TABLE III. Staining Properties of Poliovirus Φ × 174 and Polyoma.

Fixed virus concentrate	Color developed with AO staining	Nuclease susceptibility: DNA-ase	Nuclease susceptibility: RNA-ase	Proteolytic enzyme necessary
Poliovirus	Flame red	—	+	—
Φ × 174	" "	+	—	—
Polyoma	Yellow green	+	—	+

which included both plaque and hemagglutination assays.

Droplet preparations of this purified polyoma virus suspension were fixed 30 minutes in Carnoy's fluid and stained with 0.01% acridine orange at pH 3.8. The staining technic and relevant enzyme digestion tests have been described in detail(8,6). Simultaneous staining of purified preparations of bacteriophage Φ × 174(6) and poliovirus (8) was carried out as a control. The results are shown in Table III. It would appear that the nucleic acid present in polyoma is DNA, and that in the mature virus particle it exists in the double-stranded form.

It may be argued that a DNA virus replicating in the nucleus could possibly adsorb to its surface sufficient cellular DNA to give a double-stranded color reaction in the standard acridine orange test. To clarify this point, 3 unfixed droplet preparations were first incubated in DNAase (0.01% in veronal buffer) for 10 minutes, 1 hour and 2 hours respectively. They were then rinsed in distilled water, fixed in Carnoy's and stained as above. All preparations developed an equally brilliant yellow green color only slightly less in intensity than the untreated specimens. It woud appear that there is some cellular DNA adsorbed to the virus particle but that this amount is readily and rapidly removed by nuclease digestion leaving the reaction of the intact virus particle essentially unchanged.

Summary. Preparations of polyoma virus purified by CsCl isodensity centrifugation stained yellow green at pH 3.8 with 0.01% acridine orange. Pretreatment with a proteolytic enzyme was necessary before development of the stain could be inhibited by DNAase. RNAase had no effect on similar preparations. These characteristics are consistent with the identification of the nucleic acid of polyoma virus as a double-stranded DNA.

1. Di Mayorca, G. A., Eddy, B. E., Stewart, S. E., Hunter, W. S., Friend, C., Bendich, A., *Proc. Nat. Acad. Sci.*, 1959, v45, 1805.

2. Smith, J. D., Freeman, G., Vogt, M., Dulbecco, R., *Virology*, 1960, v12, 185.

3. Allison, A. C., Armstrong, J. A., *Brit. J. Cancer*, 1960, vXIV, 313.

4. Williams, M. G., Sheinin, R., *Virology*, 1961, v13, 368.

5. Bereczky, E., Dmochowski, L., Grey, C. E., *J. Nat. Cancer Inst.*, in press.

6. Mayor, H. D., Hill, N. O., *Virology*, in press.

7. Smith, K. O., Benyesh-Melnick, M., PROC. SOC. EXP. BIOL. AND MED., in press.

8. Mayor, H. D., Diwan, A. R., *Virology*, 1961, v14, 74.

Received July 10, 1961. P.S.E.B.M., 1961, v108.

Vitamin D and Urinary Amino Acid Excretion in the Rat.* (26859)

GEORGE W. ENGSTROM, HECTOR F. DELUCA, JOHN W. CRAMER AND H. STEENBOCK
(Introduced by A. E. Harper)
Department of Biochemistry, University of Wisconsin, Madison

An elevated excretion of amino acids in the urine of rachitic children was first observed by Hottinger(1). Freudenberg and Goetz(2) later demonstrated that this could be reversed by a single massive dose of Vit. D. However, Jonxis *et al.*(3), who also reported

* Published with the approval of the director of the Wisconsin Agricultural Experiment Station. We are indebted to the Wisconsin Alumni Research Foundation for financial support and to Merck Sharp and Dohme, Research Laboratories, Rahway, New Jersey, for some of the vitamins used in the experiments.

an increase in free and bound urinary amino acids in rachitic children, found that this increased excretion did not return to normal for 3 to 4 weeks after treatment with Vit. D. It was therefore obvious that this effect of the vitamin lags far behind its effect on the calcium and phosphorus levels of plasma and initiation of healing of rickets. Jonxis and Huisman(4) more recently concluded that the increased excretion was not the result of a metabolic disturbance but rather of a defective tubular reabsorption, since plasma amino acid levels were unchanged in rickets. Nevertheless these investigators considered the aminoaciduria to be a primary effect of Vit. D deficiency. Many studies on amino acid excretion have since appeared and have been carefully reviewed elsewhere (Lathem *et al.*(5), Jonxis(6), Evered(7), Harrison and Harrison(8), and Harris(9)).

