35% of normal. No changes of any significance were noted in the coagulation times.

Conclusion. It has been previously reported that methionine prolonged the bleeding and coagulation times. In this study the effects of dl-methionine on the intravenous coagulation time and prothrombin were observed in human subjects. The changes were insignificant and dl-methionine has no clinical value as an anticoagulant.

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Vitamin B_6 Group. XV. Urinary Excretion of Pyridoxal, Pyridoxamine, Pyridoxine, and 4-Pyridoxic Acid in Human Subjects.*

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The earliest investigations of the excretion of vitamin B_6 by animals were made using the chlorimide reaction for analysis.[1,2] The method as then used was not specific for the determination of pyridoxine, but since the complex nature of vitamin B_6 was then unknown, all material found by this method was called *pyridoxine*. These early investigations did establish the fact that pyridoxine was rapidly absorbed from the digestive tract and rapidly cleared in the renal pathway. Although the recovery of the ingested vitamin in the rat was 50 to 70%,[1] only 10 to 20% of the dose was recovered when pyridoxine was fed to dogs or to human subjects.[2]

Subsequent refinement of the chlorimide method showed that small amounts of some substance other than pyridoxine were excreted in the urine of the dog and man after feeding a large dose of pyridoxine.[3] It was also shown that both pyridoxine and the unknown metabolite occurred in part as conjugated forms which did not react with 2, 6-dichloroquinone chlorimide, but which could be hydrolyzed with acid to give pyridoxine and the unknown metabolite.

The demonstration of the occurrence of *pseudopyridoxine* in human urine both before and after administration of pyridoxine[4] and the subsequent characterization of *pseudopyridoxine* as pyridoxal and pyridoxamine,[5] suggested the identity of the unknown metabolite of Scudi *et al.*[3] with pyridoxal or pyridoxamine.

The main metabolic product excreted after ingestion of pyridoxine was discovered by Huff and Perlzweig and identified as 4-pyridoxic acid.[6] This compound does not produce a color with the chlorimide reagent, and is inactive in promoting growth of microorganisms in vitamin B_6-free media.

Although pyridoxal and pyridoxamine are now known to be the forms of vitamin B_6 present in largest amounts in many foodstuffs and tissues,[7,8] no information is available concerning their metabolic fate. Development of a differential assay procedure for pyridoxal, pyridoxamine, and pyridoxine[8] makes such a study feasible. Results of such an investiga-

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2 Scudi, J. V., Unna, K., and Antopol, W., *J. Biol. Chem.*, 1940, **135**, 371.

3 Scudi, J. V., Buhs, R. P., and Hood, D. B., *J. Biol. Chem.*, 1942, **142**, 323.

4 Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

5 Snell, E. E., *J.A.C.S.*, 1944, **66**, 2082.

6 Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1944, **155**, 345.

7 Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 491.

8 Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, 1948, **176**, 1157.

tion conducted with normal human subjects are presented below.

Methods. Twenty-four hour samples of urine were collected from 3 adult male subjects and used as control samples to establish a base level for vitamin B_6 excretion by individuals on a normal diet. Individual subjects were then fed 100 mg of pyridoxine hydrochloride, pyridoxamine dihydrochloride, or pyridoxal hydrochloride in one dose with approximately 200 cc of water. Urine was collected 2, 5, 8, 12, 24, and 36 hours after ingestion of the test dose, and stored under toluene in amber bottles at 7° until analysis. Two months later the experiment was repeated using the same subjects but feeding each subject a different form of the vitamin than had been fed the first time.

The pyridoxal, pyridoxamine, and pyridoxine content of these samples was determined by the differential microbiological assay which uses *Lactobacillus casei, Streptococcus faecalis,* and *Saccharomyces carlsbergensis* as test organisms.[8] The pyridoxic acid content of the samples was determined by the fluorometric method of Huff and Perlzweig,[6] and is expressed in terms of the lactone.

