VITAMIN D DEFICIENCY AND AMINO ACID EXCRETION IN THE RAT

by

John H. Grose

Presented to the Dean and
Faculty of Graduate Studies
in partial fulfilment of
the requirements for the
Degree of Master of Science.

Department of Investigative Medicine,
McGill University, Montreal.

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ABSTRACT

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The induction of hyperaminoaciduria and hyperphosphaturia was investigated in Long Evans and Holtzman rats, using rachitogenic diets low in Vitamin D and calcium or phosphorus.

Hyperexcretion of free amino acids and phosphaturia resulted from diminished net tubular absorption and occurred only in animals fed low calcium diets who developed severe hypocalcemia. The intensity of renal dysfunction was proportional to the degree of hypocalcemia below about 6.5 mg% plasma calcium; the amount of Vitamin D in the diet was immaterial in this relationship. The degree of renal dysfunction was directly proportional to the degree of parathyroid gland hypertrophy. Raising the plasma calcium to 6.5 mg%, or parathyroidectomy, both suppressed urinary hyperexcretion of amino acids and phosphorus. Excretion of bound amino acids, particularly hydroxyproline, proline and glycine was elevated in hypocalcemic animals. Parathyroid hormone excess, rather than Vitamin D deficiency per se, appears to account for impairment of membrane transport of amino acids and phosphorus in Vitamin D deficiency.

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Illustrations

ABBREVIATIONS

$Asp-NH_2$	-	Asparagine
ATP	-	Adenosine triphosphate
ATP-ase	-	Adenosine triphosphatase
Diets:		
A_1+D, HCa+D	-	Diet A_1 with Vitamin D supplement
A_1-D, HCa-D	-	Diet A_1 without Vitamin D supplement
A_2+D, LCa+D	-	Diet A_2 with Vitamin D supplement
A_2-D, LCa-D	-	Diet A_2 without Vitamin D supplement
B_1+D, NCa+D	-	Diet B_1 with Vitamin D supplement
B_1-D, NCa-D	-	Diet B_1 without Vitamin D supplement
B_2+D, LCa+D	-	Diet B_2 with Vitamin D supplement
B_2-D, LCa-D	-	Diet B_2 without Vitamin D supplement
-D	-	Rockland "D-free" Diet
N	-	Standard rat chow
DNA	-	Deoxyribonucleic acid
ECF	-	Extracellular fluid
End.	-	Endogenous
$Glu-NH_2$	-	Glutamine
HCl	-	Hydrochloric acid
PTE	-	Parathyroid extract
PTH	-	Parathyroid hormone
PTX	-	Parathyroidectomy

I. REVIEW OF LITERATURE

A. AMINOACIDURIA IN VITAMIN D DEFICIENCY

1) Observations in the human

Hottinger[1] observed in 1925 that Vitamin D deficient human infants with florid rickets have abnormally high urinary α-amino nitrogen excretion. A few years later, Freudenberg[2] reported that adequate intake of Vitamin D could reverse such an abnormality. Jonxis et al[3] also made similar observations, and by means of paper chromatography of urine amino acids, observed a two- to three-fold increase in the excretion of threonine, serine, glycine, alanine, histidine, lysine and glutamic acid. These findings were confirmed quantitatively by means of column chromatography[4]. The concentration of amino acids in the plasma of these patients was also examined and found to be within normal range[5]. The suggestion was therefore made that the excessive loss of the amino acids in D-deficient rickets was probably due to defective tubular reabsorption. When histidine was infused into rachitic patients[5] and into normal subjects, the experimental group had a five- to ten-fold greater histidine excretion, compared to the control group; the plasma histidine levels in both groups were comparable. This was regarded as further

evidence for the renal origin of the hyperaminoaciduria. Short-term ingestion of citrate and phosphate did not alter the hyperaminoaciduria[6].

Bickel et al[7] observed that six out of seven rachitic infants studied had a generalised hyperaminoaciduria; excessive excretion of glutamine, alanine, serine, glycine and cystine in particular, and also in some instances, tyrosine, lysine, threonine, leucine, valine and tryptophan, was identified by partition chromatography. After three to six weeks of Vitamin D therapy, the aminoaciduria and other biochemical disturbances had improved. These infants also had normal plasma amino acid, suggesting again that a deficit of renal conservation occurs as a result of Vitamin D lack.

Identical investigations were carried out by Biserte and coworkers[8] on twenty-one children who presented with signs of rickets. All were found to have a hyperaminoaciduria consisting of glutamine, glycine, histidine, alanine, asparagine, and sometimes β-aminoisobutyric acid; the excretion of the bound forms of proline, hydroxyproline and sarcosine was also elevated. Vitamin D diminished, but did not suppress the hyperaminoaciduria in all the cases during the period of observation. No correlation could be established between

the degree of the elevated amino acid excretion and the severity of the rickets.

Royer and collaborators[9] chromatographed urinary amino acids of rachitic children before and after Vitamin D therapy. Forty-one out of the forty-four infants investigated had the hyperaminoaciduria described above. The abnormal phenomena disappeared after a period of one to two months of vitamin treatment.

Berger and Stalder[10] studied endogenous renal clearance rates of amino acids in rachitic and normal infants. The former reabsorbed only 90-95% of the filtered load of amino acids, as compared with 98-99% efficiency of reabsorption in the normal infants. There were no significant differences in the plasma amino acid levels of the two groups of infants. The components of the aminoaciduria found in the rachitic infants were serine, glutamic acid, glycine, alanine, lysine, threonine, and in some cases, arginine, tyrosine, proline, ornithine, asparagine and aspartic acid. It was postulated that the high renal clearance of amino acids might be due to a deficit of some essential component of the renal reabsorption mechanism, resulting from Vitamin D deficiency. Similar findings and interpretation were also recorded in infants with Vitamin D

deficiency by Miguel[11], who suggested, in addition, that secondary hyperparathyroidism releasing amino acids from bone could contribute to the elevated amino acid concentration in the urine of such patients. The latter speculation was made on the basis of the known action of parathyroid hormone on the bone, the fact that secondary parathyroid hyperfunction had actually been recorded in association with Vitamin D deficiency[12], and the identification of increased amounts of urinary hydroxyproline, which was presumably of skeletal origin; this amino acid is normally present in the urine only in trace quantities.

Hanquet and Ewenbeck[13] compared the urinary amino acid excretion between rachitic and normal children of similar age. The pattern of hyperaminoaciduria observed in most instances was similar to that mentioned by the preceding authors cited. The abnormality was attributed to a possible Vitamin D deficiency in the tubular cells, which led to a diminution of certain enzymes necessary for normal tubular function. This similar view was also maintained by Bauza[14], who further postulated that Vitamin D might play an integral part in the energy component of the tubular reabsorption mechanism. In the absence of the vitamin, the efficiency of cells

responsible for the active transport of the amino acids was presumably reduced.

Very recently, Lamy and coworkers[15] reported a case of prolonged Vitamin D deficiency with the typical biochemical and radiological changes characteristic of deficiency rickets. After eighteen days of Vitamin D_2 therapy, the phosphaturia improved and the hyperaminoaciduria disappeared.

Visakorpi[16] pointed out that, although hyperaminoaciduria is regularly associated with florid rickets, this manifestation was not consistently found in mild cases. However, one patient had elevated aminoaciduria, despite normal tubular conservation of phosphorus. This observation provoked speculation that the reabsorption processes of these two substances are unrelated. Since Vitamin D effectively diminished the hyperaminoaciduria, the renal lesion was attributed to D-deficiency. Furthermore, since greater intensity of aminoaciduria was often encountered in the very young rachitic infants, it was suggested that the immature renal tubule might be more susceptible to the vitamin lack.

Chisholm and Harrison[17] noted that hyperaminoaciduria was a sensitive index of early Vitamin D

deficiency in premature infants. A highly significant correlation was also obtained between the hyperaminoaciduria and the hypophosphatemia in such groups, but no such relationship could be established with the serum calcium. The aminoaciduria, which was of a generalised type involving all the amino acids normally excreted in minute quantities, was less intense in older infants with florid rickets who had a recent sporadic Vitamin D intake. Vitamin D was thought to exert a direct effect upon some tubular mechanism common to and essential for the reabsorption of all amino acids. On the other hand, important physiological relationships could also exist between the mechanisms by which phosphorus, and amino acids are reabsorbed in the kidney tubules. Similar views regarding the renal handling of amino acids have also been expressed by Ayer and colleagues[18], Stowers et al[19], and Drummond[20].

Scriver, Kooh and Fraser[21] followed forty cases of Vitamin D deficiency rickets and noted that patients with early rickets had low serum calcium, normal serum phosphorus and no hyperaminoaciduria. Patients with more advanced rickets had normal or low serum calcium, low serum phosphorus and hyperaminoaciduria. Calcium infusion inducing mild sustained hypercalcemia was performed on two of the latter patients; the generalised

hyperaminoaciduria was suppressed transiently during the period of hypercalcemia. After Vitamin D therapy had raised serum calcium levels towards normal, hyperaminoaciduria began to disappear. These and other findings were interpreted to indicate that the hyperaminoaciduria in Vitamin D deficiency is due to secondary hyperparathyroidism. Further support for this hypothesis came from observations that hypoaminoaciduria and enhanced tubular absorption was also observed in two children and one adult with hypoparathyroidism; and patients with primary hyperparathyroidism often have hyperaminoaciduria. Parathyroidectomy performed on one infant with severe primary hyperparathyroidism suppressed an existing hyperaminoaciduria and hyperphosphaturia.

The renal origin of the hyperaminoaciduria arising from D-deficiency states in man has been confirmed by the data on endogenous clearance rates of amino acids published by Cusworth and Dent[22]. However, the precise biochemical mechanism by which the impairment of amino acid transport is brought about has yet to be elucidated.

2) Observations in the rat

Attempts have been made in the rat to duplicate the appearance of hyperaminoaciduria in Vitamin D deficiency. Engström et al[23] fed a series of D-free diets with different calcium and phosphorus contents to groups of weaned male Holtzman albino rats. After a three-week period, they found no evidence for abnormal aminoaciduria in the urine of the rachitic rats. This was evidence to them that hyperaminoaciduria is not a primary effect of Vitamin D deficiency.

Harrison[24] did not find an elevated urinary amino acid excretion in rachitic rats, but no details were available for his study.

Summary

Hyperaminoaciduria is a consistent finding at a certain stage in Vitamin D deficiency in the human. The defect has been established to be of renal origin, involving essentially all of the amino acids normally excreted in trace amounts in the urine. Many believe this renal abnormality to be directly associated with the lack of the vitamin; recent studies suggest that the defect may be due to excessive endogenous PTH secretion.

All attempts to duplicate the renal lesions in the D-deficient rat have so far been unsuccessful.

B. AMINO ACID REABSORPTION, VITAMIN D AND PTH

The urinary excretion of free amino acids by the normal human is slight. Most of the free amino acids normally present in the glomerular filtrate are very efficiently reabsorbed by the renal tubules; therefore only a small fraction appears in the urine[25]. The capacity for tubular reabsorption is limited and if the concentration of plasma amino acid is raised sufficiently in the glomerular filtrate, saturation of available transport by the tubule can be demonstrated. This is a predicted property for mediated transport and has been clearly demonstrated in the intact mammalian kidney in dog[26] and in man[27,28].

Investigations conducted in man and the dog have introduced evidence in support of separate transport mechanisms in the kidney for different groups of amino acids. These include one for the monoamino dicarboxylic acidic amino acids[29], one for the diamino monocarboxylic basic amino acids[30] including cystine, one for the imino acids and glycine[27,31], one for a large group of neutral amino acids[32], and one for the β-amino acids[33]. The principle site for amino acid transport appears to be located in the proximal segment of the renal tubule[34,35].

