.

8. Burnett, C.H., Dent, C.E.,Harper, C., Warland, B.J. Vitamin D-resistant rickets. Analysis of twenty-four pedigrees with hereditary and sporadic cases. Amer. J. Med. 36, 222-232, 1964.

9. Lyon, M.F. Gene action in the X-chromosome of the mouse. Nature 190, 372-373, 1961.

10. Albright, F., Butler, A.M., Bloomberg, E. Rickets resistant to vitamin D therapy. Amer. J. Dis. Child. 54, 529-547, 1937.

11. Dent, C.E. Rickets and osteomalacia from renal tubule defects. J. Bone Jt. Surg. 34-B, 266-274, 1952.

12. Fanconi, G., Girardet, P. Familiarer persistienrender phosphatdiabetes mit D-vitamin-resistentes rachitis. Helv. Paed. Acta 7, 14-41, 1952.

13. Williams, T.F. Pathogenesis of familial vitamin D resistant rickets. Ann. Int. Med. 68, 706-707, 1968.

14. Earp, H.S., Ney, R.L., Gitelman, H.J., Richman, R., DeLuca, H.F. Effects of 25-hydroxycholecalciferol in patients with familial hypophosphatemia and vitamin D-resistant rickets. New Engl. J. Med. 283, 627-630, 1970.

15. Boyle, I.T., Gray, R.W., DeLuca, H.F. Regulation by calcium of in vivo synthesis of 1,25-DHCC and 21,25-DHCC. P.N.A.S. 68, 2131-2134, 1971.

16. Puschett, J.B., Moranz, J., Kurnick, W.S. Evidence for a direct action of cholecalciferol and 25-HCC on the renal transport of phosphate sodium and calcium. J. Clin. Invest. 51, 373-385, 1972.

17. Hiatt, H.H., Thomson, D.D. The effects of parathyroid extract on renal function in man. J. Clin. Invest. 36, 557-565, 1957.

18. Field, M.H., Reiss, E. Vitamin D resistant rickets: the effect of calcium infusion on phosphate reabsorption. J. Clin. Invest. 39, 1807-1812, 1960.

19. Lavender, A.R., Pullman, T.N. Changes in inorganic phosphate excretion induced by renal arterial infusion of calcium. Amer. J. Phys. 205, 1025-1032, 1963.

20. Walser, M. Protein-binding of inorganic phosphate in plasma of normal subjects. J. Clin. Invest. , 501-506, 1960.

21. Rasmussen, H. Ionic and hormonal control of calcium homeostasis. Amer. J. Med. 50, 567-588, 1971.

22. Chase, L.R., Melson, G.L., Aurbach, G.D. Pseudohypoparathyroidism: defective excretion of 3',5'-AMP in response to parathyroid hormone. J. Clin. Invest. 48, 1833-1844, 1969.

## THE APPLICATION OF KNOWLEDGE TO THE TREATMENT OF X-LINKED HYPOPHOSPHATEMIA: A SUPPLEMENTAL PHOSPHATE REGIMEN IN X-LINKED HYPOPHOSPHATEMIC RICKETS

The treatment of X-linked hypophosphatemic rickets traditionally includes vitamin D in high doses. The doses effective for healing of the bone lesions can have toxic effects. Episodes of hypercalcemia and soft tissue calcification have been reported. However this treatment does not improve the low steady-state of plasma phosphorus and has almost no effect on the growth rate of the treated patients.

The supplemental phosphate was added to the vitamin D in the last ten years, as a useful adjunct to prevent hypercalcemic episodes.

The alternative viewpoint has been adopted in the present study: phosphate supplementation was employed as the major component of treatment while vitamin D was added in a relatively small dose to compensate for the hypocalcemic effects of phosphate loading.

This approach has proven to be effective in the neutralization of the mutant phenotype in X-linked hypophosphatemia. Results are reported and discussed in the following manuscript (recently submitted to The New England Journal of Medicine).

# THE USE OF PHOSPHATE AND VITAMIN D TO PREVENT DWARFISM AND RICKETS IN X-LINKED HYPOPHOSPHATEMIA

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Publication # ____ from the McGill University-Montreal Children's Hospital Research Institute.

## ABSTRACT

Eight children with X-linked hypophosphatemia (3f, 5m between 3 and 15 years of age) were treated for a total of 11,297 patient days with an inorganic phosphate salt supplement by mouth (1-4 g Pi/day in 5 divided doses given at 4 hour intervals) and vitamin $D_2$ (10-50 x $10^3$ units/day). On this regimen the mean value for serum phosphorus in the group was 4.0 mg percent and 85 percent of values were above 3.0 mg percent. The rickets healed completely and an accelerated growth rate was observed in all patients when phosphate supplements were provided continuously. Dwarfism was corrected in 3 female and 2 male subjects. The whole-blood $P_{50}$ was low (24.5 mm Hg at pH 7.4) in untreated patients but was restored to normal during phosphate treatment. There were no serious episodes of hypercalcemia and no ectopic calcification was found in association with the combined phosphate and vitamin D regimen.

Hypophosphatemia in one of its familial forms is inherited as an X-linked dominant trait[1]. This type of hypophosphatemia declares itself in early infancy[2] and rickets appear subsequently; the latter may be responsive only to large and continuous doses of vitamin D, accounting for the term "familial vitamin D resistant rickets" which is often attached to the disease. Dwarfism is the third important clinical feature of this trait[3]. All aspects of the phenotype are generally more severe and uniform in male patients than in the female, as expected of an X-linked trait.

Treatment of X-linked hypophosphatemia has always been a challenge. While vitamin D alone may heal the rickets when given in very large amounts[3,4], the effective dose is usually close to the toxic range and episodes of hypercalcemia are a significant hazard of such treatment in this disease[5-7]. Furthermore the dwarfism has almost never been corrected by vitamin D-treatment alone. Nonetheless, it has long been assumed that a primary abnormality of vitamin D-controlled mineral metabolism was responsible for this disease. We have re-examined this hypothesis[8], and in the absence of any evidence that hypophosphatemia is parathyroid-dependent, and thus might be responsive to vitamin D, we have given more emphasis to a primary defect of phosphate conservation in the kidney[9]. At present there is no way to correct the renal loss of phosphate. Therefore, we have adopted long-term, in-home phosphate replacement as the major component of a combined phosphate

and vitamin D treatment regimen which will raise the concentration of inorganic phosphate (Pi) in serum, almost to the normal range will heal the rickets, and even more important, will increase linear growth velocity so that the dwarfism can be overcome in many of our patients. This paper reports our experience of 11,297 patient treatment days with eight children who have proven X-linked hypophosphatemia. The supportive ambulatory program which makes this mode of treatment practical was described in detail previously[10].

## MATERIAL AND METHODS

The patients were eight subjects (3f, 5m between 3 and 15 years of age) with X-linked hypophosphatemic rickets. The criteria for diagnosis were: a concentration of inorganic phosphate in serum consistently below 4 mg percent after an overnight fast; rickets appearing in infancy; no hypocalcemia; no renal loss of metabolites other than phosphate; no male-male transmission of the hypophosphatemic trait; (there was mother to child transmission in all of our patients). Each subject described in this report has been followed and treated for more than a year and in one case, as long as eight years. The total period of treatment covered by the report is 11,297 patient days. The relevant clinical and biochemical features of the patients are summarized in Table I.

Follow-up: Each patient was visited at home regularly, to supervise medication, to draw venous blood samples and to describe the progress of therapy. Renal clearance studies were performed every three months. The long bones, kidneys and skull were examined radiographically during half-yearly clinic visits (or more often if indicated) for signs of rickets, hyperparathyroidism, and complications of therapy. Slit-lamp examination of the eyes were performed at least once for evidence of ectopic calcification. Blood pressure and blood pH measurements were obtained frequently.

Analytical Methods: Total calcium, inorganic phosphate (Pi), alkaline phosphatase activity and creatinine were determined by Technicon auto-analyzer methods. The amino acid and imino acid-containing oligopeptide content of urine was evaluated by two-dimensional partition and ion-exchange chromatography and endogenous renal clearance of phosphate was measured, as described previously[11,12]. Serum immuno-reactive parathyroid hormone (IPTH) was measured by Dr. Claude Arnaud using a radioimmunoassay[13] for which venous blood was collected in plastic syringes, transferred to pyrex tubes, allowed to clot and after removal of the cells, frozen at -20°C until shipment on dry ice. Whole blood oxygen pressure, at pH 7.4 and 50 percent oxygen saturation ($P_{50}$, in mm Hg), was determined by the Severinghaus method[14]. Samples were obtained from patients during phosphate treatment, and again at the same time of the day two weeks after the treatment had been stopped. The patients and their obligate heterogyzous mothers in whom $P_{50}$ and Pi were also measured, were matched

for age and sex by a paired control subject.

Measurements of linear growth were made in the standing position; the "lower segment" of total height is the distance from the top of the symphysis pubis to the heel. Measurements were plotted on copies of the charts drawn by Tanner et al[15], and by McNair and Stickler[3].

Bone density on standardized roentgenographs of the hands was determined by computerized photodensitometry of the bone image[16].

Treatment Regimen: A phosphate mixture comprising $NaH_2PO_4 \cdot 7H_2O$ (136 g/vol.); and phosphoric acid (NF85%; 58.8 g/vol.) in 1 L of tap water (Joulie's solution) was found to be most acceptable for long term treatment. The pH of this solution is 4.9, the molarity is 1725 mOsm, and it provides 30.4 mg Pi per ml. The mixture is given in multiples of 5-15 ml per dose until an average intake of 15 ml every 4 hours, five times daily is achieved. The amount taken on this time schedule is that required to maintain the average phosphorus concentration above 3.0 mg percent and is equivalent to 1-4 g Pi/day. Transient diarrhea may occur during the first days of this regimen, but careful explanation to the parents and patient, is usually sufficient to overcome its inconvenience.

Vitamin $D_2$ (10,000-50,000 IU/day) was used to offset hypocalcemia during high-phosphate intake. If secondary hyperparathyroidism appeared, phosphate was discontinued temporarily, and calcium gluconate (2-9g day) was given

in combination with vitamin $D_2$ (100,000 units/day) for 2-3 weeks, or until parathyroid gland hyperactivity abated. Cycling of treatment in this manner has prevented the clinical complications of hyperparathyroidism in our experience.

## RESULTS

Serum Pi: Serum Pi oscillates with intermittent dietary loading (Fig. 1). However the 4-hour Pi loading cycle maintained serum Pi above 3.0 mg percent in 85 percent of venous blood samples obtained from patients on this regimen. The serum Pi in the eight patients during a 12-month period in 1970-71 is given in Table II. The mean value ± 1 SD for 192 determinations during 2,725 treatment days was 4.0 ± 0.8 mg percent.

Bone: Active rickets was not found in any of our patients during regular treatment with phosphate. Coarsening of the trabecular pattern was sometimes evident but mean radiographic density measured by photodensitometry of standardized middle phalangeal x-ray images was between the 15th and 87th percentiles, for bone density in normal subjects, matched for age[16].