In this laboratory, the search for a metabolic action of Vit. D has led to a study of amino acid metabolism in rats fed various diets with and without Vit. D. This paper reports results which show clearly that aminoaciduria does not occur in rats deficient in Vit. D.

Methods. Thirty-day-old male albino rats of the Holtzman strain weighing 65-85 g were used in all experiments. They were housed in overhanging wire cages during the first 3-day period and given food and distilled water *ad libitum*. They were then placed in wire metabolism cages and were transferred to separate feeding cages for 3 one-hour periods daily during which time they had access to unlimited quantities of food unless otherwise indicated. Urine collections were made during the 7th-9th, 10th-12th, and 13th-15th days of experiment. Urine was collected under a layer of toluene and feces were retained on wire screens attached to the bottom of the cage. At the end of an experiment, the rats were killed by a blow on the head followed by severing of the carotid artery. When desired, blood was collected in 15 ml centrifuge tubes. Finally, the rachitic state of the rats was estimated by femur ash determinations and by silver nitrate staining of sectioned radii(10).

Free alpha amino nitrogen was measured by the method of Van Slyke *et al.*(11), serum alpha amino nitrogen according to the method of Hamilton and Van Slyke(12), urinary phosphorus by the method of Fiske and Subbarow(13) following digestion with concentrated nitric and perchloric acids, and creatinine according to the method of Owen *et al.*(14). Individual urinary amino acids were identified and determined quantitatively by ion exchange chromatography according to the method of Moore *et al.*(15) with the Beckman/Spinco Amino Acid Analyzer(16).

All rats were fed essentially the basal diet of Bellin and Steenbock(17) which contains D-glucose monohydrate (Cerelose) 67%, steamed egg white 18%, cottonseed oil (Wesson) 10%, roughage (Celluflour) 3%, calcium and phosphorus free salts 2%, and crystalline vitamins. In addition the rats received orally a fat soluble vitamin mixture dissolved in cottonseed oil (Wesson) which supplied 70 μg of β carotene, 875 μg of α tocopherol, and 105 μg of menadione to each rat per week. In each experiment, one-half the rats received orally 75 i.u. of crystalline Vit. D_2 (calciferol) in cottonseed oil per rat every 3 days. The desired levels of calcium and phosphorus in the diet were achieved by addition of $CaCO_3$ and/or an equimolar mixture of KH_2PO_4 and K_2HPO_4 at the expense of the Cerelose. Diet 11 was non-rachitogenic (0.45% Ca and 0.3% P), diets 23 (0.45% Ca and 0.02% P) and 34B (0.02% Ca and 0.02%P) were strongly rachitogenic while diet 11D (0.02% Ca and 0.3% P) produced the low calcium Vit. D deficiency syndrome in agreement with the results of Steenbock and Herting(10). Diet 24 (1.5% Ca and 0.12% P) was another strongly rachitogenic diet in which vitamin-free casein (General Biochemicals Inc.) plus 0.2% L-cystine served as the protein source. Severe rickets was produced by this diet in agreement with the results of Guroff(18).

Results and discussion. Although there have been numerous reports of aminoaciduria in rachitic infants and dogs, studies with rats have been lacking. In the many experiments conducted, we were unable to demonstrate a significant elevation of free alpha amino nitrogen in urine of rachitic rats above