Dent[36] has shown that abnormal amounts of amino acids in the urine could be due either to an "overflow

mechanism" or to a "renal mechanism". In the former type, there is an increase of plasma concentration of one or several amino acids, resulting in saturation of the normal transport system. In the latter mechanism, the plasma concentration of amino acids is normal or slightly reduced, and the basic abnormality resides in the tubular transport of the affected amino acids. The transport defect may be specific for a single amino acid, or involve a group of amino acids, or it may be generalised involving essentially all of the amino acids normally present in glomerular filtrate. Scriver and colleagues have recently demonstrated two examples of "combined" hyperaminoaciduria[27,33], where one amino acid whose "pre-renal" concentration is elevated and competes with other members of a specific transport system; the diseases involved the imino acid-glycine and the β-amino transport groups.

The accumulation of amino acids by kidney (and other tissues) is achieved against a concentration gradient[37,38,39] and is the result of the net balance of influx and efflux phenomena. While the components of the efflux process are less well defined experimentally, the former involves at least three distinguishable components. These are non-saturable diffusion[40], carrier-mediated diffusion with saturable binding sites having high affinity for amino acids

of the L-configuration[41], and energy-dependent accumulation against a chemical gradient[42]. How the energy component is linked to the transport system is still unknown. Energy for transport may be derived from oxidative phosphorylation since inhibition of the latter decreases the ability to concentrate solute[42]. On the other hand, the hydrolysis of the high energy compound ATP by a membrane enzyme, ATP-ase is also likely to be involved since the ionic requirements of ATP-ase for Na^+ and K^+ parallel those for the transport of some amino acids[42]. Even if there are single carriers for groups of closely related amino acids, it is quite possible that the energy source is different for each.

Experimental evidence relating Vitamin D directly to the process of amino acid transport has thus far been lacking. It is known that the vitamin is closely associated with plasma and nuclear membrane fractions[43] of kidney and intestinal epithelial cells[44]. Furthermore, Vitamin D is taken up after injection most avidly in kidney by proximal tubular cells[45]. Vitamin D also stimulates the incorporation of ^{32}P-orthophosphate into the phospholipids of intestinal mucosa and the lipids of kidney slices in the rat[46]. The significance of the latter observation and the other phenomena in relation to membrane transport of amino acids and other substrates, is unknown at present.

On the other hand, PTH is known to interfere with oxidative metabolism. PTE given in-vivo will inhibit isocitrate oxidation and phosphorylation by rat kidney mitochondria[47] and by cellular homogenates[48]. The detailed studies of Cohn et al[49] conducted with rabbit kidney mitochondria, revealed the effect of PTH given in-vivo is not limited to isocitrate; a series of other substrates are also affected, accompanied by a stimulation of ATP-ase activity. However, liver mitochondria were unaffected under identical treatment.

Of further interest is the work of Borle and Neuman[50], which showed the variety of changes of cellular activity and morphology brought about by the addition of PTH to cultures of HeLa cells. Particularly intriguing was their observation that the hormone produced a decrease in cell adhesiveness, a possible implication of the hormone altering the properties of the mammalian cell membrane. Rasmussen and Ogata[51] very recently showed that PTH alters the permeability of rat liver mitochondria membrane to K^+ and Mg^{++} ions.

C. THE ROLE OF VITAMIN D IN CALCIUM ABSORPTION BY INTESTINAL MEMBRANE

One of the primary physiological actions of Vitamin D is to improve calcium absorption from the intestine[52]. In isolated rat intestine[53], the absence of Vitamin D

depresses the transfer of calcium. The compiled studies of Schacter and coworkers[54] suggested that the transport of calcium across the intestinal wall involves two processes; penetration of the diffusion barrier of the mucosal epithelium and the uphill transport of calcium across the mucosal cell by an energy-dependent system. Recent evidence presented by Harrison and Harrison[55] indicated the participation of Vitamin D in the first step. Ogata and Rasmussen[56], on the other hand, demonstrated that oxidative metabolism is necessary for massive transport of calcium across the intestinal wall in the rat.

The persistent finding of a time lag between the administration of Vitamin D and the expression of its physiological response[57], plus the fact that the in-vitro addition of the vitamin to intestinal preparations does not promote calcium translocation across the intestinal wall[55], has led to the speculation that Vitamin D may express its action through controlling the synthesis of specific proteins thereby affecting calcium translocation. To this effect, Zull et al[58] and Norman[59] have conclusively demonstrated that actinomycin D, an inhibitor of DNA - directed RNA synthesis[60], inhibits the Vitamin D stimulated calcium absorption from the intestine of the rat and chick respectively. Norman[61] has further

shown that prolonged actinomycin D treatment could render Vitamin D treated chicks D-deficient. Recently, Wasserman and Taylor[62] reported the isolation of a protein from the supernatant of intestinal mucosa homogenate of Vitamin D_3-treated chicks. This substance, which is almost undetectable in untreated rachitic chicks, and is capable of forming a soluble complex with calcium, appeared quite simultaneously with the reinstitution of intestinal calcium absorption after Vitamin D administration. A good correlation between the rate of the intestinal calcium reabsorption and the concentration of the Vitamin D induced protein was found. Most recently, Norman[63] provided direct evidence that Vitamin D_3 also stimulates the synthesis of RNA in intestinal mucosa of the deficient chick.

The evidence presented thus far seems to favour the view that a specific protein is an essential intermediate for Vitamin D to exert its effect on calcium transport.

Bearing on this problem perhaps are the mitochondria studies. It has been shown that calcium is bound in isolated kidney mitochondria in the presence of a system which generates ATP[64-66]. The accumulated calcium is apparently deposited as a phosphate complex and Vitamin D does not affect the accumulated phase. However, when ATP

levels in the system are reduced enzymatically after calcium has been taken up, the rate of calcium release is greatly enhanced by Vitamin D. PTH further increases calcium movement out of the mitochondria. On the other hand, PTH does not produce this effect on mitochondria from D-deficient animals unless Vitamin D is added. The relationship between the observations made in the mitochondria system and that of cellular transport of calcium is not known. DeLuca and colleagues[66] suggested the mitochondria membranes may function in a similar manner to that of the cell, thus presenting a "model" response to Vitamin D. The mitochondria may serve to regulate intracellular calcium levels, which in turn yield profound cellular effects. These subcellular particles may also act as a shuttle for the transcellular transport of the calcium ion.

Although there have been reports showing that PTH promotes intestinal and renal absorption of calcium[67], the hormone probably plays a less significant role in this respect than in its effect on bone. The transport effect is apparently highly dependent on the Vitamin D status of the subject[68].

D. PLASMA CALCIUM AND PARATHYROID GLAND INTER-RELATIONSHIP

In the past decade great progress has been made in purifying and characterising calcium-raising parathyroid hormone. Relatively pure preparations of the hormone can now be obtained[69]. The bovine hormone is a single polypeptide chain consisting of approximately eighty-four amino acid residues[70]. This single molecule possesses both calcium mobilizing and phosphaturic activity[71] in the intact rat.

The first experiments conducted to establish the important role of the parathyroid glands in calcium homeostasis were performed a half century ago by MacCallum and Voegtlin[72] and by Greenwald[73]. Some years later, Ham and coworkers[74] were able to show that parathyroid hypertrophy occurred only in D-deficient rats with low plasma calcium, but not in the group with normal plasma calcium, even though both groups were rachitic. Stoerk and Carnes[75] also demonstrated a highly consistent inverse linear relationship between the level of plasma calcium and the size of the parathyroids in the rat. In a series of perfusions conducted in the dog, Patt and Luckhardt[76] presented the first direct evidence of the effect of hypocalcemia on stimulation of parathyroid hormone production. These observations led McLean and Urist[77] to postulate

the possible existence of a precise negative feedback mechanism regulating parathyroid activity and plasma calcium levels. The results of the experiments done on isolated rat parathyroid gland cultures[78] also concur with the above findings. Further evidence obtained by Au et al[79] indicated that Vitamin D alone has no direct influence on the gland activity. It is now in general acceptance that the concentration of calcium ion in the blood circulating through the parathyroid glands is the principal factor controlling the synthesis and secretion of PTH[80,81]; a high concentration of the ion suppresses synthesis and secretion and a low concentration stimulates and increases secretion.

II. PURPOSE OF STUDY

Although hyperaminoaciduria and hyperphosphaturia are well-documented observations in nutritional rickets, the precise etiology of the renal dysfunction has not yet been resolved. An acquired defect in tubular absorption clearly underlies these observations. The obvious interpretation that Vitamin D deficiency has some <u>direct</u> relation to the impairment of tubular reabsorptive function has never been proven unequivocally. On the other hand, there is recent work[21] suggesting that in man at least, the biochemical findings in Vitamin D deficiency could be attributed more to reactive hyperparathyroidism, secondary to the effect of Vitamin D deficiency upon calcium availability than to Vitamin D deficiency per se. The schematic representation of the latter proposal is as follows:

$$\downarrow \text{Vitamin } D_2 \rightarrow \downarrow Ca^{++}_{ECF} \rightarrow \uparrow PTH_{(Endog.)} \rightarrow \downarrow \text{Renal tubular reabsorption of amino acids and phosphorus, etc.}$$

If this model offers a satisfactory explanation to the biochemical abnormality, then under such circumstances, temporary suppression or extirpation of the parathyroid glands should directly counteract the disturbance in renal function. The rat was chosen to develop a model system with which to investigate this hypothesis.

The purpose of this study was two-fold:

1) To reinvestigate the possibility of inducing hyperaminoaciduria in the rat, through the use of rachitogenic diets of varying calcium and phosphorus content; and

2) to test the validity of the outlined hypothesis.

III. EXPERIMENTAL

A. MATERIALS

1) Compounds

Calcein (Fluorescein Complexone) was purchased from K and K Laboratories, Inc., New York. 1-Amino-2-naphthol-4-sulfonic acid dry powder mixture was obtained from Fisher Scientific Co. Yeast was bought from Red Star Yeast Products Co. Fibrin (hot alcohol-extracted) and salt mixture (NaCl, iodized - 50 gm; $FePO_4.2H_2O$ - 2 gm; KCl - 44 gm; $MgCO_3$ - 2 gm; $MnSO_4$ - 1.7 gm; $CuSO_4$ - 0.3 gm) were purchased from General Biochemicals Inc. Vitamin A, Vitamin D (Ostoco drops (R)), Nembutal, and calcium gluconate (10%) (Glaxo-Allenburys), were obtained from the hospital pharmacy. All the compounds used were of reagent grade.

2) Animals

Long Evans hooded rats of both sexes were obtained from Quebec Breeding Farm, St. Eustache, P.Q. for use in Study A (preliminary study). Weaned male Holtzman albino rats, weighing between 55-60 gms, were obtained from Holtzman Co., Madison, Wisconsin. The Holtzman strain is in-bred and maintained continuously through generations on diets containing negligible amounts of Vitamin D. These rats were used for Study B (formal study).

TABLE I

COMPOSITION OF VITAMIN D-FREE DIETS

	A_1	A_2	B_1	B_2
Calcium content (%)	1.2	0.02	0.4	0.02
Phosphorus content (%)	0.02	0.3	0.3	0.3
Corn starch (gm)	750	750	75	75
Dextrose (gm)	300	300	975	975
Fibrin (gm)	225	225	225	225
Yeast (gm)	60	60	60	60
K_2HPO_4 (gm)	-	17.5	17.5	17.5
$CaCO_3$ (gm)	45	-	14.25	-
Salt mixture (gm)	15	15	15	15
Corn oil (with 100 I.U. Vit. A/ml) (ml)	114	114	114	114

Diets A_1 and A_2 mixed in the lab. Diets B_1 and B_2 from Nutritional Biochemical Corp., Cleveland, Ohio.

A-diets used in pilot Study A; B-diets used in formal Study B.