Linear Growth: The average linear growth velocity for the patient group was below normal (63 ± 24 percent of the normal rate on the 50th percentile) before the initiation of phosphate treatment. At the time seven of the patients were receiving vitamin $D_2$ in the usual large doses

for treatment of vitamin D resistant rickets. The linear growth rate increased in each patients during phosphate treatment (Fig. 2). The mean value for the total patient group on phosphate treatment was double the pre-treatment rate, or equivalent to 126 ± 22 percent of the age-equivalent 50th percentile for normal children. After prolonged phosphate therapy "an accelerated" growth rate was still apparent in most of the patients (upper half of Figures 2a and 2b). The dwarfism which characterized the pre-treatment physique, is no longer present in the three female patients in the group and in two of the male patients.

The impairment of linear growth before phosphate treatment was most obvious in the lower segment, as noted by others[3]. The post-phosphate treatment acceleration of total linear growth was clearly expressed in the lower segment of our patients (Fig. 3).

Whole Blood $P_{50}$: In the absence of phosphate treatment, patients with X-linked hypophosphatemia have lower values for whole-blood $P_{50}$ and serum Pi than during treatment with phosphate (Fig. 4). The difference between their on-treatment and off-treatment $P_{50}$ values, is significant at the 0.05 level by the Wilcoxon signed-rank test[17]. The values for patients on treatment were not significantly different from the control group of age-matched healthy children. Heterozygous mothers not receiving phosphate had lower values for whole-blood $P_{50}$ and serum Pi than the age-matched control group of women. The relationship between the two groups of adult subjects parallels that for corresponding groups of

children (Fig. 4). There were no significant differences between patients and controls, in hemoglobin concentration, hematocrit or blood pH.

Complications of the Treatment Regimen: There were five episodes of hypercalcemia during 11,297 treatment days in patients on the combined phosphate and vitamin D regimen. None lasted more than two weeks and no serum calcium value exceeded 12.4 mg percent. There is no evidence of ectopic tissue calcification or of urinary calculus formation, and the glomerular filtration rate remains normal in all of our patients. The sodium phosphate salt supplement did not cause systemic vascular hypertension or acidemia.

There was no evidence of hyperparathyroidism in our patients prior to phosphate treatment[8]. However phosphate administration will lower serum calcium[18], and as expected, this is associated with elevated serum IPTH level in X-linked hypophosphatemia[8]. The relationship is reversible with reduction of phosphate intake and by supplementing calcium nutrition (Table III).

In the early phase of our treatment program, one patient (D.R.) developed severe hyperparathyroidism which did not respond to medical management; subtotal parathyroidectomy removing about 7/8th of the 4 diffusely hyperplastic glands has restored parathyroid activity to an acceptable range (serum IPTH, 14-52ml Eq/ml, normal <40), in the subsequent three years. Parathyroidectomy did not influence the phosphate transport defect in the patient.

A generalized hyperaminoaciduria was apparent, as

expected[11], when secondary hyperparathyroidism developed (Table III). The hyperaminoaciduria abated with treatment to suppress hyperparathyroidism. An iminopeptiduria which may reflect increased bone turnover[12], was evident during reversible episodes of hyperparathyroidism.

## DISCUSSION

This report documents how the phenotypic effects of the mutation causing an X-linked hypophosphatemic trait can be neutralized with considerable success. We believe that the treatment regimen described here can also benefit patients with other types of phosphate losing nephropathy. Considering that so-called vitamin-D resistant rickets was recognized as a clinical entity almost 35 years ago[19], it comes as a surprise that a reasonably effective form of treatment has taken so long to emerge. There are at least three circumstances which caused the delay, namely, problems in the differential diagnosis of hypophosphatemia; problems with interpretation of the pathogenesis of hypophosphatemia; and problems in the clinical use of dietary phosphate in large and frequent doses.

Until a decade ago, X-linked hypophosphatemia was usually included in a group of familial rickets merely classified as resistant to Vitamin D. This in itself was a misnomer because at least the rickets in this X-linked disease could be healed when sufficient doses of vitamin D were administered carefully[3,4]. The trait had also been classified on physiological grounds as one of the hypophosphatemic diseases resulting from renal tubular dysfunction[4,29-22]. The latter attempt at nosology might have encouraged more extensive use of phosphate therapy as a way to offset the presumed lesion in tubular conservation of phosphate. However, debate about the mechanism of that lesion apparently forestalled any earlier acceptance of the phosphate-replacement treatment

hypothesis.

Several interpretations of the basic defect in the X-linked form of vitamin D resistant rickets have been advanced. A primary defect of intestinal absorption of calcium was suggested by Albright[19,23] and accepted by many workers subsequently, while others have recently favored an abnormality of vitamin D metabolism[24]. However, studies have shown that hypophosphatemic patients can synthesize 25-hydroxycholecalciferol the major polar metabolite of vitamin D[25], and treatment with this form of the vitamin does not correct the abnormal phenotype[26,27]. Furthermore, any hypothesis which proposes that vitamin D-dependent calcium transport is compromised initially in X-linked hypophosphatemia, should include secondary hyperparathyroidism as the cause for the renal loss of phosphate. We now know that levels of immunoreactive parathyroid hormone are normal in untreated patients with X-linked hypophosphatemia[8].

Evidence for a defect in tubular transport of phosphate in X-linked hypophosphatemia is now quite compelling[9]. There are apparently two types of phosphate transport in human kidney and one is deficient in the disease. The other functions at its maximum capacity, but under the usual conditions this system is not able to transport sufficient phosphate to restore the body pool. How does one then interpret the well-known observations that sustained calcium infusion improves tubular transport of phosphate in X-linked hypophosphatemia[28,29]. There is evidence[9] that the

residual type of phosphate transport in the kidney of X-linked hypophosphatemic patients is sensitive to calcium, in a manner analogous to the parathyroid-independent effect of calcium on phosphate transport in normal mammalian kidney[30]. Perhaps it is for this reason that massive doses of vitamin D and their frequent association with hypercalcemia, are of therapeutic benefit in the disease. On the other hand, it has recently been suggested[31] that a newly recognized metabolite of vitamin D may be directly involved in proximal tubular transport of phosphate and that its synthesis is regulated by the ambient calcium level. There is also new evidence[32] that 25-hydroxylated vitamin-D, or a derivative, enhances tubular reabsorption of phosphate by direct action on the kidney. Thus a partial defect in the biosynthesis of the proposed phosphate transporting substance might explain vitamin D resistance and the deficit in tubular transport of phosphate in X-linked hypophosphatemia. However, until that substance can be identified, isolated and shown to be of therapeutic value, it is necessary to look elsewhere for an effective mode of treatment.

The importance of phosphate salts in bone formation, has been known for a century. Lilly, Peirce and Grant[33], in their 1935 report of bone healing induced by dietary phosphate in experimental rickets, refer to the statement made by Wegner in 1872, that elemental phosphorus increases bone formation in growing animals. It has often been shown since then, that the bone lesions of vitamin D deficiency, as well as the various forms of vitamin D resistant rickets,

can be healed by short-term phosphate supplements[34-36]. Long-term phosphate supplementation should therefore be a logical form of treatment for a condition in which the renal loss of phosphate is chronic and significant, and this form of therapy has indeed been the subject of numerous clinical studies. Some of these studies yielded negative results[37,38], probably because the administration of phosphate was inadequate. However, when the phosphate dose is sufficient, and the clinical management is aggressive, serum phosphorus can be increased and healing of long standing "vitamin-D resistant" rickets results[22,39,43]. Despite these recorded experiences, phosphate is still used more as an adjunct to vitamin D therapy to avoid vitamin D intoxication, than as a primary component of therapy.

We have shown that combined phosphate and vitamin D treatment restores bone density to normal in our patients. Albright proposed originally[44] that hypophosphatemia stimulated bone resorption and that phosphate replacement merely prevented this event. Rasmussen[45] has challenged this interpretation and he suggests that Albright's own data indicated that phosphate replacement actually stimulates bone mineralization. Recent studies in the severely hypophosphatemic rat[46], show that when bone resorption is increased, bone mineralization and matrix formation is also severely inhibited; phosphate replacement stimulates bone formation in this situation. Similar mechanisms may account for the response in bone mineralization observed after phosphate treatment in X-linked hypophosphatemia.

Phosphate loading will cause serum calcium to fall and serum immunoreactive PTH to rise[18]. We have observed a similar response in our patients. The appearance of a characteristic reversible hyperaminoaciduria[11] and iminopeptiduria[12] when serum PTH was increased in our patients treated with phosphate, indicates that kidney and bone are apparently both responsive to PTH in X-linked hypophosphatemia. It is not unreasonable to suppose that this effect on bone may actually benefit its turnover, remodelling and linear growth during phosphate treatment.

Stimulation of the linear growth rate in our series of patients is probably the most surprising finding in the study. With the exception of one patient[47], we are not aware of any publication describing a similar response in patients treated primarily with vitamin D. The only other patients in whom an accelerated growth response to treatment has been described were treated with phosphate[7,41,48]. Harrison et al[41] were the first to report a positive correlation between serum Pi and growth rate. We observed that chronic hypophosphatemia in our patients was associated with a low whole-blood $P_{50}$, indicating a shift to the left of the oxygen dissociation curve. Phosphate treatment raised both the serum Pi and whole blood $P_{50}$ to the normal range. If depletion of serum inorganic phosphate inhibits 2,3-DPG synthesis in red cells[49], thus causing a fall in whole-blood $P_{50}$, it is possible that release of oxygen to tissues would be inhibited sufficiently over long periods of time to impair growth in patients with the X-linked trait. This

hypothesis has yet to be tested. The growth failure in hypophosphatemic rickets is clearly not the result of growth hormone deficiency[51], and it cannot be the direct result of rickets[52] since the bone disease can be healed with vitamin D while growth remains impaired.

The fact that growth failure is manifest particularly in the lower segment in X-linked hypophosphatemia[3] is not surprising. Linear growth in the child after infancy, and before adolescence, is most rapid in the lower segment. The latter comprises 50% of total height in childhood but only 30 percent of total height during infancy. The combined phosphate-vitamin D treatment regimen stimulated growth equally well in the lower segment, and in total body length.

The time in which the rise and fall of the Pi concentration in serum occurs after phosphate loading by mouth, is only about 4 hours. Consequently, phosphate must be given frequently if a near-normal serum Pi is to be maintained; a burden is thus imposed on the patient who must take large amounts of a salty liquid, at frequent intervals. The in-home treatment provides continuous support to the parents who must supervise the treatment. Supervision is constant so that interest in the patient's status is tangible. Several of our patients had endured "failed-phosphate" treatment before enrolement in the current program; each one responded satisfactorily after aggressive monitoring and supervision was initiated.

Another advantage of careful supervision was demonstrated in our study. There were few episodes of toxicity

in the period of observation (11,297 patient-treatment days). Vitamin D alone, as a major treatment mode, is accompanied by hazards which include hypercalcemia and nephrotoxicity[5-7]. Phosphate supplementation can reduce the hazards of hypercalcemia[53], and our program restrained the total duration of the hypercalcemic episodes to less than 0.5 percent of patient-treatment days. In no case was the hypercalcemia of serious magnitude, and we found no evidence of ectopic calcification or renal damage in our patients.