Since a large part of the vitamin B_6 of natural materials is unavailable to the test microorganisms unless the samples are first autoclaved with acid,[9] control samples of urine were subjected to various conditions of acid hydrolysis to determine the optimal conditions for the liberation of vitamin B_6. The results (Table I) show that acid hydrolysis results in the liberation of increased amounts of vitamin B_6 in urine over that observed in unhydrolyzed samples. 0.055 N acid, the concentration commonly used for most samples[9,10,11] was found to be less effective than treatment with 0.11 N HCl or 0.55 N HCl. 0.11 N HCl was used instead of 0.55 N HCl in hydrolysis of the samples, since the amount of potassium chloride present in an assay tube after neutralization of samples treated with 0.55 N acid reaches approximately 135 mg per 5 cc of medium, which is close to the highest level of potassium chloride tolerated by *Saccharomyces carlsbergensis* and lactic acid bacteria.[12]

The samples collected were therefore treated in the following way prior to microbiological assay: 10 cc aliquots of each sample were autoclaved for 7 hours at 20 lb pressure in 180 cc of 0.11 N HCl. The samples were then neutralized with potassium hydroxide, diluted, and assayed.

TABLE I.
Liberation of Vitamin B_6 in Normal Urine by Acid Hydrolysis.

Treatment	Pyridoxal · HCl mγ/cc	Pyridoxamine · 2HCl mγ/cc
None	29	0
.055 N HCl*	78	133
.11 "	93	196
.55 "	93	210

* Autoclaved in 180 cc of acid at 20 lb pressure for 5 hr.

The limitations of the differential assay have been pointed out elsewhere,[8] especially in regard to the determination of the pyridoxine content of samples low in this form of vitamin B_6. In addition to these inherent limitations of the differential assay, it was found that the recovery of pyridoxine added to a normal urine sample was somewhat lower than could be expected from purely analytical errors.[8] Urine seemed to contain a material toxic for *Saccharomyces carlsbergensis* but not for *Streptococcus faecalis* or *Lactobacillus casei.* The presence of this toxic material results in an underestimation of whatever pyridoxine may be present in the control sample of urine, but does not affect the determination of pyridoxal or pyridoxamine in this sample. None of the results are affected in other samples, which contained much more vitamin B_6 and were therefore assayed at dilutions such that the toxic material was without effect.

Results. The distribution of the 4 compounds determined in normal urine is shown in columns *b* and *e,* Table II. The levels of

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[10] Rubin, S. J., Scheiner, J., and Hirschberg, E., *J. Biol. Chem.,* 1947, **167**, 599.

[11] Rabinowitz, J. C., and Snell, E. E., *Anal. Chem.,* 1947, **19**, 277.

[12] MacLeod, R. A., and Snell, E. E., *J. Biol. Chem.,* 1948, **176**, 39.

TABLE II.
The Urinary Excretion of Pyridoxic Acid, Pyridoxal, Pyridoxamine and Pyridoxine.

Excretion products*	Mg excreted up to 24 hr after ingestion (a)	Mg excreted 24 hr before ingestion (b)	Mg recovered (c)	Mg excreted up to 24 hr after ingestion (d)	Mg excreted 24 hr before ingestion (e)	Mg recovered (f)
	Fed 70 mg pyridoxamine. Subject A			Subject C		
4-pyridoxic acid lactone	24.60	6.48	18.12	22.37	2.59	19.78
Pyridoxal	1.57	0.102	1.47	0.985	0.058	0.93
Pyridoxamine	1.83	0.028	1.80	1.18	0.149	1.03
Pyridoxine	0.00	0.00	0.00	0.209	0.00	0.21
Total			21.39			21.95
	Fed 82 mg pyridoxine. Subject B			Subject A		
4-pyridoxic acid lactone	28.40	1.97	26.43	25.27	3.91	21.36
Pyridoxal	1.69	0.074	1.62	1.38	0.052	1.33
Pyridoxamine	0.535	0.124	0.41	0.650	0.210	0.44
Pyridoxine	8.53	0.00	8.53	7.80	0.00	7.80
Total			36.99			30.93
	Fed 82 mg pyridoxal. Subject C			Subject B		
4-pyridoxic acid lactone	59.79	3.84	55.95	53.22	2.54	50.68
Pyridoxal	1.53	0.084	1.45	1.28	0.049	1.23
Pyridoxamine	0.021	0.083	0.00	0.241	0.097	0.14
Pyridoxine	0.039	0.00	0.04	0.132	0.00	0.13
Total			57.44			52.18

