THE VITAMIN B_6 GROUP

VIII. BIOLOGICAL ASSAY OF PYRIDOXAL, PYRIDOXAMINE, AND PYRIDOXINE*

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Though various chemical and microbiological methods have been developed for the estimation of vitamin B_6 in whole (2–4) or in part (5), animal assays are still necessary for testing the accuracy of these new methods as they apply to animal nutrition. It is also important that the relative potency of pyridoxine, pyridoxamine, and pyridoxal for animals be known. Two different purified rations have been used most frequently for the rat assay. The one devised by Dimick and Schre fler (6), besides containing the usual purified constituents of a basal ration, also contains beef liver extract, while the one employed by Conger and Elvehjem (7) contains a fullers' earth filtrate of a butanol extract of 1:20 liver powder. In either case, the crude extracts contribute a definite but variable amount of vitamin B_6 to the basal ration and hence their use constitutes a distinct disadvantage. Further, neither of these two rations allows maximum growth in rats when supplemented with large amounts of vitamin B_6. Clarke and Lechycka (8) have described a biological assay for vitamin B_6 in which rats were kept on the deficient diet for a period of 4 weeks or more before feeding supplements containing the vitamin. Since the newer members of the vitamin B complex like biotin, inositol, and synthetic folic acid are now available, the present investigation was initiated with the object of devising a vitamin B_6-free ration which would permit the minimum possible growth of rats on the deficient diet and the maximum growth when optimum amounts of vitamin B_6 were added.

EXPERIMENTAL

The basal ration originally employed for the production of vitamin B_6 deficiency in rats consisted of carbohydrate 75 per cent, protein 18 per cent,

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corn oil 3 per cent, Salts 4 (9) 4 per cent, and optimum amounts of thiamine, riboflavin, calcium pantothenate, choline chloride, 2-methyl-1,4-naphthoquinone, inositol, nicotinic acid, and biotin. Male weanling rats (Sprague-Dawley) 21 days old, weighing 35 to 40 gm., were employed for these experiments. At least three animals were placed in each group and experiments on many groups were repeated several times in order to confirm the results. The values given in Tables I to V represent the average weight gain in gm. per week for the three animals of each group over a period of 4 weeks.

Several different carbohydrates and proteins were tried with the object of devising a ration which would permit the minimum growth gain in the absence of vitamin B_6. The results are given in Table I. When the diet

TABLE I

Growth of Rats on Various Vitamin B_6-Deficient Diets

Diet No.	Carbohydrate	Protein	Weight gain per wk. and range; average of 4 wks. growth
			gm.
1	Sucrose	18% casein (Smaco)	9 (6–11)
2	"	18% blood fibrin	5 (4– 5)
3	"	9% casein (Labco) + 9% blood fibrin	6 (5– 7)
4	"	10% egg albumin + 10% casein (Smaco)	10 (9–12)
5	Dextrin	18% casein (Smaco)	23 (16–28)
6	"	18% fibrin	18 (16–19)
7	Glucose	18% casein (Smaco)	9 (7–11)
8	Dextrin	18% " " + 0.5% sulfathalidine 0.025 mg.% synthetic folic acid	10 (6–16)
9	Same as (8) + 50 γ pyridoxine HCl per 100 gm. ration		23 (22–23)

contained sucrose as the carbohydrate and casein (Smaco) as the protein, the rats grew 9 gm. per week. When blood fibrin was substituted for casein the rate of gain was reduced to 5 gm. per week. This difference in growth was greater than could be accounted for by the fact that casein (Smaco) at 18 per cent level contributed 4.7 γ of vitamin B_6 per 100 gm. of ration, as compared to only 1.0 γ for blood fibrin as determined by the yeast growth method (3). The results with glucose were similar to those with sucrose, but when dextrin was used a remarkable increase in growth resulted, even though the dextrin by yeast assay contained only 0.04 γ per gm. of vitamin B_6. Dextrin is known to stimulate intestinal synthesis (10, 11) and it was therefore thought that with dextrin as the carbohydrate the intestinal flora produced more vitamin B_6 which was subsequently utilized by the rat. Several experiments indicated that this is a plausible

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explanation. Sulfathalidine, which is known to inhibit the growth of certain intestinal microorganisms, was added to the dextrin ration at 0.5 per cent level together with biotin and synthetic folic acid. The rate of growth was markedly decreased. The addition of 50 γ of pyridoxine hydrochloride per 100 gm. of ration restored the rate of growth to the original level.