TABLE II

CALCIUM AND PHOSPHORUS CONTENTS OF THE B-DIETS OBTAINED BY ANALYSIS

Diets		Calcium (%)	Phosphorus (%)
B_1	Calculated	0.40	0.30
	Analysed	0.45	0.37
B_2	Calculated	0.02	0.30
	Analysed	0.03	0.39

3) Diets

The composition of the four synthetic diets used in the two phases of the investigation is indicated in Table I. The components contain no known source of Vitamin D. The calcium and phosphorus contents of the B-diets used in the formal study were made to the specifications for mineral content by adding appropriate amounts of $CaCO_3$ and K_2HPO_4 respectively to the basic diet. The low corn starch and high dextrose contents of the B-diets is a technicality required to allow pelleting of the food. A-diets, used in the preliminary study, were made by the author and fed to the rats in powder form; the constituents were mixed in a Waring blender. A commercially available "vitamin D-free" Rockland Rat Diet (Tekland Inc., Monmouth, Ill.) (Ca = 1.45%; P = 0.92%) was also used for the breeding protocol in the preliminary study.

B. METHODS

1) Breeding D-deficient animals for the preliminary study

Long Evans hooded rats of about 3 months of age were used for breeding. During the one- to two-week period prior to mating and throughout pregnancy (three weeks), the animals were fed only the Rockland diet pellets to reduce the Vitamin D available to the offspring. The resulting litters produced by these mothers were used throughout in Study A.

2) Feeding

a) Study A

After weaning, eighty-four animals from the D-deficient litters were randomly divided into six groups with approximately equal sex distribution. Six groups of animals of equal number were put onto the following diet regimes:

i) standard Purina Rat Chow;

ii) Rockland Diet;

iii) synthetic diet A_1;

iv) A_1 + Vitamin D (3 units/gm of diet);

v) A_2 diet; and

vi) A_2 + Vitamin D (3 units/gm of diet).

The Vitamin D_2, in propylene glycol, was added to the powder food of the vitamin-supplemented groups prior to each feeding. The animals were housed seven in each cage and had unlimited access to food and water.

b) Study B

Sixty 3-week old male albino Holtzman rats were divided randomly into four groups. Diets for these groups were:

i) Diet B_1;

ii) Diet B_1 + Vitamin D (150 I.U./wk);

iii) Diet B_2;

iv) Diet B_2 + Vitamin D (150 I.U./wk).

Each of the vitamin-supplemented animals received 75 I.U. of Vitamin D_2 in 1 ml of propylene glycol, by gavage twice a week, through a K31 Pharmaseal (R) 8FR feeding tube mounted on a syringe.

3) Animal environment

A separate room protected from direct sources of sunlight was used through the entire investigation. Incandescent lighting was provided. Dark-light periods were the normal diurnal pattern. Room temperature was maintained at 73 ± 2°F and the humidity kept constant. Ordinary rat cages were used to house the animals; seven or eight animals were kept in each cage.

4) Collection of urine and blood samples

Collections were made regularly at either one-week or two-week intervals. Six rats from each of the diet groups were transferred into metabolic cages and a urine collection performed over a six-hour period approximately. The urine samples were collected into graduated cylinders and the feces were retained by wire screens and glass wool placed under the metabolic cages. All urine collections were initiated in the morning and only water was given to the animals during these periods. Immediately after each collection, all the urine specimens were acidified to pH 4 with HCl and stored at -20°C until analysed.

During the collection periods, blood samples from individual rats were also obtained. The samples were collected from the tail into heparinized capillary tubes (57 x 1.5 mm). One end of these tubes was packed with plasticene and centrifuged to separate the cells from the plasma. The tubes were then broken off at the plasma and cell junction and the plasma stored at -20°C until analyses were made.

The weights of the animals were recorded at the end of each collection period.

5) Calcium and phosphorus determination

Plasma calcium was determined fluorometrically according to the methods of Kepner and Hercules[82], modified by Turner Associates. The Clarke and Collip method[83] was used to estimate the calcium content of the diet. A micro modification of Fiske and Subbarow's method[84] was used for phosphorus determinations.

6) Identification of amino acids

a) Urine

The total nitrogen content of the urine samples was determined by the micro-Kjeldahl method[85].

i) Semiquantitative method

Aliquots equivalent to 250 µgm of total nitrogen were spotted onto 10 inch square Whatman

No. 4 filter paper and developed by ascending partition chromatography in two dimensions[86]; the first solvent was water-saturated phenol, and the second, 2,6-lutidine:water (2.2:1). After overnight drying at 40°C, the chromatograms were stained with a ninhydrin-isatin mixture[87] and heated in the oven at 80°C for ten minutes to develop the spots. The chromatograms were inspected by transmitted light on a radiological viewing screen; the intensity of each spot was estimated and scored in arbitrary units. The degree of aminoaciduria in all cases was expressed as the sum of the "units" per 250 µgm of total nitrogen.

This semi-quantitative method has been compared directly with quantitative estimation by ion exchange column chromatography and found to be reliable in the range of aminoaciduria experienced in these investigations[88].

ii) Quantitative method

Individual amino acids in urine were determined quantitatively by ion exchange elution chromatography according to recent modifications of the methods of Spackman, Stein and Moore[89],

using a Beckman Spinco Model 120 amino acid analyser[90] equipped with spherical resins for analysis of physiological fluids. Aliquots equivalent to 2 mg or less of total nitrogen were applied to the column for each analysis.

For the quantitative estimation of the bound amino acids, the samples were hydrolyzed in sealed glass vials under air with 6N HCl (final concentration) at 110°C for 24 hours. The hydrolysate was then transferred into a round bottom flask, distilled in vacuo at 40°C to remove the excess HCl. The dried hydrolysate was suspended in a small volume of distilled water and redistilled. This process was repeated four times to remove the last traces of HCl. After the final distillation, the sample was resuspended in an appropriate volume of distilled water and analysed quantitatively.

The urinary excretion rate of amino acids was expressed as a coefficient of nitrogen excretion (micromoles/100 mg total nitrogen).

b) <u>Plasma</u>

A 1 ml aliquot of plasma was treated with five volumes of 1% picric acid solution to precipitate

the protein. The mixture was centrifuged and a 5 ml aliquot of the supernatant was removed and passed through a Dowex-2 x 8 resin to remove the picric acid[90]. The resultant sample was evaporated by distillation in vacuo, resuspended in pH 2.2 sodium citrate buffer and the amino acids evaluated quantitatively according to the described procedures.

7) Roentgenograms

The animals were anaesthetised with Nembutal (0.05 mg /gm); the X-rays were taken by the Radiology department of the hospital.

8) Parathyroidectomy

A six-hour pre-parathyroidectomy urine sample was obtained from all groups of animals 24 hours before the operation. Parathyroidectomy was performed by a method using hot-wire cautery, as described in detail by Hirsh et al[91]. Post-parathyroidectomy urine samples were collected between the sixth and twelfth hour after the operations.

9) Calcium injection

Animals from the low-calcium, Vitamin D-deficient (B_2-D) group and normal calcium, Vitamin D-supplemented (B_1+D) group were used in this experiment. A four-hour pre-injection urine sample was collected from each group before the injection. Each animal was then injected

intraperitoneally with calcium gluconate suspended in 2 cc 0.9% NaCl to contain 4 mg of calcium. Six-hour post-injection urine samples were collected at two hours and at twenty hours after the injections. Blood samples were also taken from the individual rats at two hours before the injections, and at two hours and twenty hours after the injection.

10) Statistical treatment

All analyses were made according to standard statistics procedures[92].

C. RESULTS

1) Preliminary Study A

This "pilot study" was conducted in the D-deficient litters of the Long Evans rats.

a) Effect of the diets on the growth of the animals

The animals on the two D-deficient synthetic rachitogenic diets (A_1 and A_2) had very slow weight gain; growth was less retarded in the animals on the higher calcium intake. Vitamin D supplementation of the same two diets promoted growth. The animals on the Rockland pellets showed steady weight increments, but weighed slightly less than the rats on the Standard Rat Chow. The average weights of eight animals in each group on the various diets at the

twelfth week of the study were:

Standard pellets	-	380 gm
Rockland pellets	-	370 gm
A_1 + D	-	208 gm
A_2 + D	-	185 gm
A_1 - D	-	178 gm
A_2 - D	-	155 gm

One can conclude from these results that the Rockland diet contains significant, albeit small amounts, of Vitamin D.

b) Dietary influence on calcium and phosphorus metabolism

These results are presented in Figure 1. Each set of plasma values was determined from a pooled sample taken from six rats. A slight decline in the plasma concentration of calcium and phosphorus, and an increase in urinary phosphorus excretion, occurs with age in the normal animal fed on rat pellets. These values were used as controls for the other experiments.

The D-deprived animals on the low calcium diet (A_2) became extremely hypocalcemic (4-6 mg% after six weeks). The addition of Vitamin D to the same diet maintained a much higher plasma calcium level. The plasma phosphorus in these two groups of animals were within, or near, the normal range for the first ten weeks; there was a terminal lowering of the plasma phosphorus concentration to 9 mg% at the tenth

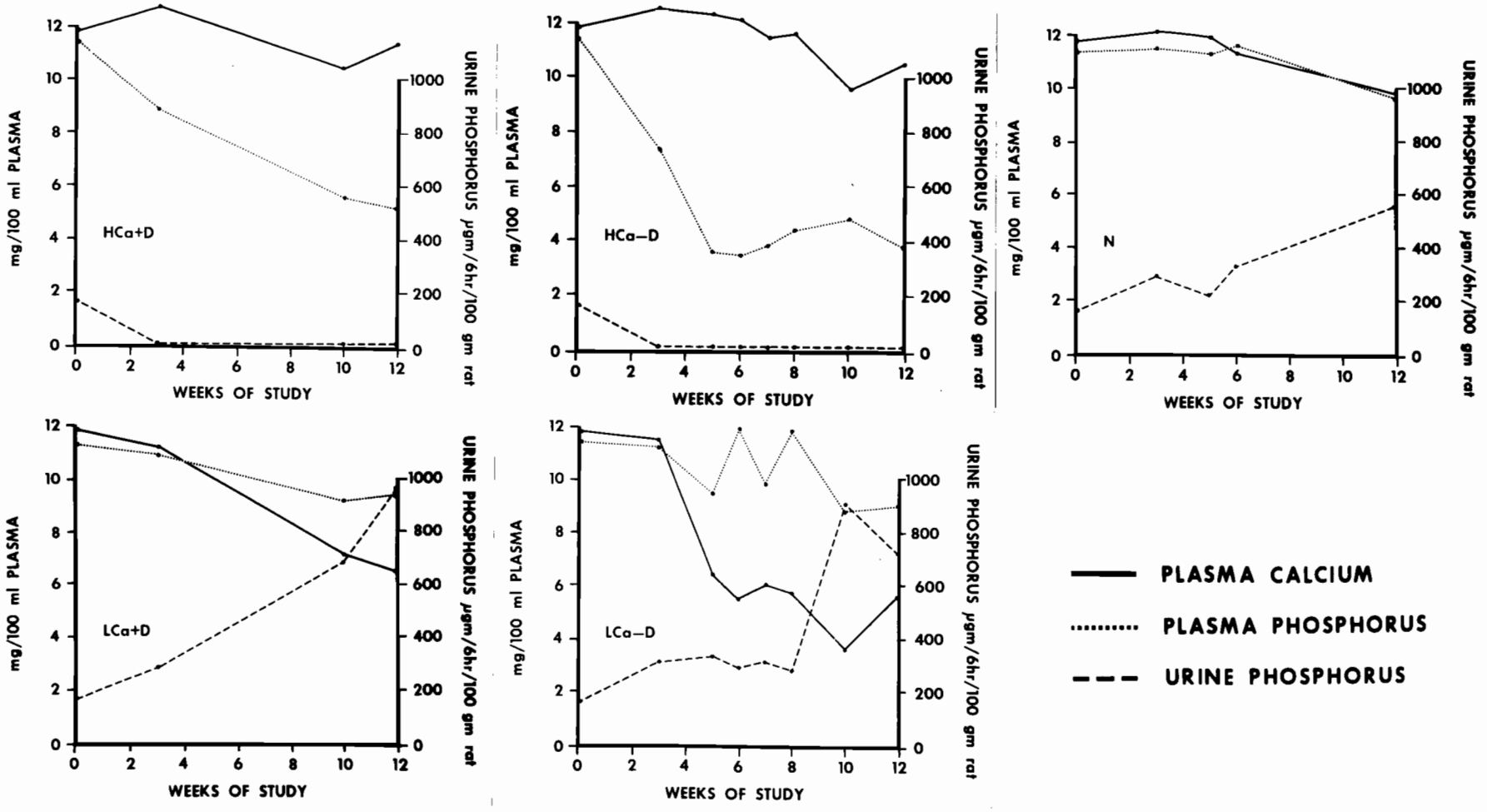


Fig. 1 Dietary influence on calcium and phosphorus metabolism in the Long Evans rats.

and twelfth weeks in the low calcium, D-deprived group. Phosphaturia increased above normal during the period of study in both groups, and more so in the D-supplemented animals.