There are other real and potential inconveniences for the patient receiving phosphate supplementation. It may cause transient diarrhea and the taste of the mixture is unpleasant to some, although Joulie's solution is preferable to the usual "neutral" phosphate salt mixture. Moreover, the sodium salt intake is high in the phosphate mixture (up to 100 mEq/day) and the Joulie solution provides an acid load. Nevertheless, we have not detected hypertension or acidemia in any of our patients over the years; diarrhea has not been a problem; and some ingenious manipulations in the pharmacy and at home have improved the taste of treatment. At the present time we believe treatment with phosphate and vitamin D provides a useful way to neutralize the clinical effects of the mutation in X-linked hypophosphatemia, until a more basic approach is discovered which will correct the defect in phosphate transport.

ACKNOWLEDGEMENTS

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This paper was presented in part to the Society for Pediatric Research, Atlantic City, Apr. 28 to May 1, 1971[54].

## REFERENCES


1. Winters, R.W., Graham, J.B., Williams, T.F., McFalls, V.W. and Burnett, C.H.: A genetic study of familial hypophosphatemia and vitamin D resistant rickets with a review of the literature. Medicine, 37:97-142, 1958.
2. Stickler, G.B.: Familial hypophosphatemic vitamin D resistant rickets: The neonatal period and infancy. Acta Paediat. Scand., 58:213-219, 1969.
3. McNair, S.L. and Stickler, G.B.: Growth in familial hypophosphatemic vitamin D resistant rickets. New Eng. J. Med., 281:511-516, 1969.
4. Williams, T.F., Winters, R.W. and Burnett, C.H.: Familial (hereditary) vitamin D-resistant rickets with hypophosphatemia. In Stanbury, J.B., Wyngaarden,J.B. and Fredrickson, D.S., eds., The Metabolic Basis of Inherited Disease. 2nd edit. New York, McGraw-Hill, 1966, pp. 1179-1204.
5. Stickler, G.B., Jowsey, J. and Bianco, A.J.: Possible detrimental effect of large doses of vitamin D in familial hypophosphatemic vitamin D resistant rickets. J. Ped., 79:68-71, 1971.
6. Paunier, L., Kooh, S.W., Conen, P.E., Gibson, A.A.M. and Fraser, D.: Renal function and histology after long-term vitamin D therapy of vitamin D refractory rickets. J. Ped., 73:833-844, 1968.

7. Moncrieff, M.W. and Chance, G.W.: Nephrotoxic effect of vitamin D therapy in vit. D refractory rickets. Arch. Dis. Childh., 44:571-579, 1969.

8. Arnaud, C., Glorieux, F. and Scriver, C.R.: Serum parathyroid hormone in X-linked hypophosphatemia. Science, 173:845-847, 1971.

9. Glorieux, F. and Scriver, C.: Loss of a PTH-sensitive component of phosphate transport in X-linked hypophosphatemia. Science, (In Press, 1972).

10. Clow, C., Reade, T. and Scriver, C.R.: Management of hereditary metabolic disease. The role of Allied Health Personnel. New Eng. J. Med., 284:1292-1298, 1971.

11. Fraser, D., Kooh, S.W. and Scriver, C.R.: Hyperparathyroidism as the cause of hyperaminoaciduria and phosphaturia in human vitamin D deficiency. Ped. Res., 1, 425-435, 1967.

12. Scriver, C.R.: Glycyl-proline in urine of humans with bone disease. Canad. J. Physiol. & Pharmac., 42: 357-364; 1964.

13. Arnaud, C., Tsao, H.S. and Littledike, T: Radioimmunoassay of human parathyroid hormone in serum. J. Clin. Invest., 50:21-34, 1971.

14. Severinghaus, J.W.: Calculation of $P_{50}$. In Altman, A. and Dittmer, D., ed., Respiration and Circulation. Bethesda, Maryland. Fed. Am. Soc. Exp. Biol. 1967, p. 205.

23. Albright, F. and Sulkowitch, H.W.: The effect of vitamin D on calcium and phosphorus metabolism; studies on four patients. J. Clin. Invest., 17:305-315, 1938.

24. Williams, T.F.: Pathogenesis of familial vitamin D-resistant rickets. Ann. Int. Med., 68:706-707, 1968.

25. DeLuca H.F., Lund, J., Rosenbloom, A.L. and Lobeck, C.: Metabolism of tritiated vit. $D_3$ in familial vitamin D resistant rickets with hypophosphatemia. J. Pediat., 70:828-832, 1967.

26. Earp, H.S., Ney, R.L., Gitelman, H.J., Richman, R. and DeLuca, H.F.: Effects of 25-hydroxycholecalciferol in patients with familial hypophosphatemia and vitamin D resistant rickets. New Eng. J. Med., 283:627-630, 1970.

27. Balsan, S.: 25-hydroxycholecalciferol: Effects in idiopathic vitamin D-resistant rickets. Calc. Tiss. Res. 4 (Suppl.) 45-46, 1970.

28. Fraser, D., Leeming, J.M., Cerwenka, E.A. and Kengeres, K.: Studies of the pathogenesis of the high renal clearance of phosphate in hypophosphatemic vitamin D refractory rickets of the simple type. Amer. J. Dis. Child., 98:586-587, 1959.

29. Falls, W.F. Jr., Carter, N.W., Rector, F.C. Jr., and Seldin, D.W.: Familial vitamin D-resistant rickets study of six cases with evaluation of the pathogenetic role of secondary hyperparathyroidism. Ann. Intern. Med., 68: 553-560, 1968.

30. Lavender, A.R. and Pullman, T.N.: Changes in inorganic phosphate excretion induced by renal arterial infusion of calcium. Amer. J. Phys., 205:1025-1032, 1963.

15. Tanner, J.M., Whitehouse, R.H. and Takoushi, M.: Standards from birth to maturity for height, height, height velocity and weight velocity in British Children I, II. Arch. Dis. Childh., 41:454-471; 613-635, 1966.

16. Colbert, C., Mazess, R.B. and Schmidt, P.B.: Bone mineral determination in vitro by radiographic photodensitometry and direct photon absorptiometry. Invest. Radiology, 5:336-340, 1970.

17. Campbell, R.C.: Statistics for Biologists. Cambridge Univ. Press. 1967, pp. 49-50.

18. Reiss, E., Canterbury, J.M., Bercowitz, M.A. and Kaplan, E.L.: The role of phosphate in the secretion of parathyroid hormone in man. J. Clin. Invest. 49: 2146-2149, 1970.

19. Albright, F., Butler, A.M. and Bloomberg, E.: Rickets resistant to vitamin D therapy. Amer. J. Dis. Child., 54:529-547, 1937.

20. Fanconi, G. and Girardet, P.: Familiarer persistienrender phosphatdiabetes mit D-vitamin-resistentes rachitis. Helv. Paed. Acta, 7:14-41, 1952.

21. Dent, C.E.: Rickets and osteomalacia from renal tubule defects. J. Bone Jt Surg., 34B:266-274, 1952.

22. Wilson, D.R., York, S.E., Jaworski, Z.F. and Yendt, E.R.: Studies in hypophosphatemic vitamin D refractory osteomalacia in adults. Medicine, 44:99-134, 1965.

31. Boyle, I.T., Gray, R.W. and DeLuca, H.F.: Regulation by calcium of in vivo synthesis of 1,25-dihydroxycholecalciferol and 21,25-dihydroxycholecalciferol. P.N.A.S., 68:2131-2134, 1971.

32. Puschett, J.B., Moranz, J. and Kurnick, W.S.: Evidence for a direct action of cholecalciferol and 25-hydroxycholecalciferol on the renal transport of phosphate, sodium and calcium. J. Clin. Invest., 51:373-385, 1972.

33. Lilly, C.A., Peirce, C.B. and Grant, R.L.: The effects of phosphates on the bones of rachitic rats. J. Nutr., 9:25-35, 1935.

34. Fraser, D., Jaco, N.T., Yendt, E.R., Munn, J.D. and Liu, E.: The induction of in vitro and in vivo calcification in bones of children suffering from vitamin D-resistant rickets without recourse to large doses of vitamin D. Amer. J. Dis. Child., 93:84-85, 1957.

35. Fraser, D., Geiger, D.W., Munn, J.D., Slater, P.E., John, R. and Liu, E.: Calcification studies in clinical vitamin D deficiency and in hypophosphatemic vitamin D refractory rickets: the induction of calcium deposition in rachitic cartilage without the administration of vitamin D. Amer. J. Dis. Child., 96:460-463, 1958.

36. Steendijk, R.: The effect of a continuous intravenous infusion of inorganic phosphate on the rachitic lesions in cystinosis. Arch. Dis. in Childh.,36:321-324, 1961.

37. Frame, B., Smith, R.W. Jr. and Fleming, J.L.: Oral phosphates in vitamin D refractory rickets and osteomalacia. Amer. J. Dis. Child., 106:147-153, 1963.

38. Stickler, G.B., Hoyles, A.B. and Rosevear, J.W.: Familial hypophosphatemic vitamin D-resistant rickets. Amer. J. Dis. Child., 110:664-667, 1965.

39. Scriver, C.R., Goldbloom R.B. and Roy, C.C.: Hypophosphatemic rickets with renal hyperglycinuria, renal glucosuria and glycyl-prolinuria. A syndrome with evidence for renal tubular secretion of phosphorus. Pediatrics, 34:357-371, 1964.

40. West, C.D., Blanton, J.C., Silverman, F.N. and Holland, N.H.: Use of phosphate salts as an adjunct to vitamin D in the treatment of hypophosphatemic vitamin D refractory rickets. J. Pediatrics., 64:469-477, 1964.

41. Harrison, H.E., Harrison, H.C., Lifshitz, F. and Johnson, A.D.: Growth disturbance in hereditary hypophosphatemia. Amer. J. Dis. Child., 112:290-297, 1966.

42. Nagant de Deuxchaisnes, C. and Krane, S.M.: The treatment of adult phosphate diabetes and Fanconi syndrome with neutral sodium phosphate. Amer. J. Med., 43:508-543, 1967.

43. Menking, M. and Sotos, J.F.: Effect of administration of oral neutral phosphate in hypophosphatemic rickets. J. Pediatrics, 75:1001-1007, 1969.

44. Albright, F., Bauer, W., Claflin, D. and Cockrill, J.R.: Studies in parathyroid physiology. III. The effect of phosphate ingestion in clinical hyperparathyroidism. J. Clin. Invest., 11:411-435, 1932.

45. Rasmussen, H.: Ionic and hormonal control of calcium homeostasis. Amer. J. Med., 50:567-588, 1971.

46. Baylink, D., Wergedal, J. and Stauffer, M.: Formation, mineralization and resorption of bone in hypophosphatemic rats. J. Clin. Invest., 50:2519-2530, 1971.

47. Schoen, E.J. and Reynolds, J.B.: Severe familial hypophosphatemic rickets. Normal growth following early treatment. Amer. J. Dis. Child., 120:58-61, 1970.

48. Tracy, W.E., Steen, J.C., Steiner, J.E. and Buist, N.R.M.: Analysis of dentine pathogenesis in vitamin D-resistant rickets. Oral Surg., 32:38-44, 1971.

49. Travis, S.F., Sugerman, H.G., Ruberg, R.L. Dudrick, S.J., Delivoria-Papadopoulos, M., Miller, L.D. and Oski, F.A.: Alterations of red cell glycolytic intermediates and oxygen transport as a consequence of hypophosphatemia in patients receiving intravenous hyperalimentation. New Eng. J. Med., 285:763-768, 1971.