Results from analysis of the urine excreted by rats maintained on sucrose and dextrin rations also confirmed the above observations. Lepkovsky and Nielsen (12) and later Miller and Baumann (13) have shown that the amount of xanthurenic acid excreted in the urine by rats kept on a diet rich in tryptophane depended on the vitamin B_6 present in the diet. More xanthurenic acid is excreted when the diet is deficient in vitamin B_6. Huff and Perlzweig (14) and later Johnson *et al.* (15) have shown that 4-pyridoxic acid is the main metabolic product of vitamin B_6 appearing in human urine. In our experiments the rats were placed in metabolism cages fitted

TABLE II

Urinary Excretion Studies on Vitamin B_6-Deficient Diets

	4-Pyridoxic acid excreted during 24 hrs.	Xanthurenic acid excretion per gm. food ingested
	γ	γ
Sucrose-basal + 18% fibrin......	36	815
Dextrin-basal + 18% "	54	267

with glass funnels, and urine was collected for a period of 24 hours. Xanthurenic acid was estimated in a suitable aliquot of the urine sample, essentially according to the method of Miller and Baumann (13). After the conversion to lactone, 4-pyridoxic acid was estimated fluorometrically in the Coleman photofluorometer (14). The results, given in Table II, show that more 4-pyridoxic acid and less xanthurenic acid are excreted by animals on the dextrin diet than by those on the sucrose diet, indicating that the former animals are obtaining more vitamin B_6 than the latter. Since the two rations are equally deficient in vitamin B_6, the supposition is that the intestinal flora are stimulated to produce the extra vitamin B_6 in animals fed the dextrin diet.

From these results it is evident that dextrin cannot be used as a source of carbohydrate in the deficient ration and that blood fibrin is better than casein as the protein component of the ration. Experiments to determine the influence of biotin, inositol, and folic acid on the growth of rats receiving this deficient ration were carried out next. The results are given in Table III. Folic acid did not have any significant effect when added at a level of 25 γ per 100 gm. of ration. However, on rations with casein as the pro-

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tein, the addition of biotin and inositol appeared to have a slight stimulating effect. These substances were consequently added to all the subsequent deficient rations.

The diet finally selected for the bioassay of vitamin B_6 has the following composition: sucrose 75 gm., blood fibrin 18 gm., Salts 4, 4 gm., corn oil 3 gm., thiamine 0.2 mg., riboflavin 0.3 mg., nicotinic acid 2.5 mg., calcium pantothenate 2 mg., 2-methyl-1,4-naphthoquinone 1 mg., inositol 10 mg., choline chloride 100 mg., and biotin 0.01 mg., per 100 gm. of diet. Halibut liver oil diluted 1:2 with corn oil was fed at a level of 2 drops per week, with α-tocopherol included at 0.5 mg. per drop. After a depletion period

TABLE III

Influence of Biotin, Inositol, and Synthetic Folic Acid on Growth of Vitamin B_6-Deficient Rats

Diet No.		Growth gain per wk. and range; average of 4 wks. growth
		gm.
1	Sucrose-basal + 18% casein without inositol and biotin	7 (6– 8)
2	Same as (1) + inositol, 10 mg. per 100 gm.	8 (7– 8)
3	" " (1) + biotin, 20 γ per 100 gm.	8 (7–10)
4	" " (1) + inositol and biotin	9 (8–10)
5	Sucrose-basal + 18% fibrin + biotin + inositol	5 (4– 5)
6	Same as (5) + *p*-aminobenzoic acid + synthetic folic acid, 25 γ per 100 gm. ration	5 (4– 7)
7	Sucrose-basal + 18% fibrin + 50 γ per 100 gm. pyridoxine HCl with inositol and biotin	20 (18–21)
8	Same as (1) + synthetic folic acid, 25 γ per 100 gm., + *p*-aminobenzoic acid + 50 γ per 100 gm. pyridoxine HCl	21 (18–24)

of 2 weeks, the rats are weighed, divided evenly with respect to weights into groups of three, and placed on diets containing different amounts of vitamin B_6. Fig. 1 shows the average weight gain per week plotted against increasing concentration of pyridoxine hydrochloride in the ration. The growth response is approximately linear up to 75 γ of pyridoxine hydrochloride for 100 gm. of diet. Twice this amount was required to permit maximum growth of over 30 gm. per week.