* All products are expressed in terms of the free bases. Pyridoxic acid is determined and expressed as the lactone. The molecular weight of the free bases are almost equal (pyridoxal = 167, pyridoxamine = 168, pyridoxine = 169, 4-pyridoxic acid lactone = 165). Expressed in this way, weights are directly comparable as molar quantities without significant error.

these compounds were not particularly constant. Of the 4, pyridoxic acid was quantitatively the most prominent, accounting for from 91 to 98% of the total. Of the 3 forms of vitamin B_6, pyridoxamine was usually present in highest concentrations, although pyridoxal was sometimes present in higher concentrations. Some pyridoxal was always found in the urine. The presence of pyridoxine could not be demonstrated in any of the normal urine samples; but, as noted above, this result may be due to the limitations of the method of analysis and does not constitute satisfactory evidence that pyridoxine is not excreted under normal conditions.

When pyridoxamine was fed, a significant rise in the pyridoxal as well as in the pyridoxamine content of the urine was noted; in fact, the amount of the administered pyridoxamine recovered as pyridoxal was equal to the amount recovered as pyridoxamine. The amount of pyridoxine indicated as being formed was not significant. The principal excretion product was pyridoxic acid, which accounted for 85% of the measured excretion products for subject A and 90% for subject C.

After feeding pyridoxine, the major part of the vitamin B_6 excreted was in the form of pyridoxine, but 14 to 15% of the activity appeared as pyridoxal. The pyridoxamine of the urine accounted for only 4 to 5% of the vitamin B_6 activity of the urine. In this case 71 and 69% of the excretion products measured appeared as pyridoxic acid.

When pyridoxal was fed, larger amounts of pyridoxic acid were recovered than in either of the two preceding instances. However, the

total amount of vitamin B_6 in the urine was somewhat lower. 82 and 98% of this was pyridoxal.

Fig. 1 illustrates the fact that after feeding each of the 3 forms of vitamin B_6, a large increase in the level of the form fed occurs within 2 to 5 hours after administration of the dose, and that this level returns to the normal value within 8 hours. Similar results, not shown in this graph, were also obtained in connection with the excretion of the forms of the vitamin other than the one fed. The pyridoxic acid levels of the urine, shown in Fig. 2, increase in a much more pronounced manner, and levels did not return to normal until 12 hours after ingestion of the dose.

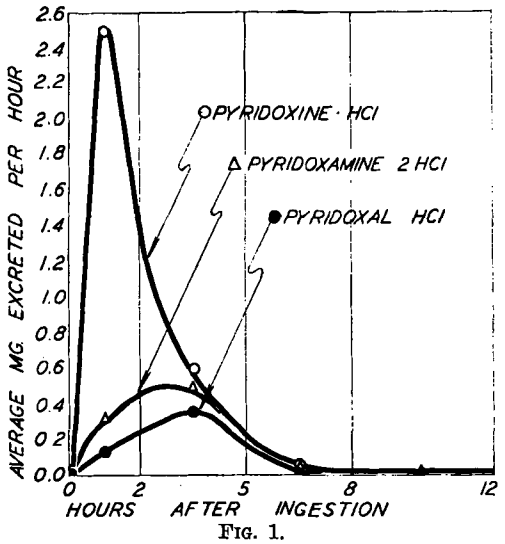

FIG. 1.

The Excretion of Vitamin B_6. The total amount of the indicated form of the vitamin found in the urine samples is plotted against the average time interval, after ingestion of the dose, during which the sample was collected.

○ Fed 100 mg of pyridoxine HCl.
△ Fed 100 mg of pyridoxamine 2HCl.
● Fed 100 mg of pyridoxal HCl.

The levels of the forms of the vitamin other than the one fed are not shown.

The amount of the test dose recovered was characteristic of the form of the vitamin fed, judging from the agreement of the results obtained in the two experiments, in which different subjects were fed the same compound. The highest recovery of the test dose, 70% and 64%, was obtained after feeding pyridoxal. The amount of pyridoxine recovered as the 4 products measured was 45 and 38% while the smallest recovery, 31 and 31%, was obtained after feeding pyridoxamine. These results may be inferred from Fig. 2, since the major component of the excretion products is pyridoxic acid.