50. Brewer, G.J. and Eaton, J.W.: Erythrocyte metabolism: interaction with oxygen transport. Science, 171: 1205-1211, 1971.

51. Gerschberg, H., Neumann, L.L. and Mari, S.: Studies on vitamin D-resistant rickets. Effects of human growth hormone. Metabolism, 13: 636-649, 1964.

52. Steendijk, R.: Studies on growth in refractory rickets. J. Pediatrics, 60:340-345, 1962.

53. Goldsmith, R.S. and Ingbar, S.N.: Inorganic phosphate treatment of hypercalcemia of diverse etiologies. New Eng. J. Med., 274:1-7, 1966.

54. Glorieux, F., Arnaud, C., Clow, C., Goldman, H., Reade, T. and Scriver, C.: X-linked hypophosphatemic rickets: A PTH-insensitive transport defect responsive to phosphate. Ped. Res., 5:392, 1971.

TABLE I

| Patient | Sex | Birthdate | Age at Diagnosis (yr) | Height (percentile)[b] | Plasma Values[a] Ca (mg%) | Pi (mg%) | Renal Clearance of Pi[c] (ml/min/1.73m$^2$) | Status of Serum Pi[a] (mg%) | Mother Bone Disease |
|---|---|---|---|---|---|---|---|---|---|
| M.Ch. | F | 7/ 4/60 | 6 6/12 | <3rd | 9.6 | 2.3 | 43 | 2.8 | o |
| S.V. | F | 25/ 7/60 | 2 3/12 | <3rd | 9.2 | 2.6 | 51 | 1.8 | + |
| I.B. | F | 14/ 8/62 | 5 10/12 | <3rd | 9.1 | 2.2 | 67 | 2.3 | o |
| D.R. | M | 20/12/56 | 7 10/12 | <3rd | 10.2 | 2.6 | 68 | 1.5 | + |
| E.M. | M | 25/ 6/59 | 0 8/12 | <3rd | 9.3 | 3.3 | 67 | 1.5 | + |
| L.A. | M | 16/ 6/59 | 3 | <3rd | 10.4 | 3.0 | 70 | 3.0 | o |
| J.B. | M | 26/ 9/62 | 3 1/2 | <3rd | 10.0 | 2.2 | 39 | 2.3 | o |
| M.C. | M | 1/11/67 | 2 3/12 | <3rd | 9.7 | 2.2 | 58 | 1.8 | + |

a) Determinations done on a morning blood sample, after an overnight fast.

b) Derived from Tanner's charts (see Figure 2).

c) Normal $C_{Pi}$ <16ml/min/1.73m$^2$.

TABLE II

CONTROL OF SERUM Pi DURING 12 CONSECUTIVE MONTHS OF PHOSPHATE REPLACEMENT IN CHILDREN WITH X-LINKED HYPOPHOSPHATEMIA.

| PATIENT | AGE (yrs) | SERUM Pi (mg %) Mean ± SD | HOME VISITS (number/12 months) |
|---|---|---|---|
| M.Ch. | 10 | 3.7 ± 1.1 | 30 |
| S.V. | 10 | 2.8 ± 0.4 | 35 |
| I.B. | 9 | 4.5 ± 0.6 | 18 |
| D.R. | 13 | 4.2 ± 1.0 | 35 |
| E.M. | 11 | 3.5 ± 1.3 | 28 |
| L.A. | 11 | 5.1 ± 1.4 | 24 |
| J.B.[a] | 8 | 3.2 ± 0.6 | - |
| M.C.[b] | 3 | 4.9 ± 0.9 | (7) |
| | Mean | 4.0 ± 0.8 | 28.3 |

All blood samples (excepting patient J.B.) obtained in the home or at school by visiting nurse (T.R.). The frequency of visits is indicated. Serum was separated from the clot within 2 hours of sample collection.

a) Domiciled 90 miles from Montreal; regular visits to local hospital were substituted for home visits by our team. Fifteen hospital visits were recorded.

b) For six-month period (April-Oct., 1971) calculation of home visit frequency not included in calculation of mean frequency for the group.

TABLE III

EFFECT OF PHOSPHATE TREATMENT ON PARATHYROID ACTIVITY IN X-LINKED HYPOPHOSPHATEMIA

| PATIENT | High Phosphate Regimen[a] | | | | Moderate Phosphate Regimen[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | SERUM Pi (mg%) | SERUM CALCIUM (mg%) | SERUM IPTH (µlEq/ml) | URINE AMINO ACIDS[c] | SERUM Pi (mg%) | SERUM CALCIUM (Mg%) | SERUM IPTH (µlEq/ml) | URINE AMINO ACIDS[c] |
| M.C. | 5.4 | 8.9 | 290 | general increase | 3.9 | 10.4 | 40 | normal |
| L.A. | 5.0 | 9.3 | 100 | general increase | 3.8 | 11.4 | 67 | normal |
| E.M. | 4.4 | 7.4 | 750 | general increase | 3.9 | 10.6 | 43 | hyper-glycinuria |
| J.B. | 3.4 | 9.4 | 90 | hyper-glycinuria | 3.4 | 10.0 | 36 | normal |
| Average | 4.5 | 8.7 | 307 | increased | 3.7 | 10.6 | 46.5 | normal |

Normal serum values: Pi, >4.0 mg%; calcium, 9-11 mg%; IPTH, <40 µlEq/ml.

a) Pi supplement, 3-4 g/day; Vitamin D, <50,000 units/day

b) Pi supplement, 1-3 g/day; Vitamin D, 100,000 units/day x 3 weeks or more

c) Amino aciduria assessed by partition chromatography[11].

FIGURE 1

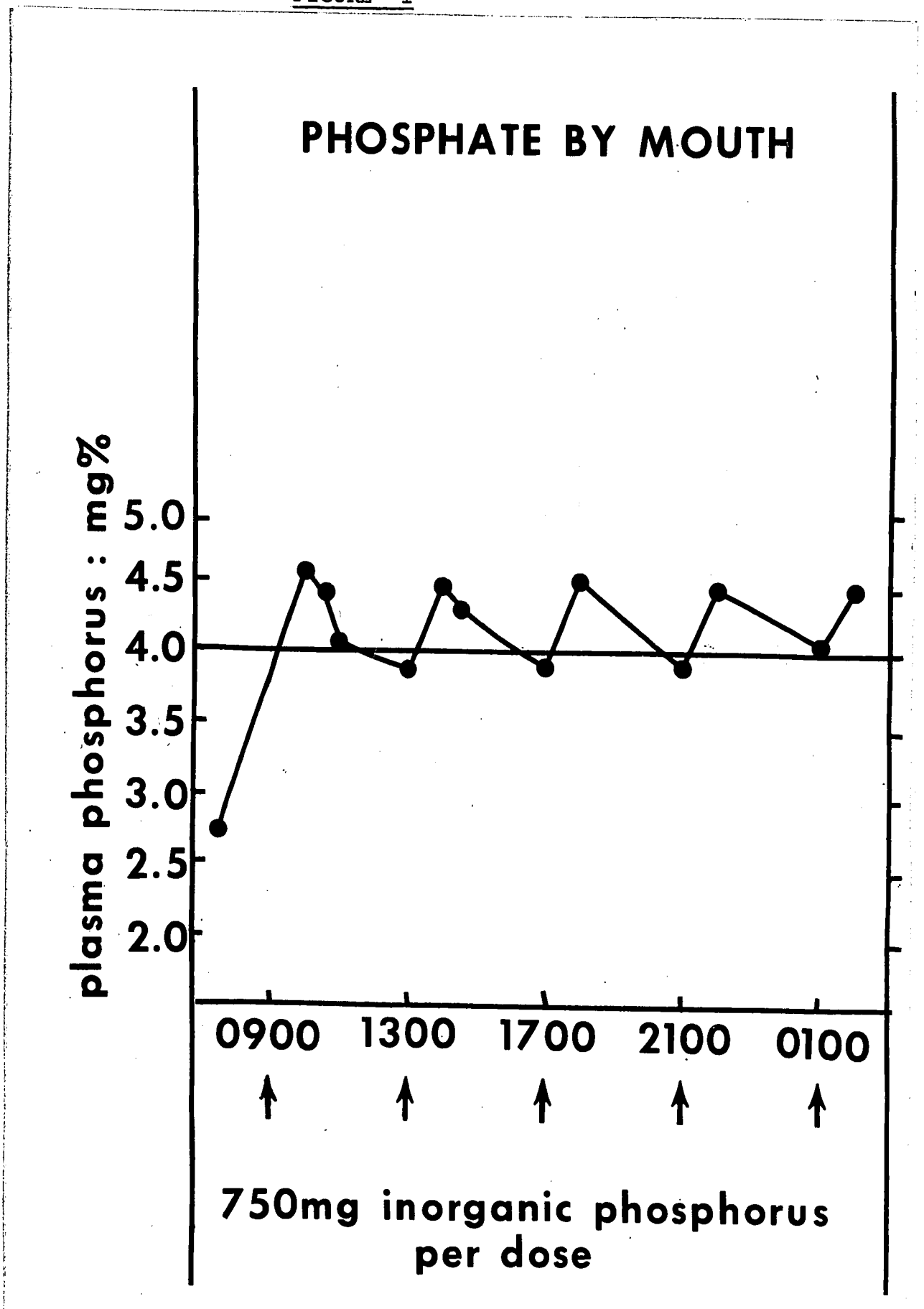
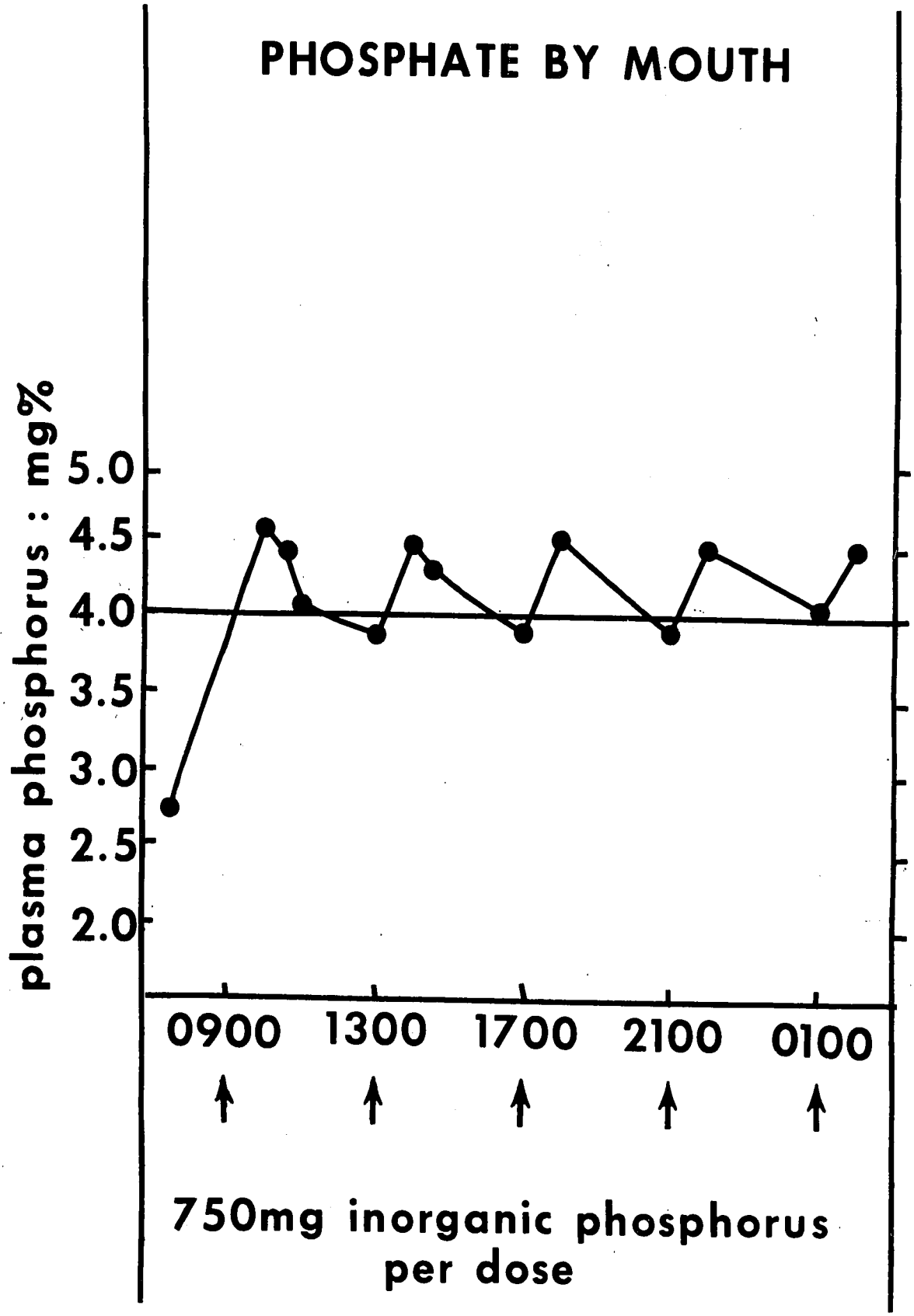