The plasma calcium concentration in the animals on high calcium diets (A_1), either with or without D-deprivation, remained within the normal range. A decline in plasma phosphorus concentration occurred in both groups of animals because the diet is low in phosphorus content (Table I). The rate of decrease was more gradual in the D-supplemented group and a relatively higher plasma phosphorus was found at twelve weeks in this group than in the D-deprived group. The urinary phosphorus excretion was neglible in both groups.

At the twelfth week of the study, the concentrations in plasma of calcium and phosphorus, in the animals on the Vitamin D-free Rockland pellets were both normal (Ca - 10.1 mg %; P - 9.8 mg %). The phosphorus excretion in these animals (770 µg / 6 hr/100 gm rat) was comparable to the other animals on high phosphorus intakes (Diets A_2 and Rat Chow).

c) Semi-quantitative estimation of the aminoacidurias

The degree of aminoaciduria at the twelfth week of study in the different groups of animals is summarised in Figure 2. Taurine is expressed separately because it is the dominant component (40-50%) of normal aminoaciduria in the rat. The

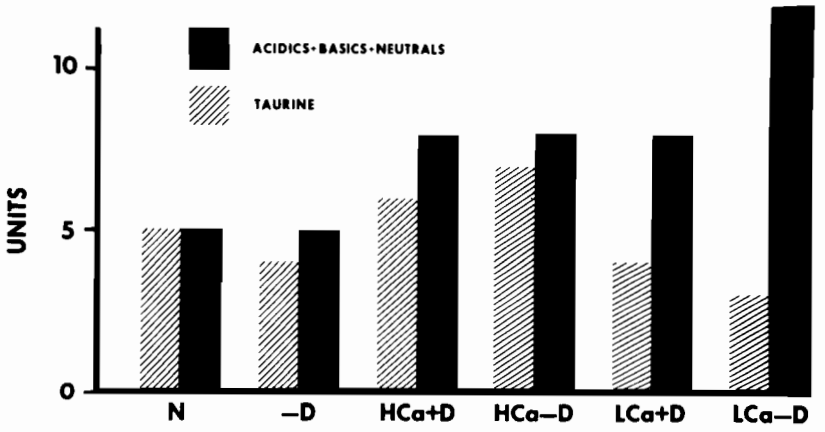


Fig. 2 Semiquantitative estimation of the aminoaciduria in the Long Evans rats at the twelfth week of Study A.

test groups can be divided into three categories of aminoaciduria. The highest excretion (twice normal) is observed in animals who are hypocalcemic and particularly when D-deprived. The animals on rachitogenic diets and without severe hypocalcemia (i.e. Diet A_2+D, and both A_1 low phosphorus diets) had modest hyperaminoaciduria. The third category is normal aminoaciduria found in chow fed and Rockland diet D-deprived animals.

d) Radiological findings

Rickets were indicated by a decrease in the bone density of the long bones and widening of uncalcified zone of the epiphysis. These findings were visible in the D-deficient animals on either low calcium or low phosphorus intakes; the lesions were more severe in the latter animals. In the other animals, no bone abnormality was observed (Figure 3).

e) Parathyroidectomy effect on aminoaciduria and phosphaturia

At the twelfth week of the study, parathyroidectomy was performed on three groups of animals. The removal of the glands in eight animals on the low calcium D-deprived diet revealed greatly hypertrophied glands in each animal. Phosphaturia decreased

1 = HCa-D: 2 = HCa+D; 3 = N; 4 = LCa-D; 5 = LCa+D.

Fig. 3 Roentgenogram of the Long Evans rats at the ninth week of Study A.

from 723 µg /6 hr/100 gm rat, to 550 µg /6 hr/ 100 gm rat in the first twelve hours after the operation. Urinary excretion of acidic and neutral amino acids was 31.7 µmoles/100 mg total nitrogen pre-operatively and fell to 18.5 µmoles/100 mg total nitrogen in the urine samples collected post-operatively. This decline in aminoaciduria is accounted for mainly by free hydroxyproline (absent post-operatively), threonine, serine, proline, glutamic acid, glycine, alanine and methionine. Five animals died within eighteen hours with tetany and convulsions.

The operation was performed on seven rats on the D-supplemented low calcium diet A_2. The para-thyroid glands were much less hypertrophied than in the first group. In this group and in the control group fed on chow, there was no significant change in aminoaciduria and phosphaturia.

Resume

It is evident that diets very low in calcium or phosphorus and in Vitamin D are capable of inducing hypocalcemia or hypophosphatemia respectively in the rats. Rickets occur in such animals.

Since diet clearly influences the rate of phospha-turia and perhaps of aminoaciduria also, comparisons

of urinary excretion of these compounds in the different groups of animals is difficult. It did appear, however, that the very hypocalcemic D-deprived animal was prone to hyperaminoaciduria and perhaps to hyperphosphaturia, particularly relative to its plasma phosphorus levels.

Vitamin D supplementation partially offsets the effect of a low calcium diet with reference to the changes in plasma and urine composition; it completely prevents rickets.

The occurrence of hyperaminoaciduria only in the low-calcium, D-free animals, the enlargement of parathyroids in these animals, and the failure to produce abnormal aminoaciduria with other forms of D-deprivation (Rockland diet and Diet A_1-D), suggests that aminoaciduria in D-deficiency may be parathyroid dependent, according to the level of calcium in the extracellular fluid. In order to investigate this further, a second study was undertaken in the "classic" D-deficient Holtzman rats in whom the environmental variables were narrowed.

2) Study B

Male Holtzman rats were used throughout this study. The diets varied only in calcium content and Vitamin D (Tables I and II).

a) Effect of the diets on the growth of the rats

Weight gain was poorest in the animals receiving the low calcium, D-deprived diet (B_2) (Figure 4). An improved rate of weight gain, which however was still abnormal, resulted when Vitamin D was added to the B_2 diet. D-deprivation with normal calcium intake (Diet B_1) produced considerable growth

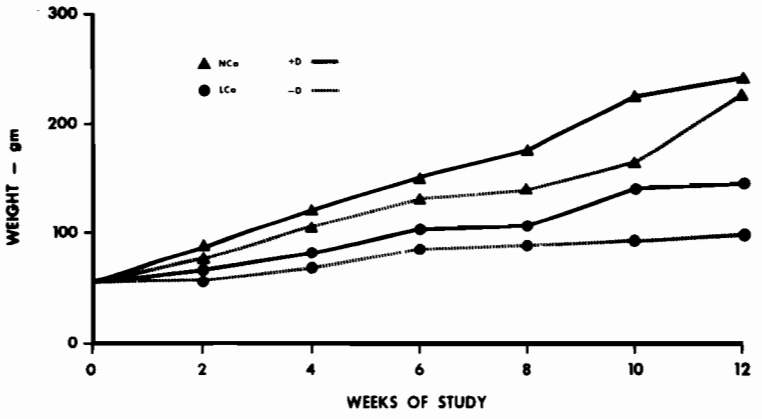


Fig. 4 Effect of dietary calcium and Vitamin D on the weights of the Holtzman rats.

retardation, indicating a satisfactory technique in withholding the vitamin. The weight increments in the group receiving an 0.4% calcium diet with added Vitamin D were used as control values. Whether anorexia, secondary to hypocalcemia produced by calcium deprivation, played a significant role in the growth failure was not ascertained, since pair-feeding experiments could not be done with the available facilities.

b) Dietary influence on calcium and phosphorus metabolism

i) Calcium in Plasma

The most severe hypocalcemia was observed in the animals given a low-calcium, D-free diet (Figure 5); a lesser degree of hypocalcemia also occurred on the same B_2 diet supplemented with Vitamin D. In the D-deprived group, the plasma calcium fell rapidly by the second week of study. At the fourth and eighth weeks, the calcium level increased slightly and was maintained between 5 - 5.5 mg % throughout the last four weeks of the study. A steady decline in plasma calcium occurred in the D-supplemented animals, until the sixth week, after which the level fluctuated between 6 and 7 mg %.

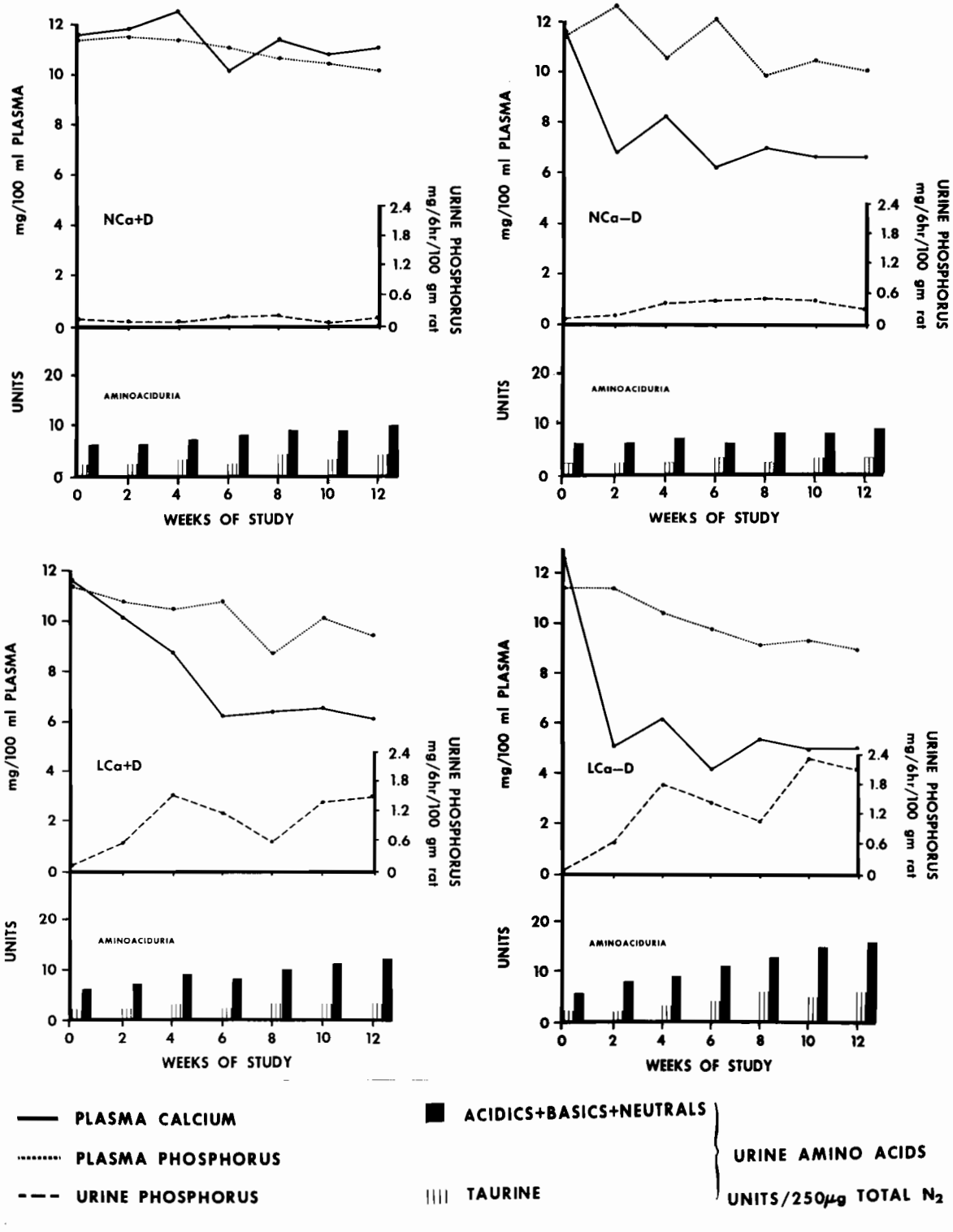


Fig. 5 Effect of the diets on calcium and phosphorus metabolism and aminoaciduria in the Holtzman rats.