Discussion. The above comparison of the amount of the test dose recovered as excretion products is of interest, since with pyridoxine and pyridoxamine, less than half of the administered dose was recovered. With pyridoxal, the most highly oxidized form of the vitamin, the recovery was higher because of the increased excretion of 4-pyridoxic acid which resulted when this form was fed.

A number of explanations for these low recoveries might be postulated. It is possible, for example, that the absorption of the various forms of the vitamin from the tract is incomplete. This explanation, however, is not in accord with the observations of Scudi, *et al.*,[2] who showed with both dogs and human beings that the total urinary excretion of vitamin B_6 following administration of large doses of pyridoxine (50 to 100 mg) was closely similar whether the vitamin was given orally or by intravenous injection. This indicates rather conclusively that absorption of test doses of this size from the tract is complete. The amounts of "pyridoxine" (total chlorimide reacting substances) which they found excreted following ingestion of 100 mg of pyridoxine hydrochloride approximate closely the amount of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) found in the urine of our subjects. When limited amounts of the three forms of the vitamin are fed to deficient rats, chicks, or dogs apart from the diet, they show equal growth-promoting activities,[13,14] again indicating equal absorption.

A second possible explanation for low recoveries would be that the ingested dose was being stored in the tissues. However, the subjects used were ingesting normal diets and thus were presumably not deficient in vitamin B_6. Consequently, no great storage of the ingested vitamin would be expected, and

[13] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **165**, 55.

[14] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 284.

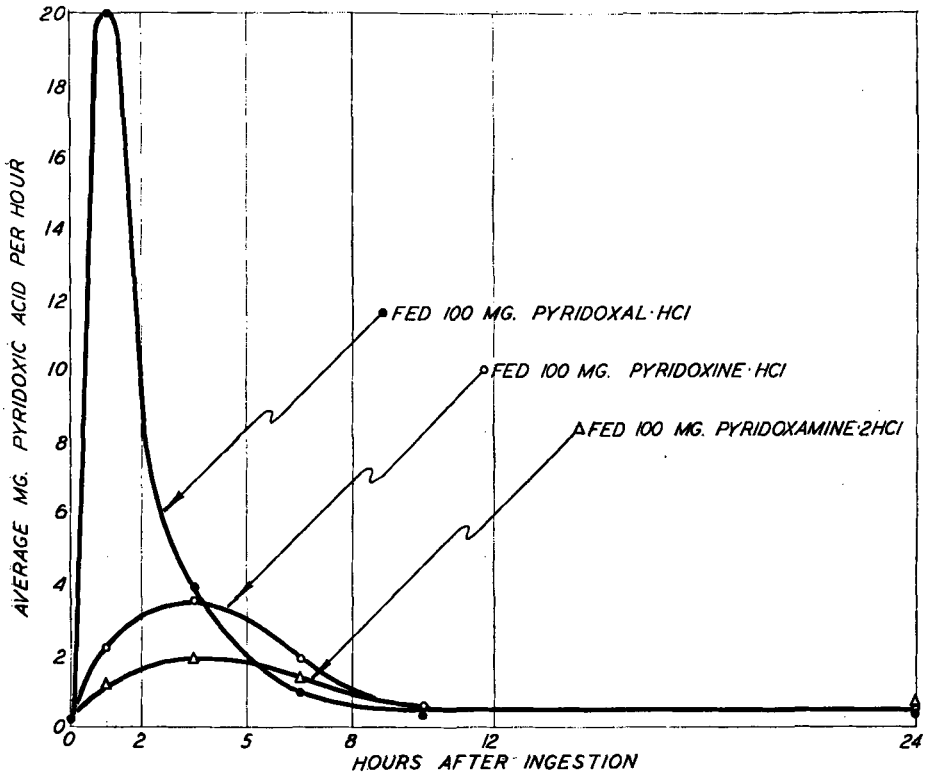

FIG. 2.