PHOSPHATE BY MOUTH
plasma phosphorus : mg%
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0900
1300
1700
2100
0100
750mg inorganic phosphorus
per dose

FIGURE 1


PHOSPHATE BY MOUTH
plasma phosphorus : mg%
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0900
1300
1700
2100
0100
750mg inorganic phosphorus per dose

LEGEND

Figure 1: Effect of repeated loading by mouth with an inorganic phosphate mixture on the concentration of inorganic phosphate in serum of a male patient with X-linked hypophosphatemia. The diurnal timing and amount of phosphorus administered in the mixture is indicated.

FIGURE 2 (left)

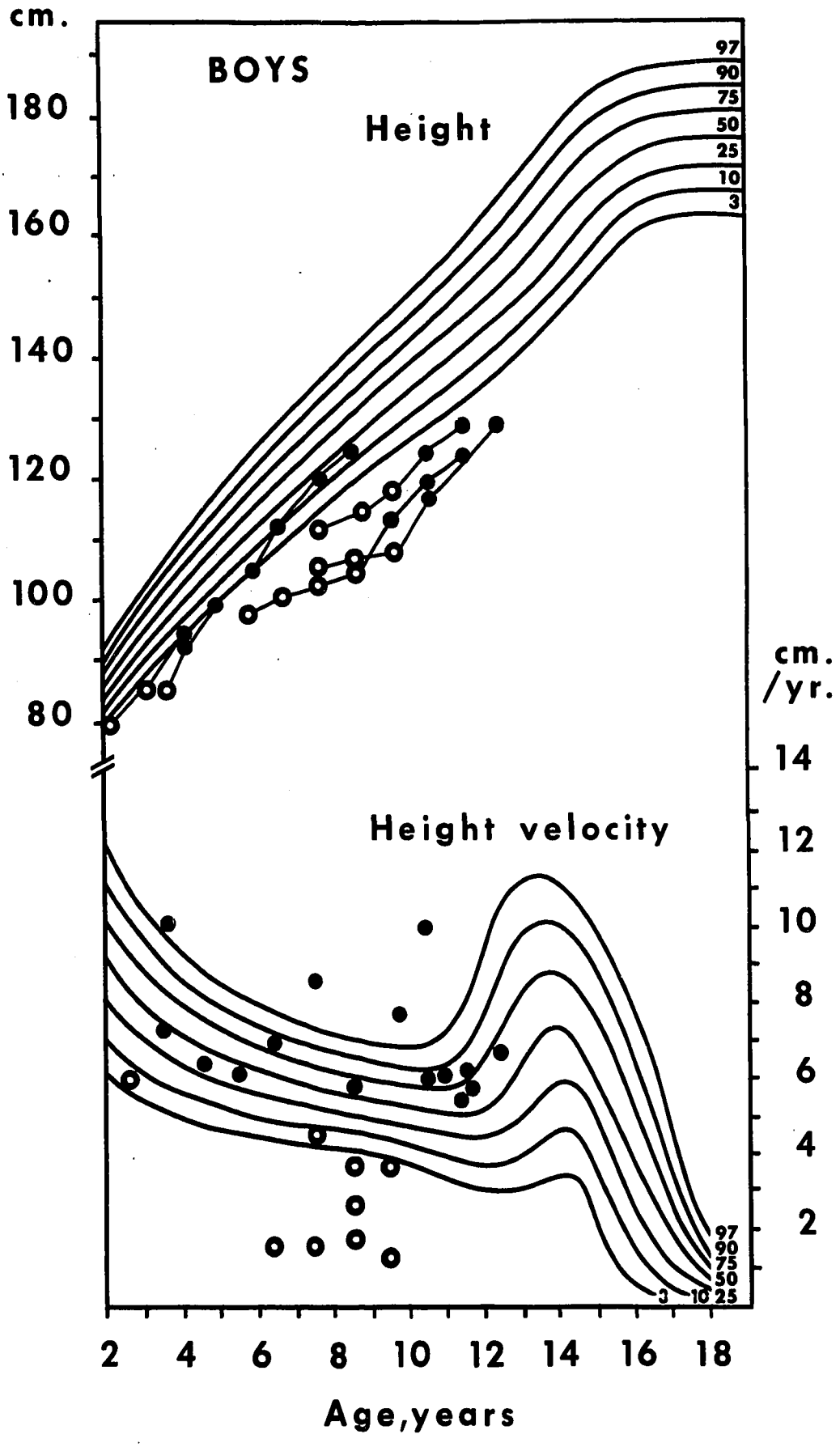


FIGURE 2 (left)

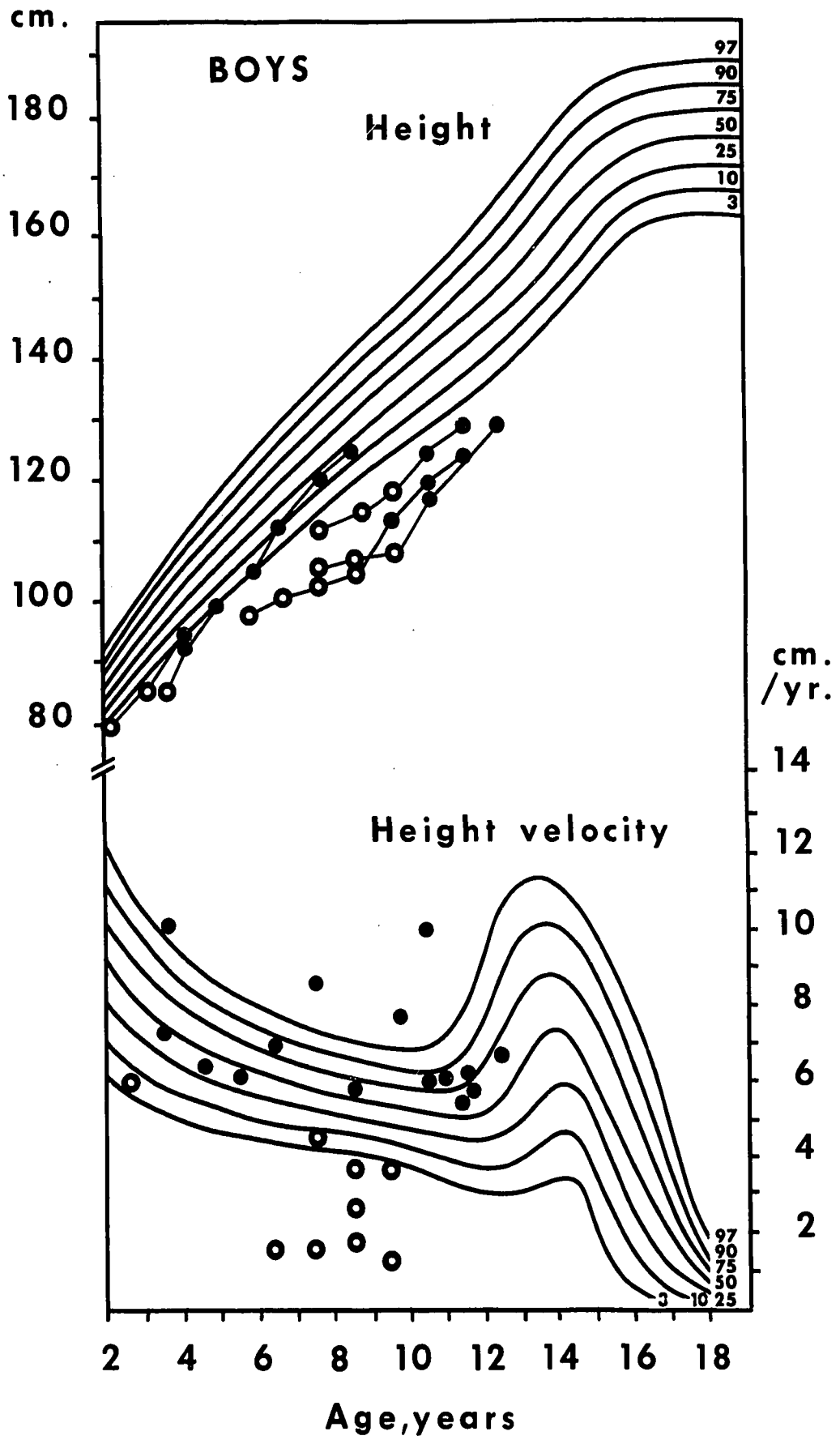


FIGURE 2 (right)