Hypocalcemia occurred also in the D-deprived animals on a normal calcium intake, but the level did not fall below 6.5 mg %. The plasma calcium levels in this group of animals were significantly higher ($P < 0.001$) than the corresponding values in the (LCa+D) group during the last eight weeks of the study (Table IIIa).

ii) Plasma phosphorus

A steady decrease in plasma phosphorus was indicated in all four groups; the levels in the animals fed a low calcium, D-deprived diet tended to be lower than in the group on normal calcium intake, supplemented with Vitamin D (Figure 5 and Table IIIb).

iii) Urinary phosphorus

The animals on the B_2 diet had a higher phosphaturia than the animals on the B_1 diet; the highest excretion values were those from the (LCa-D) group (Table IIIb and Figure 5).

Urinary phosphorus of the (NCa-D) animals was consistently higher than the control (NCa+D) animals. Note that the phosphorus content of the diets of these four groups of animals are practically identical (Table II).

TABLE IIIa

STATISTICAL ANALYSES* FOR DIFFERENCES BETWEEN THE PLASMA CALCIUM LEVELS IN THE (NCa-D) GROUP AND (LCa+D) GROUP WITHIN THE SIXTH AND TWELFTH WEEK PERIOD OF STUDY B

	Plasma Ca (mg.%)											
Group	6th week			8th week			10th week			12th week		
NCa-D	6.2	6.2	6.6	7.4	7.2	7.1	6.4	6.9	6.7	6.7	6.9	6.9
	6.3	6.5	6.1	6.8	7.0	6.8	6.8	6.6	6.9	7.0	6.6	6.2
LCa+D	5.9	6.2	6.4	6.8	6.5	6.3	6.3	6.5	6.9	6.5	6.2	6.3
	6.1	6.3	6.2	6.5	6.4	6.1	6.4	6.6	6.1	6.4	6.3	6.1

* An analysis of variance of the data in the above table provided the following results:

Item	Sum of Squares	N	Mean Square	Variance Ratio	Probability
Total	5.40480	47			
Treatment(T)	1.50521	1	1.50521	30.49	0.001
Sample(S)	1.52730	3	0.50910	10.31	0.01
T/S interaction	0.39729	3	0.13243	2.68	Not sig.
Error	1.97500	40	0.04937		

The difference between the means for the two treatments (6.70000 - 6.34580) is highly significant. In addition, there are highly significant differences among the means for the four sample periods; inspection of the data shows that this is primarily due to the large increase in the measurement between the six and eight week samples. There was no evidence in the analysis of interaction between the treatments and the sample periods; inspection of the data shows that treatment A has a higher value at each of the four sample periods. (Treatment A = NCa-D; Treatment B = LCa+D).

TABLE IIIb

EFFECT OF THE DIETS ON AMINOACIDURIA AND PHOSPHORUS METABOLISM IN THE HOLTZMAN RATS AND A STATISTICAL EVALUATION* OF THE RATES OF DECLINE IN PLASMA PHOSPHORUS IN THE DIFFERENT GROUPS OF ANIMALS

	NCa+D			NCa-D			LCa+D			LCa-D		
Period	Phosphorus[+]		Amino-[++]	Phosphorus		Amino-	Phosphorus		Amino-	Phosphorus		Amino-
Weeks	Plasma	Urine	aciduria	Plasma	Urine	aciduria	Plasma	Urine	aciduria	Plasma	Urine	aciduria
0	11.4	0.09	6	11.4	0.09	6	11.4	0.09	6	11.4	0.09	6
2	11.5	0.08	6	12.6	0.12	6	10.8	0.54	7	11.5	0.66	8
4	11.4	0.06	7	10.6	0.42	7	10.5	1.50	9	10.5	1.82	9
6	11.1	0.15	8	12.1	0.45	6	10.8	1.15	8	9.9	1.40	11
8	10.7	0.17	9	9.9	0.48	8	8.7	0.57	10	9.2	1.05	13
10	10.5	0.05	9	10.5	0.43	8	10.1	1.35	11	9.4	2.30	15
12	10.2	0.12	10	10.1	0.30	9	9.4	1.44	12	9.0	2.10	16

[+] Plasma phosphorus in mg % (average value of six rats); urine phosphorus in mg /6 hr/100 gm rat.

[++] units/250 µg total nitrogen (taurine not included).

* The linear regressions of plasma phosphorus level on time were calculated for each of the four groups; the rates of decrease per two week period for each group, and the level of significance in each case are as follows: NCa+D - 0.21785 ($P<0.001$); NCa-D - 0.31428 (not significant); LCa+D - 0.33571 ($P<0.05$); LCa-D - 0.45357 ($P<0.001$).
The significant regressions from groups NCa+D, LCa+D and LCa-D were combined into one analysis of variance. The results showed that the difference between the rate of decrease for group NCa+D and that for group LCa-D was <u>almost</u> significant at P 0.05; there was no significant difference between the rates for groups NCa+D and LCa+D.

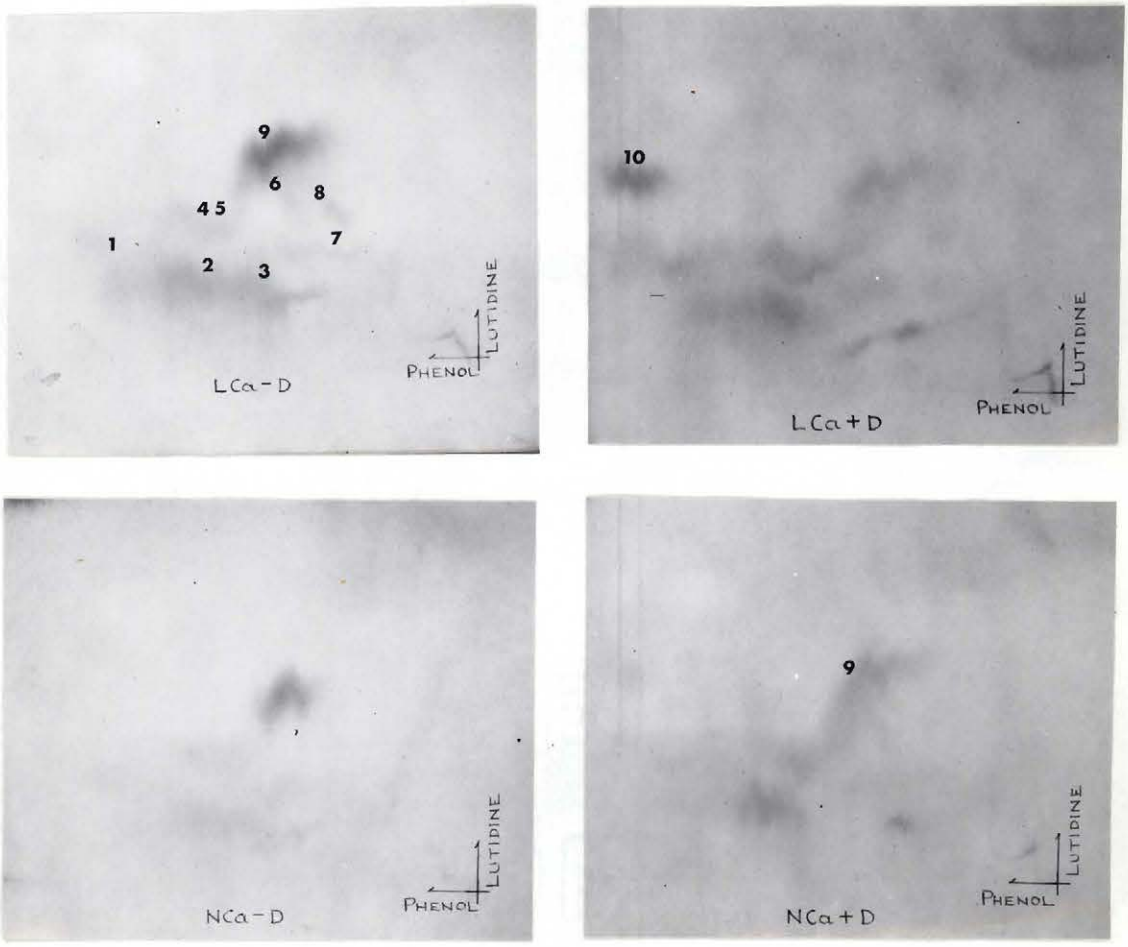
c) Effect of diet on amino acid excretion

The semi-quantitative data presented in Figure 5 and Table IIIb indicate emergency of hyperaminoaciduria in the low calcium, D-deprived group at the fourth week, when compared with the control group (NCa+D) at the eighth week; aminoaciduria in the former group was greater than the maximum value achieved in the other three groups at any time. Abnormal aminoaciduria (by comparison with control group at any age), appeared in the (LCa+D) animals at the tenth week. The aminoaciduria in animals in the (NCa-D) and the control group was similar and did not increase as described above. In all four groups, the amino acid excretion increased to some extent with age. Figure 6 shows the paper chromatographic pattern of the urine samples from the four groups of animals at the twelfth week of the study.

d) Quantitative estimation of the urine and plasma amino acids at the twelfth week of the study

i) Free amino acids in the urine

Animals on low calcium, D-deprived diets had the highest total amino acid output (column A, Figure 7). The other calcium deprived group also had hyperaminoaciduria. The (NCa-D) group had a slightly lower amino acid excretion than the



1 = Histidine; 2 = Arginine + Lysine + Ornithine;
3 = Glutamine; 4 = Alanine; 5 = Threonine; 6 = Glycine;
7 = Glutamic acid; 8 = Serine; 9 = Taurine; 10 = "Food spot".

Fig. 6 Urine chromatograms of the Holtzman rats at the twelfth week of Study B.

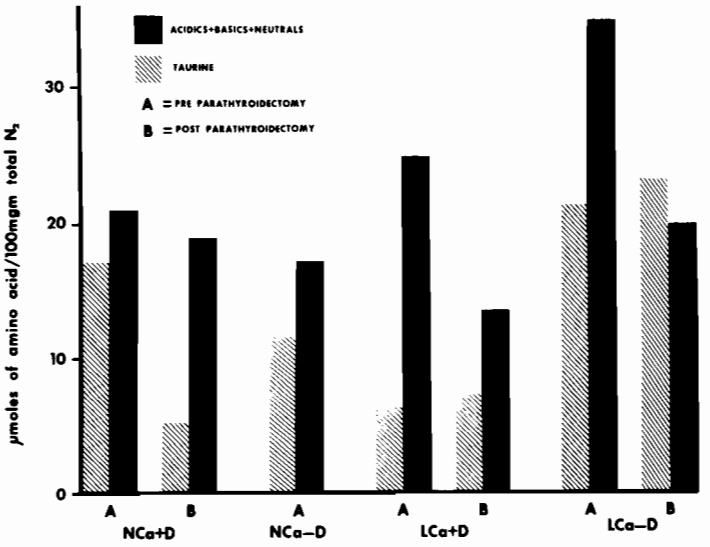


Fig. 7 Effect of parathyroidectomy on the total amino acid excretion in the Holtzman rats at the twelfth week of Study B.

control group. These data corroborate the semi-quantitative estimations (Figure 5).