TABLE I. Urinary Free Alpha Amino Nitrogen from Rats Fed Various Diets with and without Vitamin D.*

	mg α-amino nitrogen/100 g rat/3 days				mg α-amino nitrogen/mg creatinine/3 days			
	Ad libitum		Pair fed		*Ad libitum*		Pair fed	
	−D	+D†	−D	+D†	−D	+D†	−D	+D†
Diet 24 (1.5% Ca + .12% P)‡	3.3	3.7	3.1	3.3	.6	.4	.6	.4
" 23 (.45% Ca + .02% P)	6.3	5.3	—	—	.6	.5	—	—
" 34B (.02% Ca + .02% P)	4.4	4.4	4.7	4.2	.6	.5	.6	.5
" 11D (.02% Ca + .3% P)	2.1	3.3	1.9	2.1	.3	.4	.3	.3
" 11 (.45% Ca + .3% P)	3.3	4.0	3.1	4.4	.5	.6	.5	.6

* Values are avg from at least 6 animals fed the various diets for 12 days.
† Seventy-five i.u. of vit. D_2 (calciferol) in Wesson oil was given each rat every 3 days.
‡ Protein source was casein plus 0.2% L-cystine.

that in the urine of their Vit. D supplemented controls, in contrast to a previous report(19). Typical results of those obtained are shown in Table I. It is clear that only slight elevations were found in urinary alpha amino nitrogen when the potent rachitogenic diets 23 and 34B were fed. In a further effort to simulate dietary conditions associated with infantile rickets more closely, the protein source was changed to casein plus cystine and calcium level was elevated to insure rachitogenesis. Again, no aminoaciduria was evident (diet 24). These results contrast sharply with those obtained with human rickets in which a 3- to 4-fold elevation of alpha amino nitrogen occurs(20).

The problem of changes in food consumption and animal size is a major consideration in studies such as these despite the expression of amino nitrogen values on an equivalent rat weight basis. To overcome such criticism, rats on the various diets tested were pair-fed with their Vit. D supplemented controls and amino nitrogen excretion was expressed on either a rat weight basis or a urinary creatinine basis. In these experiments (Table I) it was even more evident that aminoaciduria does not occur in rat rickets (diets 23, 34B or 24) or when either a non-rachitogenic (No. 11) or a low Ca (No. 11D) diet is fed. Our results, therefore, do not support the belief of Jonxis *et al.*(4) that aminoaciduria is a primary effect of Vit. D deficiency.

The difference between human and rat rickets with regard to aminoaciduria aroused some interest. The existence of a defective tubular reabsorption of phosphate in rachitic infants and dogs has been known for some time(21). In agreement with others(22), results shown in Table II demonstrate that excessive urinary excretion of phosphate is not found in rachitic rats. It may be a coincidence that rats and humans differ in both amino acid and phosphate reabsorption in rickets; however, a possible relationship between phosphate and amino acid reabsorption in the kidney tubules may provide insight into this problem.

It appeared possible that aminoaciduria in rat rickets might be masked by excessive excretion of a single amino acid which might be unaffected by Vit. D. The amino acids from rachitic and Vit. D supplemented rats were therefore determined qualitatively and quantitatively by ion exchange chromatography. Jonxis and Huisman(23) have reported large losses of serine, threonine, glycine, alanine, histidine, lysine, and bound glutamic acid in urine of rachitic children. Our results shown in Table III clearly reveal that in rat rickets no marked increases occur that might be classified as an aminoaciduria. It should be noted that the relatively large increase in cystine excretion occurred with the diet (No. 24) supplemented with 0.2%

TABLE II. Urinary Phosphorus of Rats Fed Rachitogenic Diets with and without Vitamin D.*

	mg/rat/6 days	
	−D	+D†
Diet 23 (.45% Ca + .02% P)	.8	1.0
" 34B (.02% Ca + .02% P)	1.0	1.0

* Values are avg from 8 rats fed their respective diets for 12 days.
† Seventy-five i.u. of vit. D_2/rat every 3 days.

TABLE III. Urinary Amino Acids from Rats Fed Rachitogenic Diets with and without Vitamin D.*