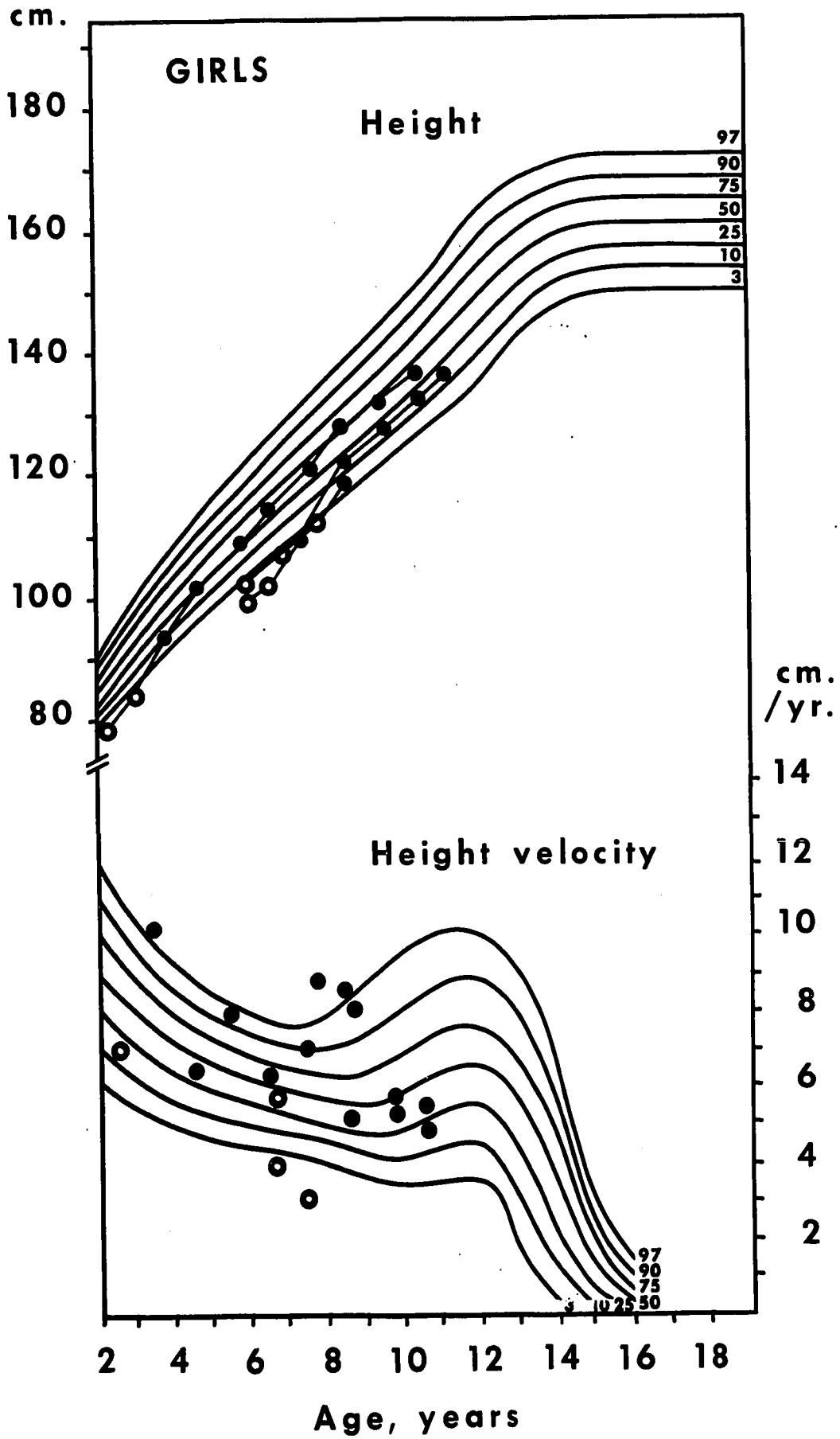
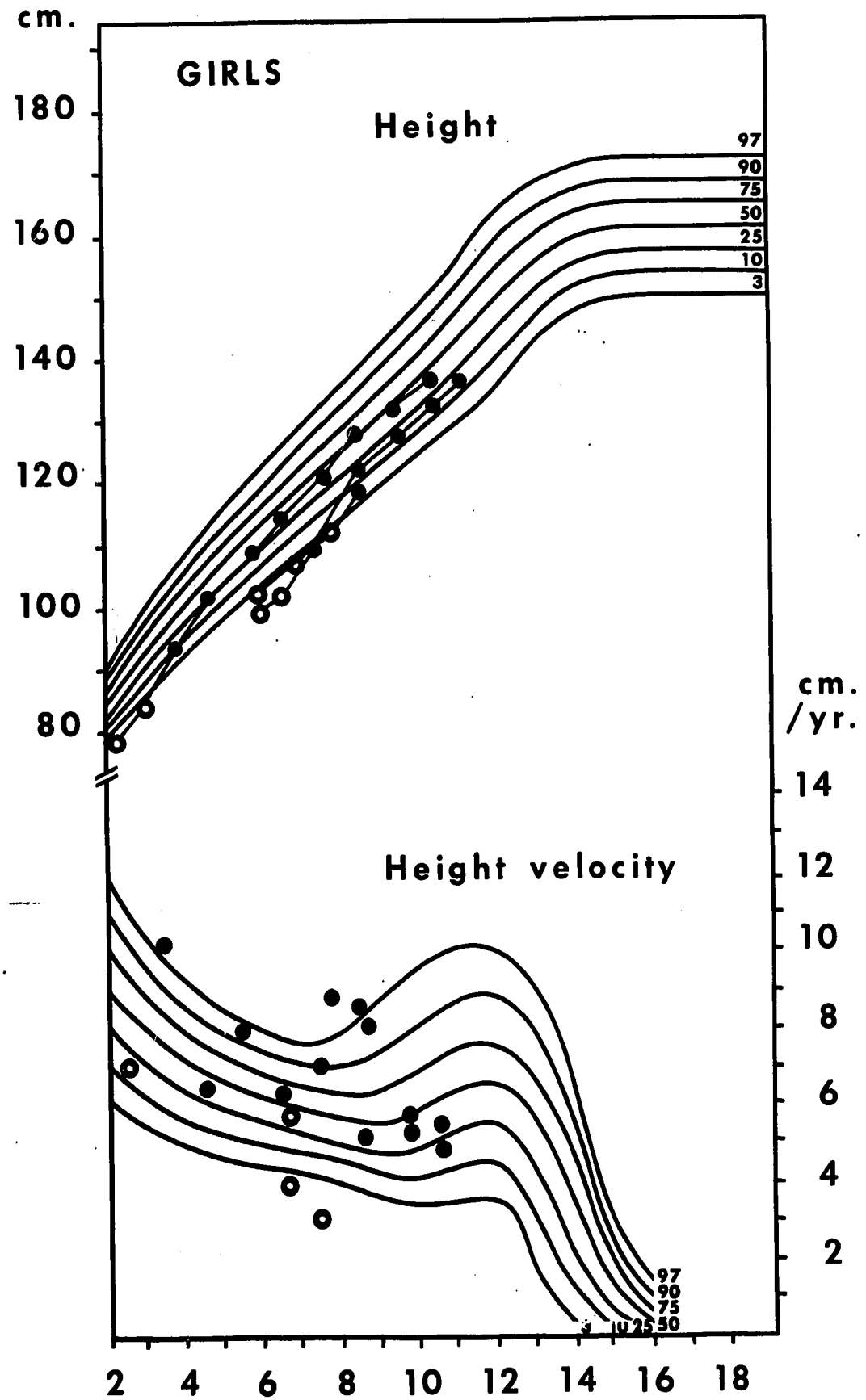


FIGURE 2 (right)



LEGEND

Figure 2: Growth charts for boys (left) and girls (right) before (open circles) and during (closed circles) phosphate supplementation. Vitamin D alone was the major form of treatment given before the combined regime was begun. The upper graphs show growth as a linear measurement related to age. The lower graphs indicate the rate of growth (gain in height per year). Graphs show percentiles for a normal reference population as described by Tanner et al[15].

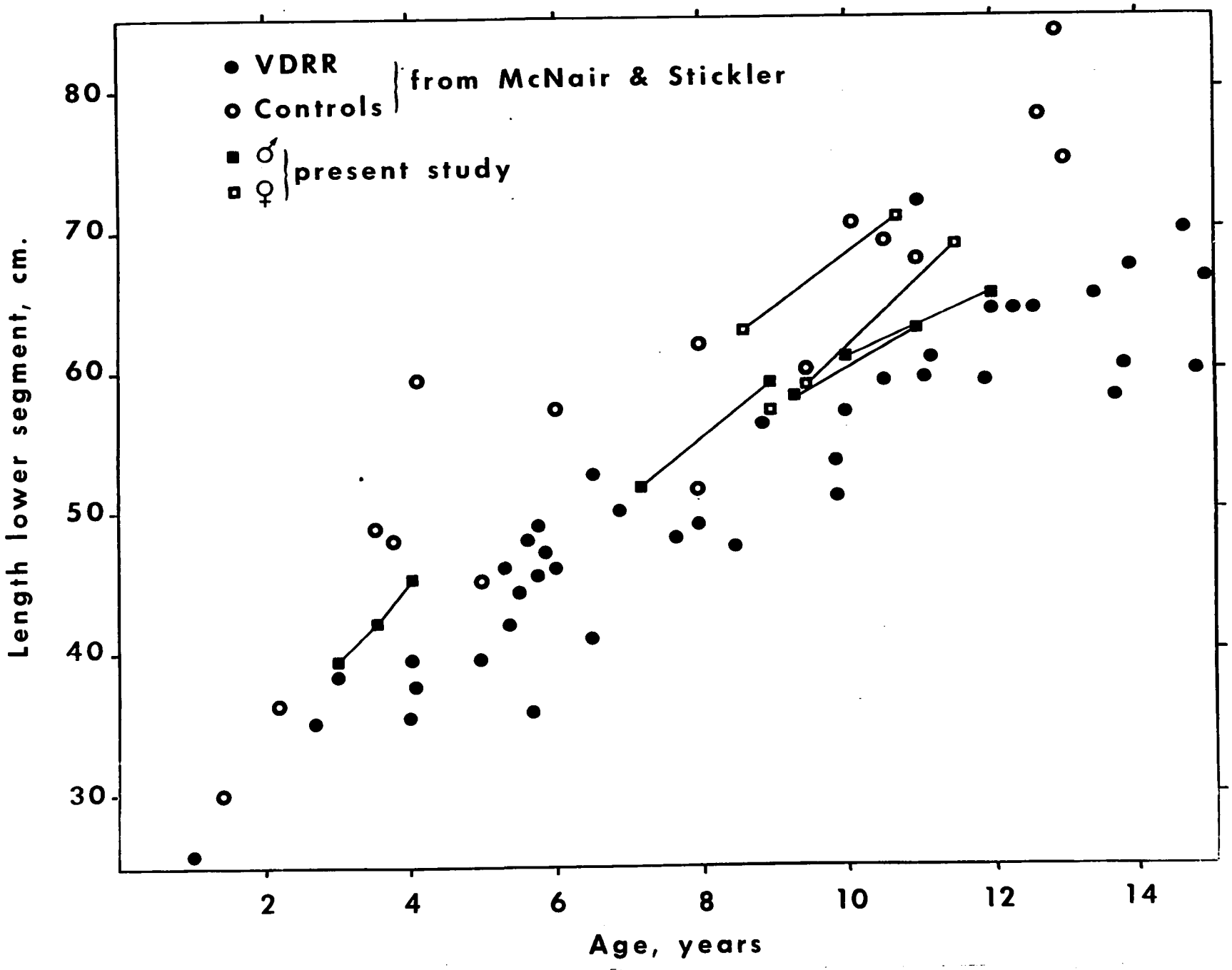

VDRR
Controls
from McNair & Stickler
♂
♀
present study
Length lower segment, cm.
80
70
60
50
40
30
2
4
6
8
10
12
14
Age, years