Comparison of Activities of Pyridoxine, Pyridoxamine, and Pyridoxal—Snell and Rannefeld (16) compared the activities of the members of the vitamin B_6 group by feeding them orally, separate from the ration, to deficient rats. They found that pyridoxine, pyridoxamine, and pyridoxal possessed equal activities within the limits of experimental error. Miller and Baumann (17) later reported that pyridoxamine and pyridoxal were

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much less active than pyridoxine for mice and rats. The vitamin supplements in their experiments were added to the ration, a procedure which they found did not result in any destruction of pyridoxamine or pyridoxal. Luckey *et al.* (18) in experiments with chicks also found pyridoxamine and pyridoxal less active than pyridoxine. In view of these conflicting results the following experiments were carried out to test the potencies of the three compounds. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, and pyridoxal hydrochloride were administered by three different methods. In one group the three compounds were each mixed in 100 gm. of ration

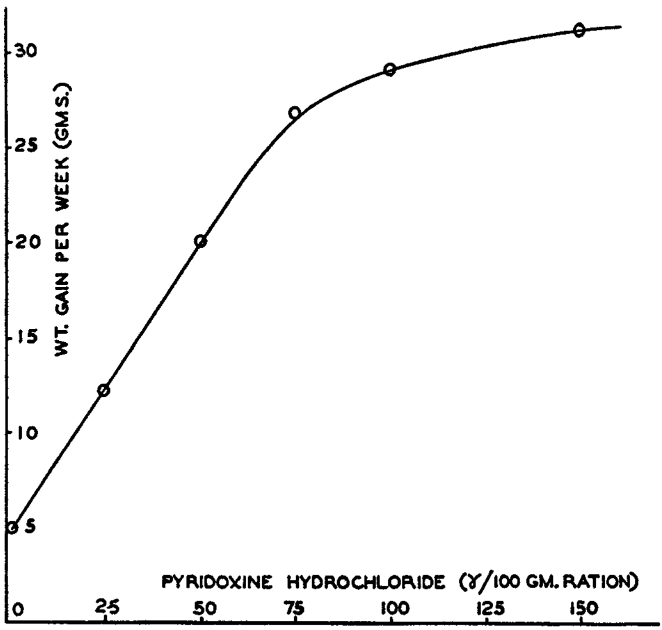

FIG. 1. Growth response of rats to pyridoxine hydrochloride on the improved ration.

at equimolar levels equivalent to 50 γ of pyridoxine hydrochloride. In the second group, the rats were fed daily molar supplements equivalent to 5 γ of pyridoxine hydrochloride by dropper. In the third group, these same amounts were injected intraperitoneally. The results (Table IV) show a marked decrease in the growth of rats on pyridoxamine and pyridoxal when fed in the ration, whereas all compounds were equally active when fed by dropper or by intraperitoneal injection. Similar results (Table IV) were obtained with chicks. Day-old white Leghorn chicks were fed the deficient ration (Table IV, foot-note) and were kept in heated cages with raised screen bottoms, seven chicks being used in each group.

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All the chicks on the deficient diet died in 2 weeks without gaining any appreciable weight. The results obtained when pyridoxine, pyridoxamine,

TABLE IV

Comparison of Activities of Pyridoxine Hydrochloride, Pyridoxamine Dihydrochloride, and Pyridoxal Hydrochloride

	Average weekly gain of rats in gm.			Average weight of chicks* at 3 wks. in gm.		
	In ration, 50 γ per 100 gm.	Fed by dropper, 5 γ per rat per day	Intra-peritoneal injection, 5 γ per rat per day	In ration, 100 γ per 100 gm.	Fed by dropper, 7.5 γ per chick per day	Intra-peritoneal injection, 7.5 γ per chick per day
Pyridoxine HCl	20	24	22	124	106	111
Pyridoxamine 2HCl (equimolar)	15	21	21	74	103	107
Pyridoxal HCl	14	25	26	91	111	112

* The basal ration used for the production of vitamin B_6 deficiency in chicks had the following composition: cerelose 61 gm., blood fibrin 18 gm., gelatin 10 gm., Salts 4, 5 gm., $CaHPO_4 \cdot 2H_2O$ 1 gm., *l*-cystine 0.3 gm., thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., calcium pantothenate 2.0 mg., choline chloride 150 mg., nicotinic acid 5.0 mg., biotin 0.02 mg., inositol 100.0 mg., synthetic folic acid 0.125 mg., vitamin D_3 0.004 mg., α-tocopherol 0.03 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., vitamin A 1700 I.U. This ration differed from the one used by Luckey *et al.* (18) in that cerelose replaced dextrin and synthetic folic acid was substituted for norit eluate of 1:20 liver powder.