From Figure 6 and Table IV it is evident that the hyperaminoaciduria in the (LCa-D) group is accounted for primarily by the amino acids hydroxyproline, aspartic acid, glycine, alanine,

TABLE IV

FREE URINARY AMINO ACID EXCRETION IN THE DIFFERENT GROUPS OF RATS AT THE TWELFTH WEEK OF STUDY B

	μmoles of amino acid per 100 mgm total N_2					
	Control			Test		
	NCa+D		NCa-D	LCa-D		LCa+D
Amino Acids	(i)	(ii)		(i)	(ii)	
Taurine	17.10	18.10	11.70	21.25	21.70	6.52
Acidics + Neutrals						
Hydroxyproline	-	-	-	0.47	0.83	0.42
Aspartic acid	-	0.21	0.15	0.55	0.86	0.31
Threonine	5.57	3.26	3.05	4.10	4.30	4.69
Serine	0.99	1.20	1.65	1.70	1.96	1.74
Asp-NH_2 + Glu-NH_2	1.44	1.28	1.3	1.80	2.59	1.14
Proline	-	0.61	-	0.60	0.97	0.55
Glutamic acid	0.88	0.52	0.45	1.05	1.36	1.38
Glycine	2.52	2.78	3.6	4.05	4.96	4.16
Alanine	1.01	1.09	1.70	1.75	2.47	1.36
Valine	trace	0.30	0.25	0.32	0.32	0.35
Methionine	0.34	0.52	0.75	1.18	1.20	0.53
Isoleucine	0.12	0.16	0.18	0.73	0.12	trace
Leucine	0.51	0.18	0.15	0.72	0.06	0.24
Tyrosine	0.16	0.24	0.10	0.33	0.46	0.38
Phenylalanine	0.10	0.16	0.25	0.22	0.46	0.17
β-alanine	0.27	0.39	0.14	0.73	0.59	0.33
β-aminoisobutyric a.	0.41	0.55	0.44	0.26	0.77	0.71
Sub-total	14.32	13.45	14.16	20.56	24.28	18.46
Basics						
Ornithine	0.45	0.44	0.22	0.37	0.89	0.27
Lysine	5.49	5.05	2.28	11.60	9.11	5.30
1-methylhistidine	0.28	0.37	0.05	0.39	0.09	0.35
Histidine	0.92	0.68	0.38	1.19	0.66	0.56
Arginine	0.67	0.17	0.19	0.81	0.53	0.23
Sub-total	7.81	6.71	3.12	14.36	11.28	6.71
TOTAL*	22.13	20.16	17.28	34.92	35.56	25.17

* Exclusive of Taurine.

and lysine; serine, glutamic acid, tyrosine, phenylalanine and asparagine + glutamine, were also elevated. In the (LCa+D) group, only hydroxyproline, glycine, aspartic acid, serine, glutamic acid and tyrosine were increased. Although serine, alanine, methionine and phenylalanine were excreted in slightly higher amounts in the (NCa-D) animals, the total amount of amino acid excreted by this group is less than in the control group. Hydroxyproline was not detected in the control and (NCa-D) samples. Taurine excretion does not seem to bear any relationship to the aminoaciduria in all groups.

ii) Bound amino acids in the urine

In all hydrolyzed samples, there was an increase in amino acids after hydrolysis (Figure 8). The difference between amino acids after hydrolysis and before, represents the "bound" fraction. The bound fraction was considerably greater in the (LCa-D) group than the control group. The bound aminoaciduria was less in the urine specimens from the (LCa+D) and (NCa-D) groups.

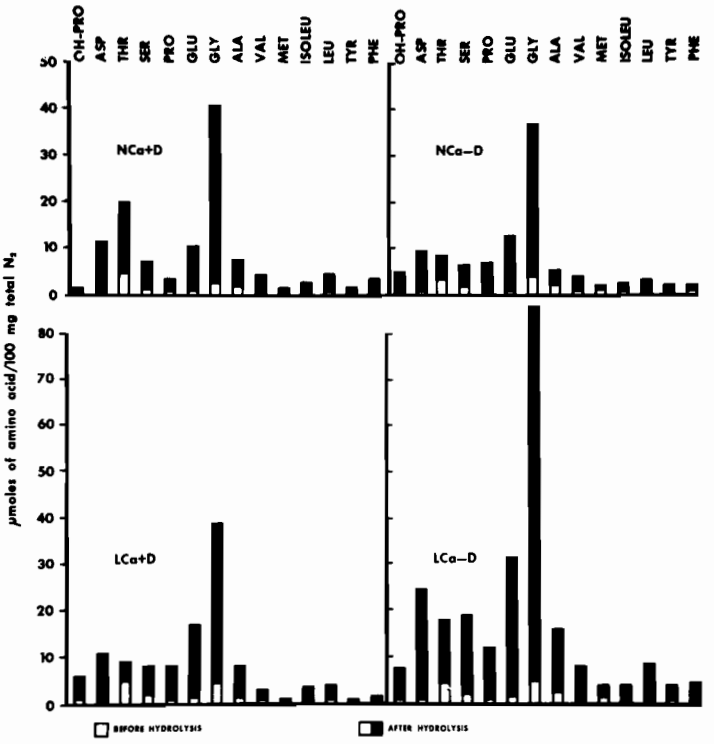


Fig. 8 Amounts of individual acidic and neutral amino acids in the twelfth week urine samples of the Holtzman rats before and after hydrolysis.

The total amounts of the individual amino acids measured after hydrolysis are indicated in Table V. The (LCa-D) sample has a two- to three-fold increase in most of the amino acids over those of the control sample. The bound fractions of hydroxyproline, proline and glycine

TABLE V

TOTAL AMOUNTS OF NEUTRAL AND ACIDIC AMINO ACIDS ESTIMATED AFTER HYDROLYSIS OF THE URINE SAMPLES LISTED IN TABLE IV

	µmoles of amino acid per 100 mgm total N_2					
	Control		Test			
	NCa+D		NCa-D	LCa-D		LCa+D
Amino Acids	(i)	(ii)		(i)	(ii)	
Hydroxyproline	0.60	2.29	5.40	7.65	7.79	6.29
Aspartic acid	12.10	10.07	9.75	25.42	24.12	10.82
Threonine	12.90	8.52	8.72	18.98	17.61	9.13
Serine	7.59	6.51	6.74	17.56	21.14	8.06
Proline	3.0	3.86	6.97	8.55	16.07	8.82
Glutamic acid	16.77	4.62	13.35	32.90	30.50	17.20
Glycine	42.65	39.00	38.97	96.34	75.97	39.35
Alanine	8.25	6.48	5.30	12.45	19.94	8.55
Valine	4.21	4.28	4.05	8.56	7.72	3.22
Methionine	1.45	1.83	2.13	5.34	2.55	0.81
Isoleucine	2.91	2.72	2.43	4.57	4.20	3.77
Leucine	5.47	4.23	3.38	8.29	8.82	4.14
Tyrosine	2.06	1.83	2.08	3.98	4.50	1.11
Phenylalanine	3.77	3.09	2.06	4.59	4.48	1.70

are of particular interest in view of their presumed origins from collagen[93].

iii) Free amino acids in the plasma

Each set of values shown in Table VI was obtained from plasma samples pooled from six rats. The total amount of amino acids in the plasma of the (LCa-D) and (LCa+D) groups are significantly lower than in the control (NCa+D) group. These low values are the results of decreases in the amino acids threonine, serine, asparagine + glutamine, proline, glycine, ornithine and lysine (that is, those amino acids particularly elevated in urine). Only a slight variation was seen in the (NCa-D) sample. Note that free hydroxyproline was detected in plasma only in the (LCa-D) group of animals.

e) Effect of parathyroidectomy

Parathyroidectomy was performed on six rats from the control (NCa+D) group, eight rats from the (LCa+D) group, and ten rats from the (LCa-D) group, at the twelfth week of the study.

Parathyroidectomy was well tolerated by the control animals, but induced a traumatic effect in all the animals from the (LCa-D) and (LCa+D) group.

TABLE VI

PLASMA AMINO ACIDS IN THE DIFFERENT GROUPS OF RATS AT THE TWELFTH WEEK OF STUDY B

	µmoles of amino acid per ml of plasma			
	Control	Test		
Amino Acids	NCa+D	NCa-D	LCa+D	LCa-D
Taurine	0.59	0.27	0.42	0.47
Acidics + Neutrals				
Hydroxyproline	trace	trace	trace	0.01
Aspartic acid	0.09	trace	0.1	0.05
Threonine	0.48	0.45	0.29	0.30
Serine	0.43	0.58	0.26	0.27
Asp-NH_2 + Glu-NH_2	0.48	0.29	0.21	0.22
Proline	0.22	0.29	0.12	0.11
Glutamic acid	0.28	0.61	0.45	0.29
Glycine	0.62	0.83	0.34	0.39
Alanine	0.56	0.52	0.43	0.52
Valine	0.22	0.23	0.22	0.25
Methionine	0.09	0.07	0.09	0.10
Isoleucine	0.16	0.09	0.06	0.18
Leucine	0.23	0.17	0.24	0.26
Tyrosine	0.07	0.15	0.05	0.07
Phenylalanine	0.10	0.06	0.09	0.11
Sub-total	4.03	4.34	2.95	3.13
Basics				
Ornithine	0.22	0.31	0.13	0.12
Lysine	0.65	0.75	0.44	0.39
Histidine	0.07	0.05	0.07	0.07
Arginine	0.15	0.11	0.13	0.17
Sub-total	1.09	1.22	0.77	0.75
TOTAL	5.71	5.83	4.14	4.35

Between the second and fifth hour after the operations these animals developed tetany; two rats from the (LCa+D) group, and three rats from the (LCa-D) group died. Calcium (4 mg, as gluconate in 2 cc of 0.9% NaCl), was injected intraperitoneally into each of the surviving animals. Although the parathyroidectomized control animals did not develop tetany, these animals were also injected with the same amount of calcium.

i) Size of glands

During the process of extirpation, the size of the two parathyroid glands in each animal was carefully studied. The largest glands were observed in the animals from the (LCa-D) group; less prominent were those from the (LCa+D) animals. The parathyroid glands of the animals from the control (NCa+D) group were barely visible under the dissecting microscope. A visual assessment of the gland-sizes is indicated in Table VII.

ii) Effect on plasma calcium

The level of plasma calcium in all the groups of animals measured prior to calcium injection, was lowered by performing a parathyroidectomy (Table VIII).

TABLE VII

VISUAL APPEARANCE OF PARATHYROID GLANDS

Group		Appearance of Glands*
(LCa-D)	(10)**	easily visible to naked eye
(LCa+D)	(8)	just visible to naked eye
(NCa+D) (Control)	(6)	invisible to naked eye; just visible under dissecting microscope.

* Two visible in each animal.

** Number of animals observed.

TABLE VIII

EFFECT OF PARATHYROIDECTOMY ON

PLASMA CALCIUM AND URINARY PHOSPHORUS EXCRETION

	Plasma Ca(mg%)+		Urine P (mg/6 hrs/100 gm rat)	
Group	Pre-PTX	Post-PTX	Pre-PTX	Post-PTX
NCa+D	10.2	6.7	0.14	0.12
LCa+D	6.3	4.9	1.45	0.43
LCa-D	5.0	4.3	2.05	0.32

+ Average value of six animals.