	μmoles/100 g rat/3 days			
	Diet 23		Diet 24‡	
	−D	+D†	−D	+D†
Aspartic acid	1.4	3.1	1.4	2.1
Threonine	3.5	3.3	5.0	4.9
Serine, glutamine & asparagine	9.2	5.9	7.9	6.8
Glutamic acid	11.0	12.0	6.9	7.0
Proline, citrulline	4.1	2.9	4.8	3.4
Glycine	14.7	17.5	10.9	11.9
Alanine	6.8	6.7	5.7	6.6
Methionine	2.3	1.6	2.0	1.9
Valine	1.4	.8	1.1	.9
Cystine	—	2.2	47.7	23.2
Isoleucine	1.8	1.3	2.2	1.3
Leucine	1.6	1.6	1.5	1.3
Tyrosine	1.2	.9	1.1	1.0
Phenylalanine	1.2	.9	.8	1.1
Lysine, ornithine	1.7	3.2	4.6	6.0
Histidine	5.7	5.6	1.4	2.2
Arginine	3.0	3.2	2.7	3.2
Methionine sulfoxides	16.0	12.7	3.9	3.6
Total	86.6	85.4	111.6	87.5

* Values represent one analysis of a pooled sample from 10 rats fed their respective diets for 10-12 days.

† Seventy-five i.u. of vit. D_2/rat every 3 days.

‡ Casein plus 0.2% cystine served as protein source.

L cystine. Since no significant effect of Vit. D deficiency on excretion of this amino acid occurred with all the other diets tested, which contain adequate amounts of cystine in the protein form, this was not looked upon as an aminoaciduria associated with rickets. Similar results, not shown, were also obtained with the other diets (Nos. 34B, 11 and 11D) tested.

Summary. Free alpha amino nitrogen as well as individual amino acids have been determined in urine of rats fed various diets with and without Vit. D. In contrast to infantile rickets, no aminoaciduria occurs in Vit. D deficiency whether strongly rachitogenic or non-rachitogenic diets are fed. Furthermore, a rachitogenic casein diet also produced no aminoaciduria.

1. Hottinger, A., Hottinger, G., *Mschr. f. Kinderh.,* 1932, v52, 204.

2. Freudenberg, E., Goetz, H., *Annales Paediatrici,* 1939, v153, 233.

3. Jonxis, J. H. P., Huisman, T. H. J., *Lancet,* 1952, v263, 1015.

4. ———, *ibid.,* 1954, v267, 513.

5. Lathem, W., Baker, K., Bradley, S. E., *Am. J. Med.,* 1955, v18, 249.

6. Jonxis, J. H. P., *Helv. Paed. Acta,* 1955, v10, 245.

7. Evered, D. F., *Biochem. J.,* 1956, v62, 416.

8. Harrison, H. E., Harrison, H. C., *J. Am. Med. Assn.,* 1957, v164, 1571.

9. Harris, H., *Brit. Med. Bull.,* 1957, v13, 27.

10. Steenbock, H., Herting, D. C., *J. Nutrition,* 1955, v57, 449.

11. Van Slyke, D. D., Fadyen, D. A. M., Hamilton, P. B., *J. Biol. Chem.,* 1943, v150, 251.

12. Hamilton, P. B., Van Slyke, D. D., *ibid.,* 1943, v150, 231.

13. Fiske, C. H., Subbarow, Y., *ibid.,* 1925, v66, 375.

14. Owen, J. A., Iggo, B., Scandret, F. J., Stewart, C. P., *Biochem. J.,* 1954, v58, 426.

15. Moore, S., Spackman, D. H., Stein, W. H., *Anal. Chem.,* 1958, v30, 1185.

16. Spackman, D. H., Stein, W. H., Moore, S., *ibid.,* 1958, v30, 1190.

17. Bellin, S. A., Steenbock, H., *J. Biol. Chem.,* 1952, v194, 311.

18. Guroff, G., Ph.D. Thesis, Univ. Wisconsin, Citric Acid Metabolism in Relation to Vit. D and Calcification.

19. Cramer, J. W., Engstrom, G. W., Steenbock, H., *Fed. Proc.,* 1957, v16, 383.

20. Harrison, H. E., *Pediat.,* 1954, v14, 285.

21. Harrison, H. E., Harrison, H. C., *J. Clin. Invest.,* 1941, v20, 47.

22. Nicolaysen, R., Eeg-Larsen, N., Ciba Foundation symposium on bone structure and metabolism. Mode of action of vitamin D., 1956, pp 175-184.

23. Jonxis, J. H. P., Huisman, T. H., *Lancet,* 1953, v265, 428.

Received July 10, 1961. P.S.E.B.M., 1961, v108.