FIGURE 3

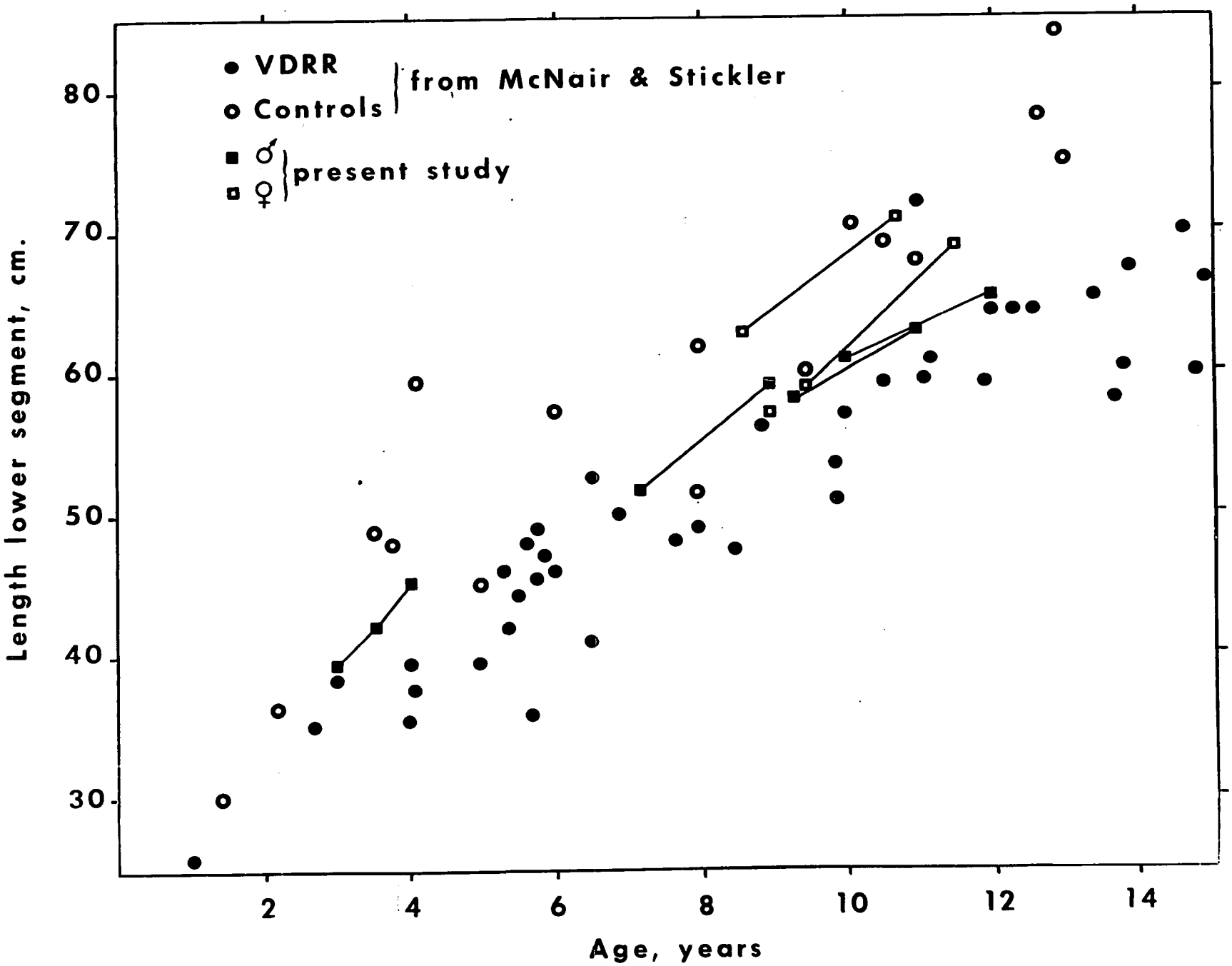

VDRR
Controls
from McNair & Stickler
♂
♀
present study
Length lower segment, cm.
80
70
60
50
40
30
2
4
6
8
10
12
14
Age, years

FIGURE 3

## LEGEND

Figure 3: Growth in the lower segment of patients treated with phosphate and vitamin D, compared with lower-segment growth of patients treated with vitamin D alone as reported by McNair and Stickler[3].

FIGURE 4

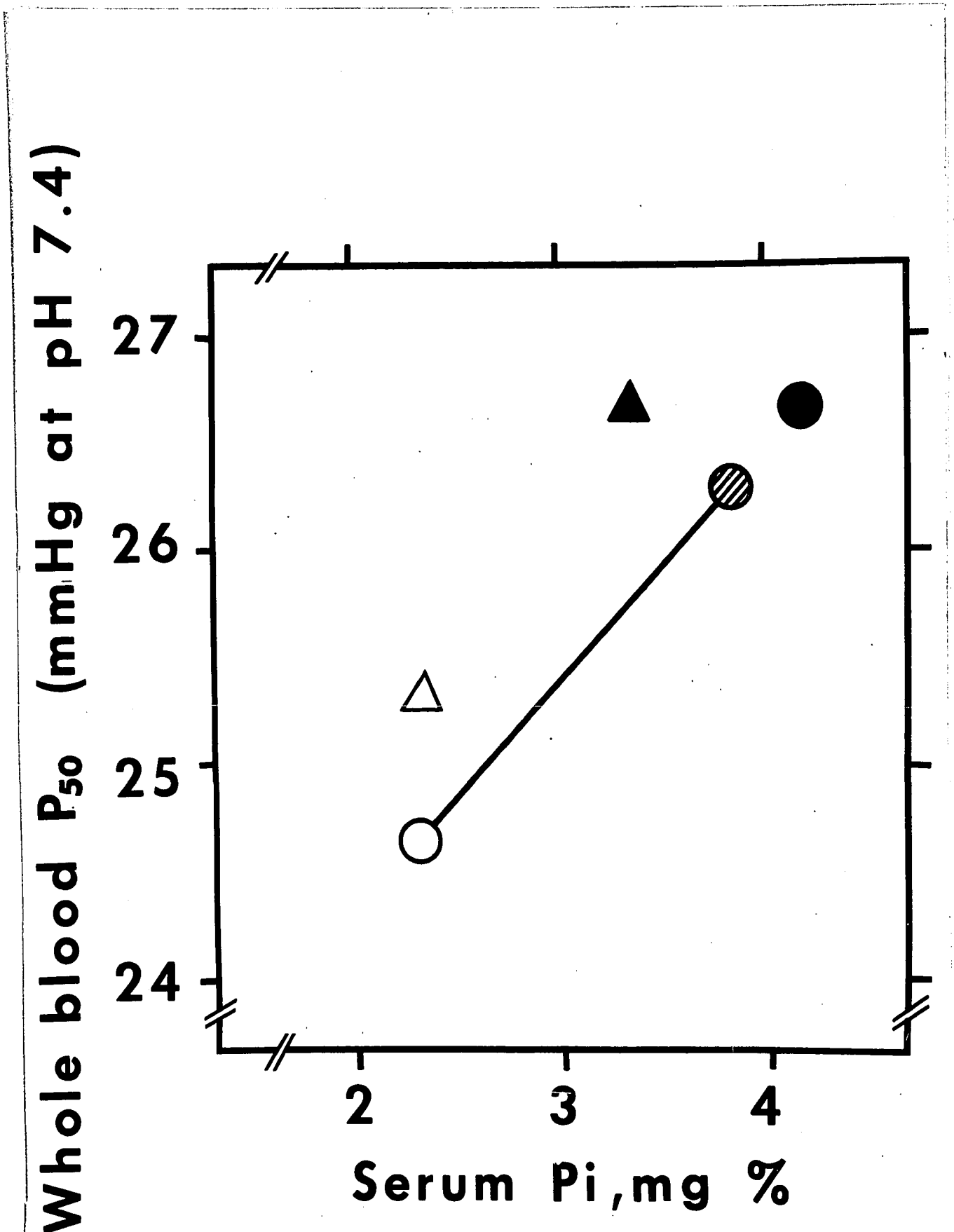
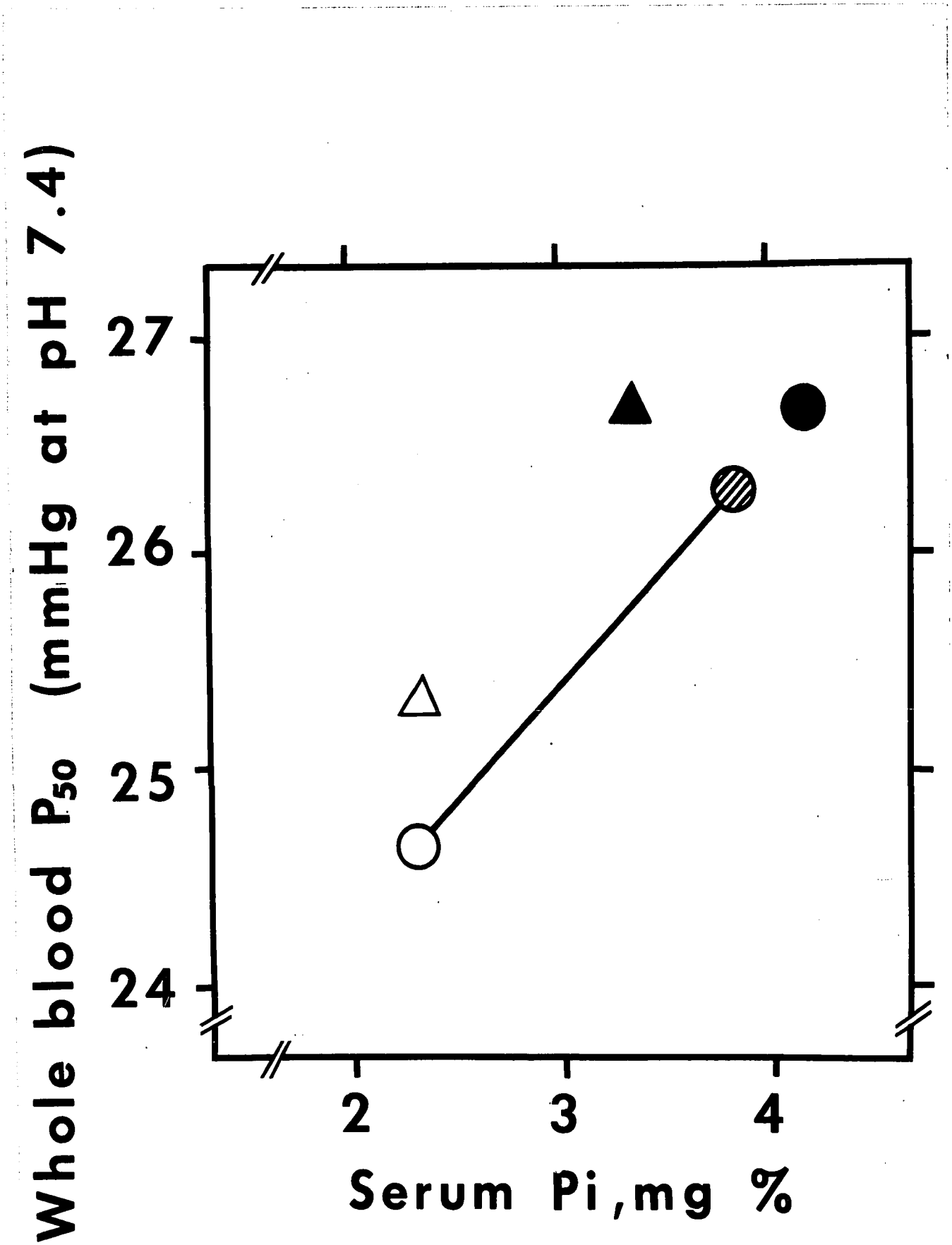


FIGURE 4



LEGEND

Figure 4: Relationship between serum phosphorus (Pi) and whole blood $P_{50}$ value in 8 untreated adult female heterozygotes (Δ) and 8 untreated children (O) with X-linked hypophosphatemia. Controls for adults (▲) and children (●) were individually matched for age and sex. Phosphate treatment of children with the disease (Ø) raises the serum Pi and whole blood $P_{50}$.