Post-operative plasma phosphorus levels were not determined because the blood samples were hemolysed.

iii) Effect on aminoaciduria

The amino acid excretion before parathyroidectomy and in the six to twelve hour period after operation is shown in Figure 7. Urinary amino acids decreased strikingly in the (LCa-D) rats; the amino acid excretion in the (LCa+D) rats was also lowered, but to a lesser degree. Only slight changes were seen in the control group. Table IX shows that urinary excretion of hydroxyproline, aspartic acid, asparagine + glutamine, proline, glycine, alanine and methionine was particularly influenced by parathyroidectomy in the (LCa-D) group. The excretion of the basic amino acids was generally decreased; the most pronounced change was observed with lysine. In the (LCa+D) rats, only hydroxyproline, threonine, glutamine + asparagine, glycine and methionine excretion was significantly decreased. Amino acid excretion was only slightly altered in the control group and this effect can probably be attributed to the operative procedure and other manipulations.

TABLE IX

EFFECT OF PARATHYROIDECTOMY ON THE URINARY AMINO ACID EXCRETION IN THE DIFFERENT GROUPS OF RATS AT THE TWELFTH WEEK OF STUDY B

	µmoles of amino acid per 100 mgm total N_2								
	Control			Test					
	NCa+D			LCa-D			LCa+D		
Amino Acids	Pre-PTX+	Post-PTX	△	Pre-PTX+	Post-PTX	△	Pre-PTX	Post-PTX	△
Taurine	17.6	5.72	-11.88	21.5	23.40	+ 1.90	6.52	7.44	+0.92
Acidics + Neutrals									
Hydroxyproline	-	-	-	0.65	-	- 0.65	0.42	-	-0.42
Aspartic acid	0.10	trace	-	0.71	trace	- 0.71	-	-	-
Threonine	4.51	3.42	- 1.09	4.20	4.83	+ 0.63	4.70	2.48	-2.22
Serine	1.10	0.86	- 0.24	1.83	2.58	+ 0.75	1.74	1.16	-0.58
Asp-NH_2 + Glu-NH_2	1.36	1.25	- 0.11	2.20	0.33	- 1.87	1.14	0.08	-1.06
Proline	0.31	-	- 0.31	0.79	trace	- 0.79	0.55	-	-0.55
Glutamic acid	0.70	1.26	+ 0.56	1.21	0.58	- 0.63	1.38	2.16	+0.78
Glycine	2.65	3.1	+ 0.45	4.51	3.43	- 1.08	4.16	2.75	-2.41
Alanine	1.05	0.81	- 0.24	2.11	1.35	- 0.76	1.36	1.02	-0.34
Valine	0.15	0.26	+ 0.11	0.32	0.73	+ 0.41	0.35	0.36	+0.01
Methionine	0.43	0.37	- 0.06	1.19	0.55	- 0.64	0.53	0.17	-0.36
Isoleucine	0.14	0.53	+ 0.29	0.43	0.94	+ 0.51	trace	0.07	+0.07
Leucine	0.35	0.02	- 0.33	0.39	0.59	+ 0.20	0.24	0.14	-0.10
Tyrosine	0.20	0.21	+ 0.01	0.35	0.39	+ 0.04	0.38	0.44	+0.06
Phenylalanine	0.13	0.10	- 0.03	0.34	0.24	- 0.10	0.17	0.53	+0.36
β-alanine	0.33	0.19	- 0.14	0.66	0.75	+ 0.09	0.33	0.27	-0.06
β-aminoisobutyric acid	0.48	0.15	- 0.33	0.56	0.88	+ 0.32	0.71	0.49	-0.22
Net change			- 1.46			- 4.28			-6.49

.... cont'd

Table IX continued.....

Amino Acids	µmoles of amino acid per 100 mgm total N_2								
	Control			Test					
	NCa+D			LCa-D			LCa+D		
	Pre-PTX+	Post-PTX	Δ	Pre-PTX+	Post-PTX	Δ	Pre-PTX	Post-PTX	Δ
Basics									
Ornithine	0.45	0.25	- 0.20	0.63	0.17	- 0.46	0.27	0.19	-0.08
Lysine	5.27	4.95	- 0.32	10.36	1.06	- 9.30	0.53	0.43	-0.10
1-Methylhistidine	0.33	0.27	- 0.06	0.29	0.19	- 0.10	-	0.03	+0.03
Histidine	0.80	0.58	- 0.22	0.93	0.52	- 0.41	0.56	0.50	-0.06
Arginine	0.42	0.75	+ 0.33	0.67	0.18	- 0.49	0.23	0.45	+0.22
Net change			- 0.47			-10.76			+0.01
Total net change*			- 1.93			-15.04			-6.48

* Exclusive of Taurine.

\+ Mean values from columns (i) and (ii) in Table IV.

Note the complete disappearance of hydroxyproline after parathyroidectomy in the urines of the (LCa-D) and (LCa+D) groups.

iv) Effect on urinary phosphorus

Urinary phosphorus excretion in the (LCa-D) and (LCa+D) groups decreased after parathyroidectomy. A similar effect was not observed with the control animals (Table VIII).

f) Effect of calcium injection on amino acid and phosphorus excretion

This experiment was conducted at the sixteenth week of the study. A special group of six animals had to be used because of the loss of the animals on the low calcium, D-deprived diet. Animals selected randomly from the low calcium, D-supplemented group were taken off Vitamin D at the twelfth week. Calcium gluconate (4 mg calcium) was injected four weeks later intraperitoneally in test animals and in the usual control group.

i) Plasma calcium levels fell in the special test group from 6.5 mg% approximately at the twelfth week, to 5.2 mg%.

ii) Rapid calcium injection induced a rise in plasma calcium in the control (NCa+D) rats, as well as the (LCa-D) rats (Table X).

TABLE X

EFFECT OF CALCIUM INJECTION
ON PLASMA CALCIUM AND URINARY PHOSPHORUS EXCRETION

	Plasma Ca(mg%)+			Urine phosphorus (µg /hr/100 gm rat)		
Group	A	B	C	A	B	C
NCa+D	10.5	11.8	10.2	273	243	292
LCa-D	5.2	6.5	5.1	790	263	867

A = pre-injection;

B = 2-8 hours post-injection;

C = 20-26 hours post-injection.

+ Average value of six rats.

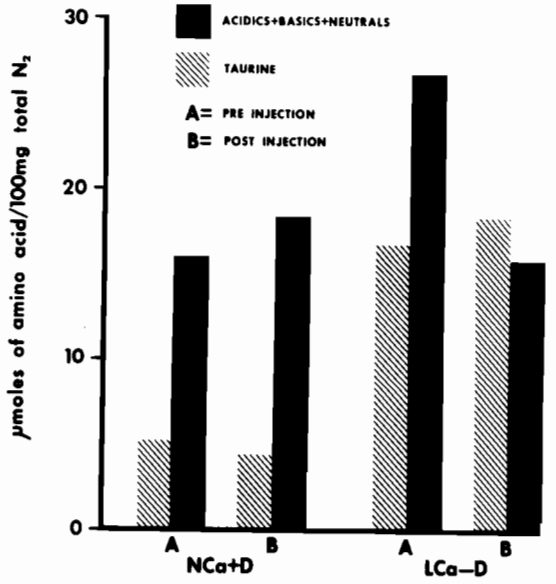


Fig. 9 Effect of calcium injection on the total amino acid excretion in the Holtzman rats at the sixteenth week of Study B.

iii) Hyperaminoaciduria was apparent in the (LCa-D) group (Figure 9). This was suppressed by the calcium injection (Figure 9 and Table XI). The decrease in excretion affected hydroxyproline, glutamic acid, glycine, methionine, tyrosine, lysine and histidine in particular. Twenty hours after the injection, hyperaminoaciduria was again apparent in the low calcium animals (Table XI). Calcium injection had no suppressive effect on the aminoaciduria of the animals in the control group.

iv) Hyperphosphaturia, present in the (LCa-D) rats, was also suppressed by the calcium injection (Table X). Twenty hours after the calcium administration, the hyperphosphaturia had resumed.

TABLE XI

EFFECT OF CALCIUM INJECTION ON URINARY AMINO ACID EXCRETION IN THE CONTROL AND TEST GROUP AT THE SIXTEENTH WEEK OF STUDY B

	µmoles of amino acid per 100 mgm total N_2						
	Control			Test			
	NCa+D			LCa-D			
Amino Acids	A	B	Δ	A	B	Δ	C
Taurine	5.07	4.56	-0.51	16.65	18.50	+1.85	20.55
Acidics + Neutrals							
Hydroxyproline	-	-	-	0.24	-	-0.24	0.32
Aspartic acid	trace	-	-	trace	-	-	trace
Threonine	3.35	4.73	+1.38	7.40	4.85	-2.55	8.91
Serine	1.35	1.23	-0.12	1.87	1.62	-0.25	4.06
Asp-NH_2 + Glu-NH_2	0.58	0.69	+0.11	0.91	0.85	-0.06	0.73
Proline	0.43	0.35	-0.08	trace	trace	-	trace
Glutamic acid	0.90	1.08	+0.18	1.44	0.59	-0.85	1.81
Glycine	3.58	4.21	+0.63	3.43	2.48	-0.95	3.52
Alanine	1.10	0.91	-0.19	1.49	1.34	-0.15	2.08
Valine	0.29	-	-0.29	0.26	0.33	+0.07	0.79
Methionine	0.62	0.87	+0.25	1.56	0.72	-0.84	1.72
Isoleucine	0.28	0.38	+0.10	-	-	-	-
Leucine	0.08	0.08	-	0.11	0.15	+0.04	0.38
Tyrosine	0.13	0.18	+0.05	0.41	0.16	-0.25	0.47
Phenylalanine	0.09	0.09	-	0.06	0.05	-0.01	0.40
β-alanine	0.23	0.13	-0.10	0.33	0.15	-0.18	0.29
β-aminoisobutyric a.	-	0.06	+0.06	0.19	0.12	-0.07	0.54
Net change			+2.98			-6.29	
Basics							
Ornithine	0.26	0.28	+0.02	0.55	0.32	-0.23	0.33
Lysine	2.29	2.48	+0.19	3.10	1.96	-1.14	2.50
1-methylhistidine	0.10	trace	-0.10	0.33	0.31	-0.02	0.19
Histidine	0.25	0.19	-0.06	3.01	0.16	-2.85	0.41
Arginine	0.21	0.23	+0.02	0.47	0.49	+0.02	0.34
Net change			+0.03			-4.22	
Total net change*			+3.01			-11.51	

A = pre-injection;

B = 2-8 hours post-injection;

C = 20-26 hours post-injection.

* Exclusive of Taurine.

IV. DISCUSSION

The findings of the present investigation indicate that severe hypocalcemia and growth retardation can be induced in rats fed on a diet which is very low in calcium content; Vitamin D supplementation to these animals had only a slight beneficial effect on the hypocalcemia and growth rate. In animals fed a normal calcium diet deprived of Vitamin D, hypocalcemia and growth retardation was also apparent; Vitamin D supplementation was able to reverse these abnormalities. Hyperaminoaciduria and hyperphosphaturia occurred only in the animals fed low-calcium diets (A_2-D) and B_2; the excretory abnormality was less intense in the Vitamin D-supplemented animals (B_2+D). Normal aminoaciduria was observed in the D-deprived animals on normal calcium intakes.

Since there was a considerable variation in the components of the diets used in Study A, an accurate interpretation of the aminoaciduria and phosphaturia was difficult in that study. The formal Study B was therefore undertaken to avoid this difficulty and the effect of variation only in calcium or Vitamin D intake on the hyperaminoaciduria and hyperphosphaturia was reinvestigated. The elevated excretion of amino acids and phosphorus observed in the (LCa-D) and (LCa+D) animals cannot be attributed to the dietary differences.