TABLE V

Comparative Activities for Rats of Pyridoxine Hydrochloride and Synthetic Codecarboxylase

	In ration		Intraperitoneal injection	
	Amount fed*	Average gain and range	Amount injected*	Average gain and range
	γ per 100 gm.	*gm. per wk.*	*γ per rat per day*	*gm. per wk.*
Pyridoxine HCl	40	16 (12–20)	2.5	15 (14–16)
Synthetic codecarboxylase (pyridoxal phosphate)	125	11 (10–12)	7.8	15 (14–18)

* The preparation of pyridoxal phosphate used contained 32 per cent of pyridoxal, and was consequently used at levels equimolar with pyridoxine. The barium present was removed as barium sulfate before administration.

and pyridoxal were mixed in 100 gm. of ration at a level of 100 γ, given by dropper at 7.5 γ per chick per day and injected intraperitoneally, at the same level are given in Table IV. There was a marked decrease in the

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growth of chicks in the first group when pyridoxal and pyridoxamine were mixed in the ration, while the growth was approximately the same in all cases in which vitamin supplements were fed by dropper or injected intraperitoneally. The lower activity of pyridoxal and pyridoxamine when fed in the ration cannot be attributed to destruction during storage, since yeast assays on the amounts of vitamins present in the rations under the storage conditions used (not more than 10 days at 4°) showed no destruction of pyridoxine, pyridoxal, or pyridoxamine.

Codecarboxylase (phosphorylated pyridoxal), which has been synthesized and purified as the barium salt by Gunsalus and coworkers (19), was compared with pyridoxine hydrochloride for its relative activity for the rat. The coenzyme,[1] which contained 32 per cent of pyridoxal after hydrolysis as determined by spectrum analysis (19), was mixed with the ration or injected intraperitoneally in quantities calculated to furnish amounts of pyridoxal equimolar with the pyridoxine supplied to the control groups. The results are given in Table V. Like pyridoxal hydrochloride, codecarboxylase was less active than pyridoxine when mixed in the ration, but had the same activity as pyridoxine when injected intraperitoneally.

DISCUSSION

Snell (5) showed by differential microbiological assay methods with yeast (*Saccharomyces carlsbergensis*), *Streptococcus faecalis*, and *Lactobacillus casei* that natural materials contain varying proportions of pyridoxine, pyridoxamine, and pyridoxal. These compounds possess equivalent activities towards yeast and also towards animals, if fed by a dropper or by injection. However, if the supplements are mixed in the ration, pyridoxamine and pyridoxal show considerably less activity than pyridoxine for both rats and chicks. The explanation for these results is at present unknown. A possible explanation is that pyridoxal and pyridoxamine are more susceptible to destruction or utilization by intestinal bacteria than is pyridoxine. When exposed to bacterial action, as when fed in the ration, less of these compounds might be available for the animal. When fed by a dropper, absorption is rapid, and such influences could not enter to alter the true comparative activities of the three compounds. In support of this hypothesis are the known facts that lactic acid bacteria constitute a large proportion of the total intestinal flora (20, 21), that several of these organisms require vitamin B_6 for growth (16), and that, although these organisms utilize pyridoxal and pyridoxamine readily, they are in general unable to utilize pyridoxine (16). Whatever the true explanation, it is evident that when natural materials in which most of the vitamin B_6 is

[1] We are indebted to Dr. I. C. Gunsalus for a pure specimen of the barium salt of pyridoxal phosphate.

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present as pyridoxal and pyridoxamine are fed with the ration, a considerably lower figure for the vitamin B_6 content might result from rat assay than would be obtained by yeast assay. When pyridoxine is the major constituent of the B_6 group present, the same values should be obtained. Considering further that methods of extraction of vitamin B_6 from natural materials for microbiological and chemical assay are not entirely satisfactory, it is evident that bioassay with the rat is still of great value in assessing the vitamin B_6 activity of dietary constituents used in animal nutrition.

SUMMARY

A deficient diet for the assay of vitamin B_6 with the rat is described which is based on sucrose as the carbohydrate and blood fibrin as the protein. In the absence of vitamin B_6, the diet gives the least growth in weanling rats of any tried; maximum growth (over 30 gm. per week) is obtained when 150 γ of pyridoxine hydrochloride are added to 100 gm. of ration. When dextrin is substituted for sucrose, considerable growth occurs on the deficient diet. On the dextrin diet, urinary excretion of xanthurenic acid is decreased, while the amount of 4-pyridoxic acid excreted increases. The addition of sulfathalidine to such a ration eliminated its growth-promoting effects; growth is resumed if vitamin B_6 is fed. These results indicate that on the dextrin diet intestinal flora synthesize considerable amounts of vitamin B_6, which is then utilized by the rat.

A standard curve which is approximately linear up to 75 γ of pyridoxine hydrochloride in 100 gm. of ration has been obtained on the improved basal diet. The relative activities of pyridoxine, pyridoxamine, and pyridoxal vary with the way in which the supplements are given. When mixed in the ration, pyridoxamine and pyridoxal are less active than pyridoxine; when given by dropper or injected intraperitoneally, they all possess equivalent activities. Similar results have been obtained with chicks.

The growth-promoting potency of synthetic pyridoxal phosphate (codecarboxylase) corresponds to its pyridoxal content. Like pyridoxal, it is less active than pyridoxine when mixed in the ration, but is equally active when injected intraperitoneally.

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