The excretion of pyridoxic acid (expressed as 4-pyridoxic acid lactone) following ingestion of various forms of vitamin B_6.

the fact that excretion levels rapidly returned to basal levels indicates that no great storage occurred. A third explanation for these low recoveries would be that still unidentified metabolic products which are without vitamin activity are formed from vitamin B_6 and excreted. From the considerations outlined above, this hypothesis seems most likely. It finds additional experimental support in the observation of Scudi, *et al.*[2] that following injection of 50 mg of pyridoxine hydrochloride in dogs, only 18% of this dose could be recovered in the urine. Yet, according to Huff and Perlzweig,[15] dogs do not excrete 4-pyridoxic acid. Unidentified excretion products, or complete oxidation within the body of considerable portions of the vitamin are thus indicated.

Pyridoxal is the only form of the vitamin which appears in the urine in greatly increased amounts as the result of feeding all 3 compounds. This further emphasizes the position of central importance indicated for this compound by direct assay of animal tissues,[8] by its universal availability to all microorganisms tested,[16] and by its occurrence in the only known catalytically active form of vitamin B_6, pyridoxal phosphate.[17]

Finally, it should perhaps be emphasized that the metabolic changes described above are those which occur when large amounts of vitamin B_6 are superimposed on a normal diet which already supplies the relatively small amounts of vitamin B_6 presumably required by man. Whether the small quantities normally ingested are metabolized in the same manner cannot be decided from these data.

Summary. The known metabolic products

[15] Huff, J. W., and Perlzweig, W. A., *Science*, 1944, **100**, 55.

[16] Snell, E. E., and Ranncfeld, A. N., *J. Biol. Chem.*, 1945, **157**, 475.

[17] Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1948, **161**, 311.

of vitamin B_6—pyridoxal, pyridoxamine, pyridoxine and pyridoxic acid—were measured in normal human urine and in the urine of human subjects each fed one of the 3 forms of the vitamin.

The chief product found, regardless of the form fed, was pyridoxic acid. Pyridoxal gave rise to significantly higher amounts of this product than did pyridoxine or pyridoxamine. No evidence could be obtained showing the conversion of pyridoxal or pyridoxamine to pyridoxine. When pyridoxal or pyridoxine was fed, the chief form in which the vitamin occurred in the urine was the form fed. However, when pyridoxamine was fed both pyridoxal and pyridoxamine were excreted in approximately equal amounts. Ingestion of pyridoxine also greatly increased the amount of pyridoxal and pyridoxamine excreted.

The excretion of all products was very rapid. The largest amounts of each of the compounds were found in samples collected 2 and 5 hours after ingestion of the dose. The levels of pyridoxic acid returned to normal values after 12 hours, while the vitamin levels had returned to normal within 8 hours. The amount of the dose recovered varied with the form fed. The highest recovery, 70%, was obtained when pyridoxal was fed; 45% of the pyridoxine was recovered, while only 31% of the pyridoxamine could be recovered. Together with published data which indicate that complete absorption of large doses of vitamin B_6 occurs, these findings suggest that a large proportion of the vitamin B_6 was converted to products still unknown.

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Loss of Body Protein and Antibody Production by Rats on Low Protein Diets.*

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Previous studies in this laboratory have demonstrated that prolonged severe deficiency of protein without other dietary restriction results in a reduced capacity of the rat to produce antibodies to sheep erythrocytes,[1] Friedlander's bacillus,[2] pneumococci,[3] and most recently a parasitic nematode.[4] Concomitant with the reduction of antibody production there was shown to be a reduction in the capacity to form leukocytes of both the granulocytic and lymphocytic series.[5] Associated with these phenomena there is a reduction in the resistance of animals to infection with virulent organisms.[3] Furthermore this reduction in resistance was shown to be due largely to the inability of the animals to fabricate antibodies since the depleted animals, when passively immunized, survive the infection as well as the normally nourished controls. Having established the fact that protein depletion of long duration reduces the rate of antibody formation and the resistance to infection it then becomes of interest to investigate the rate of decay of the antibody forming capacity with time under conditions

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1 Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. Surg.*, 1944, **120**, 514.

2 Woolridge, R. L., unpublished observations.

3 Wissler, R. W., *J. Infect. Dis.*, 1947, **80**, 264.

4 Woolridge, R. L., unpublished observations.

5 Asirvadham, M., *J. Infect. Dis.*, 1948, **83**, 87.