## COMMENTARY

Detailed discussions are given in the published and pending manuscripts which form the body of this thesis. Only a general perspective to the thesis work is presented in the following commentary.

The products of gene action are represented by the polypeptide chains. These essential constituents of the mammalian organism are ubiquitous. For instance, they are found in the membranes where as "carriers" they are essential to the transfer of organic solutes across the cellular membrane; as enzymes and hormones they regulate cellular metabolism; in the body fluids they regulate the osmotic pressure and selectively bind certain small molecules. The size, shape and specific function of a protein originates partly in the primary amino acid sequence. This sequence depends on the nucleotide sequence of the gene which codes for the polypeptide chain. Therefore a mutation in the structural gene will be expressed as an inherited variation in protein structure. Some will be easily detected if they produce important chemical or clinical disturbance but some will be more difficult to characterize because they are incompatible with life or,on the other hand,bear no significant functional disturbance and thus may not come readily to attention. All these concepts are now part of the central dogma in molecular biology and were alluded to with great prescience by Garrod in his book "Inborn Factors of Disease" published in 1931.

In his first descriptions of inborn errors of metabolism, Garrod placed emphasis on the enzyme defects producing a block in a metabolic pathway. Sarcosinemia studied in the first part of this thesis is a good example of this type of mutation. The presumed block affects sarcosine oxidase and leads to the accumulation of sarcosine in the organism. The normal enzyme exhibits tissue specificity for it is found only in the liver and the kidney. The genetic trait, sarcosinemia is apparently harmless and therefore appears of limited clinical interest. The mental retardation reported in some cases seems to be purely coincidental. However from the biochemical genetic viewpoint, many aspects of hypersarcosinemia are of significance. The phenotype of the homozygote and the heterozygote has been clearly defined. The other interesting fact concerns the tubular transport of sarcosine. The incidental discovery of the mutant individual studied in this thesis program was made by screening urine amino acids by partition chromatography. Since the plasma concentration of sarcosine was abnormally high, the mechanism for the hypersarcosinuria was classified as a "prerenal" or "overflow" aminoaciduria. By infusion methods in the blocked catabolic mutant and in a normal individual, the maximum reabsorption capacity was demonstrated to be intact in the proband. Therefore the transport system for sarcosine in the kidney tubule is not affected by the mutation or by the high filtered load of sarcosine. Moreover since the kidney is one of the only two tissues where sarcosine oxidation normally takes place, the proband who is in effect a blocked catabolic mutant for sarcosine oxidation provided the opportunity to

study the transport of sarcosine independent of its metabolism in kidney. The independence of these two events observed in vivo was confirmed with an in vitro system in rat kidney. The in vivo studies have indicated that sarcosine (N-methyl-glycine) is transported primarily by systems serving proline, hydroxyproline and glycine uptake. The competitive inhibition and exchanges observed among that group of substrates in vitro suggest that the main carrier available for sarcosine transport in kidney is the high capacity system shared by imino acids and glycine. Sarcosine is not normally present in significant amounts in the extracellular fluid and glomerular filtrate and it does not have a transport system of its own. When transport is required, it parasitizes the transport systems of other substrates. The relevance of this concept with respect to drug transport was touched upon in the relevant publication (Section IV).

The other problem investigated in this thesis was: X-linked hypophosphatemia. This trait can probably be included among the inborn errors of membrane transport. The impaired phosphate reabsorption has a "renal" mechanism. The hyperphosphaturia is not due to "overflow" as in the hypersarcosinuria. There is a chronic depletion of the inorganic phosphate pool as a consequence of the renal leak. The impairment of phosphate transfer across the cellular membrane in the kidney could reflect the genetic absence of a carrier protein or might involve abnormalities in the coupling of the transport process to the source of energy. The latter mechanism is thought to be involved in the Fanconi syndrome where there is

a generalized defect for reabsorption of various solutes including glucose, amino acids,potassium,bicarbonate and phosphate. Because transport defect in X-linked hypophosphatemia is highly selective, it is appealing to suggest a defect in the binding of the ion to a specific reactive site. Precedence for this interpretation is found in certain genetically determined defects of phosphate transport which have been identified in microorganisms (Medveczky, N. and Rosenberg, H., BBA 241, 494, 1971.). The data presented in this thesis indicate that the saturable mode of phosphate transport present in the normal human kidney is defective in the mutant for X-linked hypophosphatemia. The defect is complete in the affected male and variably expressed in the heterozygous female. This is compatible with the dose effect of a single gene located on an X-chromosome. However, in the male patient, a mode of transport for phosphorus is retained. This component of Pi transport is apparently saturable but because of its low capacity it is unable to maintain an inorganic phosphate concentration compatible with a normal phenotype. This transport component is not affected by parathyroid hormone but can apparently be modulated by the calcium ion concentration. On the basis of these findings, we propose that transport of phosphorus in human kidney involves at least two components. The net secretion phosphorus which is sometimes observed in the hemizygous individuals, is apparently influenced by calcium. Tubular efflux of inorganic phosphate may thus occur on the second component of phosphate transport.

The data do not allow one to determine whether the two components of phosphate transport are located at the same or at opposite poles of the tubular epithelial cell. We also do not know whether other tissues in man have comparable modes of phosphate transport that could be affected by the X-linked mutation.

Some aspects of the mutant phenotypes particularly in the heterozygous female are difficult to explain solely on the basis of a renal transport defect. For example, the absence of dwarfism and bone disease in some females presenting with constant hypophosphatemia merits further study.

The data presented here give strong support to the presence of a tubular transport defect in X-linked hypophosphatemia. They do not allow, however to determine whether the defect is primary, or secondary to an, as yet, unidentified factor. The independence from parathyroid hormone activity has been established but the role of vitamin D and its recently identified polar metabolites, is far from clear. It is known that hypophosphatemic mutants are able to synthesize 25-hydroxycholecalciferol, the circulating or "hormonal" form of the vitamin D. The possibility that 21,25-dihydroxycholecalciferol, a vitamin D metabolite synthesized in the kidney of normocalcemic rats, is involved in tubular phosphate reabsorption, has been recently suggested (see References in Section VI). There is however no correlation between the plasma phosphate concentration and the concentration of 21,25-$(OH)_2D_3$ in the rat. The hypothesis that a partial defect in the synthesis of that substance could play a role in the phosphate transport

defect identified in X-linked hypophosphatemia and explain the resistance to vitamin D cannot be tested until the vitamin D metabolite is made available for clinical trial.

The information gained from the characteristics of phosphate transport in human kidney indicated a mode of treatment more successful than previously reported attempts employing vitamin D as the principle pharmacologic agent. In the absence of a way to correct the primary defect, it was logical to try to offset the mutant gene by a continuously augmented dietary intake of inorganic phosphate to offset renal loss. The possibility to restore the body pool in the absence of an efficient phosphate transport system may be due to the presence in the cell membrane of a non saturable component for phosphate transport as documented in the red blood cell by Schrier (see References in Section Vc). The positive results obtained are rather unique in the literature and allow one to consider it as the best available method to neutralize the effect of the X-linked mutation.

## CLAIMS TO ORIGINAL WORK

1. In Sarcosinemia:

   1. Dietary folic acid does not influence the plasma level of sarcosine in the hypersarcosinemic subject. Therefore the mutation probably does not involve a deficiency of the acceptor of the "one-carbon moeity" during oxidation of sarcosine to glycine.
   2. Delineation of homozygous and heterozygous phenotypes is possible by examination of plasma sarcosine and glycine after sarcosine load. An autosomal recessive inheritance for sarcosinemia is clearly suggested.
   3. Sarcosine dehydrogenase is normally absent in cultured human skin fibroblasts and leukocytes. (These studies were made before the same conclusions were reported by other investigators).
   4. Renal tubular transport of sarcosine is normal in the blocked catabolic mutant state known as sarcosinemia. Renal uptake of sarcosine is, therefore, not influenced by its own catabolism.
   5. Sarcosine transport in kidney is mediated and concentrative and there is interaction with glycine and proline transport.
   6. Sarcosine has no apparent transport system of its own. It parasitizes the low affinity high capacity transport system shared by imino acids and glycine, and the specific high affinity low capacity transport systems for L-proline and glycine respectively.

2. In X-linked hypophosphatemia:

A. Transport studies

1. A normal level of immunoreactive parathyroid hormone in X-linked hypophosphatemic rickets was proven for the first time.
2. Tubular reabsorption of phosphorus is normally saturable. Reabsorption is reduced and completely saturated in the mutant hemizygotes. Phosphate transport is not affected by the level of circulating parathyroid hormone in this circumstance.
3. Tubular transport of phosphorus in human kidney involves at least two components: one, is PTH-sensitive, and is responsible for about two-thirds of the total capacity; the other is insensitive to PTH but can be modulated by calcium ion concentration.
4. Hemizygotes for X-linked hypophosphatemia are completely lacking the PTH-sensitive transport system for phosphate.
5. Intravenous calcium infusion rapidly enhances net reabsorption of phosphorus by the system revealed in the mutant hemizygote.
6. Net tubular secretion of phosphorus can occur in the mutant hemizygote. This net flux from plasma to the tubular lumen, is proportional to the plasma phosphate concentration and can be suppressed by a rapid intravenous calcium infusion.
7. The heterozygous females show variable expression of the"transport phenotype" fully expressed in the hemizygotes.

8. Intravenous infusion of bovine PTH does not influence phosphate reabsorption in mutant hemizygotes in contrast to the normal response. The response in heterozygotes is intermediate between the normal and the hemizygote range.
9. PTH has the expected effect on calcium excretion in urine in the mutant phenotype.
10. Excretion of adenosine 3',5'-monophosphate is normal in X-linked hypophosphatemic subjects.

B. Relaxed selection in X-linked hypophosphatemia

1. Continuous phosphate supplementation in the diet maintains a satisfactory level of phosphate in blood in the mutant phenotype.
2. Reversible secondary hyperparathyroidism is induced by phosphate therapy.
3. Prolonged phosphate therapy accelerates the growth rate. Normal height has been achieved in 5 of our 8 dwarf patients. (The rickets also heal and good bone mineralization is achieved as shown by other investigators).
4. Whole blood $P_{50}$ is influenced by the changes in plasma phosphate concentration. The value is abnormal in the affected patients untreated and is corrected towards normal during treatment.