First, the dietary intake was probably less than in the control animals. Secondly, the plasma levels of phosphorus and amino acids were not higher than the control (NCa+D) group (Figure 5 and Table VI). The increase in the renal excretion of these substances can be most easily accounted for by decreased reabsorption by the kidney tubules of filtered substrate. The animals which had the highest degree of aminoaciduria, (LCa-D) group, (Table IV), and phosphaturia (Table IIIb), had the lowest plasma levels of amino acids and phosphorus. A similar, but less pronounced relation between urine and plasma levels is also seen in the (LCa+D) animals.

The effect of Vitamin D deprivation in animals on an adequate calcium intake (NCa-D) group, was apparent; despite adequate dietary calcium, the plasma calcium level was lower than in the control group (NCa+D), indicating an impairment in calcium absorption. The aminoaciduria and plasma amino acids in this group of animals at the twelfth week of study was comparable however with those of the control animals (Tables IV and VI), and the urinary phosphorus excretion was slightly elevated (Table IIIb). Statistical analyses showed that the plasma levels in this group of animals was significantly higher than those in the (LCa+D) group during the last eight weeks of the study (Table IIIa).

The "bound" fraction of the amino acids was significantly higher in the (LCa-D) group than in the other groups (Table V). Since a dietary cause of this variation is unlikely, the elevation of the "bound" fraction in urine may be the result either of decreased reabsorption by the renal tubules of peptides, or an increased peptide turnover and prerenal excretion, or both. The particular elevation in excretion of bound glycine, hydroxyproline and proline suggests the finding reflects higher turnover of bone collagen[93,94]. Walker et al[95] have demonstrated that PTE stimulates a collagenolytic factor in bone in-vitro, which releases bound hydroxyproline.

In the human afflicted with Vitamin D deficiency, renal dysfunction exists comparable to that described here in the rat, which has been attributed directly to deficiency of the vitamin in the kidney tubule[17]. However, the evidence obtained in the present study suggests that the latter possibility is unlikely.

On the basis of the criteria of growth retardation and hypocalcemia[96], the animals in the (NCa-D) and (LCa-D) groups of Study B were D-deficient. However, the latter group of animals had a much higher degree of aminoaciduria and phosphaturia (Figure 5 and Table IV), despite presumably identical levels of D-deprivation. Conversely, in the (NCa+D) and (LCa+D) animals receiving adequate Vitamin D intakes,

there was obvious aminoaciduria and phosphaturia only in the animals on the low calcium intake. If Vitamin D is the factor directly controlling the reabsorption of phosphorus and amino acids, their urinary excretion rates should not be different in either of the pairs of groups (with Vitamin D and without Vitamin D).

Inspection of the plasma calcium levels of the four groups of animals in Study B (Figure 5) revealed that, excepting the control (NCa+D) animals, the three other groups of animals became hypocalcemic after the fourth week of the study. The most severely hypocalcemic (LCa-D) group had the greatest aminoaciduria and phosphaturia; the (NCa-D) group with a lesser degree of hypocalcemia, had only a much less phosphaturia and no hyperaminoaciduria. Only the dietary calcium content was different in these two groups of animals. Despite Vitamin D supplementation, the (LCa+D) animals exhibit abnormal aminoaciduria and phosphaturia; these animals are extremely hypocalcemic. Finally, it was seen that, when significantly lower hypocalcemia (Table IIIa) can be produced by restricting calcium intake instead of Vitamin D, the phosphaturia and hyperaminoaciduria is greater in the more hypocalcemic animals. It seems then that the renal dysfunction is more closely related to the level of calcium in plasma, than to the intake of Vitamin D per se.

The probability that hypocalcemia itself is the factor directly responsible for the renal defect was considered. This is unlikely in view of the finding that parathyroidectomy performed on the (LCa+D) and (LCa-D) animals caused a further decrease in the existing hypocalcemia in both groups; at the same time, the hyperaminoaciduria and phosphaturia were greatly diminished. If the renal dysfunction had any direct relationship to the low plasma calcium, then the further drop in plasma calcium levels should intensify the renal defect, rather than lessening it (Tables VIII and IX, and Figure 7).

Parathyroid gland activity in the rat is known to be highly dependent upon the plasma calcium values[81]. Gland enlargement, coupled with increased activity, is associated with varying degrees of hypocalcemia irrespective of the Vitamin D status of the animals. In the present study the most hypocalcemic (LCa-D) animals had the largest parathyroid glands (Table VII) and these animals had the highest amino acid and phosphorus excretions; the slightly less hypocalcemic (LCa+D) animals (Figure 5) had a lesser degree of parathyroid gland hypertrophy and a less intense hyperaminoaciduria and hyperphosphaturia. The normocalcemic control animals (NCa+D) had normal parathyroid glands and no hyperaminoaciduria or hyperphosphaturia.

The question was then asked, if renal dysfunction is not Vitamin D-dependent and is parathyroid-dependent, can it be temporarily suppressed by induction of transient hypercalcemia. Calcium injection did in fact produce this response (Tables X and XI, and Figure 9). This and the preceding observations suggest that the abnormal renal dysfunction is the direct consequence of hyperactive parathyroids and that the magnitude of the aminoaciduria and phosphaturia bears some direct relationship with the degree of glandular hyperfunction.

The hyperaminoaciduria and the hyperphosphaturia in the (LCa-D) group was suppressed when the plasma calcium was raised to 6.5 mgm%. This suggests that a plasma calcium level below 6.5 mgm% is required to initiate the endogenous parathyroid response which inhibits tubular function. This finding may also explain why the (NCa-D) animals with a plasma calcium level of 6.7 mgm% at the twelfth week of the study did not have hyperaminoaciduria.

Thus far, evidence has been presented to indicate that the renal dysfunction is brought about by a secondary hyperparathyroidism mediated through hypocalcemia. A phosphaturic response to PTH has been demonstrated in the rat[97] and the dog[98]. Recent studies by Leeming and Fraser[99], and Ney et al[100] showed that the hyperphosphaturic response to

D-deficiency in the dog can be abolished by parathyroidectomy.

The effect of PTH on amino acid transport has not been recorded. In-vitro experiments indicated that: i) PTE and PTH both inhibit the oxidation of the substrates in the Krebs cycle in kidney cell preparations of the rat[47,48] and of the rabbit[49]; ii) PTH stimulates an ATP-ase activity (not dependent on Na^+ and K^+ ions) in liver mitochondria[101] and rabbit kidney preparations[49]. Since active transport of amino acids is dependent on the energy derived from oxidative metabolism[42], it is expected that a PTH-induced inhibition of cellular energy metabolism could lead to an impairment of amino acid transport in the organism.

PTH is also known to affect calcium and magnesium transport. The magnesium content in the kidney mitochondria in the D-deficient animal is significantly lower than in D-fed controls[102]; parathyroidectomy in the D-deficient animal eliminates this difference. Studies in-vitro also demonstrated that PTH alters Mg^{++} ion transport in isolated mitochondria[51]. It is also known that PTH injected into the rat decreases calcium "clearance"[103] and parathyroidectomy increases urinary calcium excretion[104]. The Mg^{++} ion dependent and Ca^{++} ion inhibited Na^+ plus K^+-activated ATP-ases from membrane preparations have been implicated to participate in the active transport of amino acids in-vitro[42]. Recent

results obtained from rabbit kidney cortex preparations indicate that Ca^{++} ion inhibits the enzyme activity via competition with Mg^{++} for the substrate ATP; the substrate hydrolysed by the enzyme is the Mg^{++}-ATP complex[105]. In instances of elevated endogenous PTH levels, the imbalance of two ions Mg^{++} and Ca^{++} at the kidney tubules could then lead to an inhibition of the membrane enzyme and indirectly interfere with amino acid transport. The important role of these two ions in active transport processes have been emphasized by Quastel[106]. In the present study, parathyroidectomy corrected the defect in amino acid transport, and it is possible that this is brought about through the redistribution of the Mg^{++} and Ca^{++} ions in the kidney cells.

The finding of elevated quantities of hydroxyproline in the urine of the (LCa-D) and (LCa+D) animals is of interest. This amino acid is known to reflect collagen turnover[94]. PTE injected into the human increased the excretion of hydroxyproline[107] and tracer studies conducted in the rat indicated that the increased amounts of this amino acid excreted is of bone origin[108]. The finding of increased amounts of free and bound hydroxyproline in the (LCa-D) groups (Tables IV and V) implied that endogenous PTH is acting effectively on the bone sites despite Vitamin D-deprivation. Toverud[109] was able to show that PTH injection does increase

the plasma Ca in D-deficient rats and Rasmussen et al[110] also reported similar findings with high doses of PTH. Bone was considered to be the source of the calcium elevation in these experiments.

The greatly enlarged parathyroid glands in the severely hypocalcemic (LCa-D) animals in both the preliminary Study A and the formal Study B were presumably indicative of a maximum response to offset the low plasma calcium levels induced by the diet. The further drop in the plasma calcium which occurred after parathyroidectomy in these animals (Table VIII), indicates that this glandular response was exerting an effect on the bone pool of calcium.

Thus far, evidence is presented in full support of the proposed hypothesis that the hyperaminoaciduria and hyperphosphaturia demonstrated in the experimental rat is the direct consequence of a secondary hyperparathyroidism in response to the diet-induced hypocalcemia. The lesser degree of hyperaminoaciduria and hyperphosphaturia in the Vitamin D-supplemented, hypocalcemic rat, is not related to a direct effect of the vitamin in the renal tubules, but rather to the higher calcium level and lesser degree of hyperparathyroidism in these animals. A defect in phosphorus reabsorption is apparent at an earlier stage of hypocalcemia than the

defect in amino acid reabsorption mechanism, suggests that phosphaturia is a more sensitive index to PTH levels than is hyperaminoaciduria. The inability of Engström et al[23] to demonstrate an elevated aminoaciduria in their D-deficient Holtzman rats may be attributed to their short follow-up period of only three weeks. Although no information was given to indicate the plasma calcium levels of their animals, an inadequate degree of hyperparathyroidism can be postulated to explain the discrepancy between their study and the present one.

V. SUMMARY

1. Rachitic lesions were induced in the rat through the use of two D-deficient diets, one with a low calcium content and the other with a low phosphorus content.
2. The animals fed low calcium diets with or without Vitamin D, and the normal calcium diet deprived of Vitamin D, developed hypocalcemia. Vitamin D supplementation corrected the hypocalcemia only in the animals given the normal calcium diet.
3. Growth retardation was observed in the D-deficient animals. The smallest weight gain was observed in the animals fed the low calcium diet; Vitamin D supplementation to calcium deprived animals caused only a slight increase in body weight.
4. Hyperexcretion of free amino acids and phosphorus was demonstrated only in severely hypocalcemic rats (<6.5 mg%), irrespective of the Vitamin D status of these animals. Hyperphosphaturia appeared earlier than hyperaminoaciduria in the temporal progression of tubular dysfunction.
5. Abnormal excretion rates were apparently related to impaired net tubular absorption of solutes.
6. The more severely hypocalcemic, Vitamin D-deprived animals had enlarged parathyroid glands; the intensity of hyper-

aminoaciduria and hyperphosphaturia was directly proportional to the degree of parathyroid enlargement, which was in turn inversely proportional to the plasma calcium level.

7. The excretion of bound amino acids was two-three times higher in the most hypocalcemic animals, compared to the control group. The principle components of the bound fraction were hydroxyproline, proline and glycine.
8. Calcium injection, which raised serum calcium above 6.5 mg%, produced a temporary suppression of the renal dysfunction in hypocalcemic animals. Parathyroidectomy completely abolished the renal dysfunction. Control groups were not influenced by these procedures.
9. It was concluded that hyperfunction of parathyroid glands, and not absence of Vitamin D, causes impairment of renal tubular transport in Vitamin D deficiency.

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