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The Effect of Vitamin B_6 on the Growth and the Blood Picture of the Rat

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In continuing studies of rat growth on highly purified diets (Copping, Crowe & Pond, 1951) it seemed useful to test the value of the purified basal diet for biological estimation of vitamins of the B complex. A preliminary study was made with vitamin B_6. As the literature at that time contained apparently conflicting reports on the blood picture in vitamin B_6-deficient rats a haematological study was also undertaken.

EXPERIMENTAL

Animals. The 222 rats used in these experiments were of the Lister Institute black-and-white stock from a colony now maintained at Queen Elizabeth College and behaving, whenever biological tests have made comparisons possible, in the same manner as the original stock. The rats were taken at weaning at 23 days of age, weighing 35–45 g. They were placed in separate cages with open-grid floors and were given the purified basal diet with all vitamin supplements except vitamin B_6. Litters of six to eight rats were used and, when graded doses of vitamin B_6 were given, due regard was paid to the distribution of litter-mates and sexes throughout the various groups. In preliminary tests, series C_3, C_4 and C_6, it was observed that larger differences in the growth response to graded doses of vitamin B_6 were obtained if the animals were deprived of the vitamin for 3 weeks before they were dosed. On the other hand, in series C_5 the effect of this deprivation on the blood picture was found to be negligible. In this series four animals in each group were given the graded doses

from weaning, and the remaining four were deprived for 3 weeks before they were dosed. In series C_7 rats were kept without vitamin B_6; ten survived for 11 weeks, four died within this time, fourteen received curative doses after 7–9 weeks, and six control rats received vitamin B_6 for 11 weeks. In later series, C_8 and C_{10}, no animals underwent a period of deprivation.

Diets. The basal diet in all experiments consisted of casein 20, sucrose 60, arachis oil 12, lard 3 and salt mixture 5, parts. The casein was vitamin-low casein prepared by Genatosan Ltd and was found to be satisfactory for inclusion in highly purified diets. This basal diet was fed as a dry powder in deep pots, and most of the animals ate it readily without undue scattering. Vitamins A and D were given as Adexolin (Glaxo Laboratories Ltd) diluted with arachis oil so that one drop provided 120 i.u. vitamin A and 20 i.u. vitamin D. One drop was given weekly.

The B-vitamins were given in an aqueous mixture containing in a daily dose of 1 ml.:

Thiamine	10 μg	Riboflavin	40 μg
Nicotinic acid	0·5 mg	Pantothenic acid	100 μg
Biotin	0·2 μg	Folic acid	2 μg
p-Aminobenzoic acid	0·5 mg	Inositol	0·5 mg
Choline	1·5 mg		

Vitamin B_6 as a solution of pyridoxin hydrochloride was administered separately from a dropping pipette calibrated to give the required doses of 2·5, 5·0 and 10·0 μg. In series C_{10} vitamin B_{12} was given from an Agla micrometer syringe (Burroughs, Wellcome and Co.) in a dose of 1 μg once weekly.

Vitamin E was given as a weekly dose of 1 mg α-tocopheryl acetate in arachis oil in series C_3, C_4, C_5 and C_6, but was omitted from series C_7 and C_8. In series C_{10} the effects of vitamin E and vitamin B_{12} were investigated.

The blood studies were made on rats that had the experimental diets for 7 weeks in three series and for 11 weeks in one series. For comparison, litter-mates of the rats having the purified diet were kept on a normal stock diet of mixed grains, meat, fish, bread and milk.

Sampling of blood. In experiments C_5 and C_7 the animals were anaesthetized with ether. In experiments C_8 and C_{10} a 3·3% solution of Nembutal (Abbot Laboratories Ltd, London) was injected intraperitoneally, 0·25–1·0 ml. according to the weight of the animal.

A sample of blood from the tail was taken directly into a white-cell diluting pipette. The chest was then opened. About 0·75 ml. blood was withdrawn from the right ventricle. Sodium fluoride was used as the anticoagulant in series C_5, C_7 and C_8, and heparin in C_{10}. Blood from the heart was used for smears, red- and white-cell counts, haemoglobin estimation, and, in series C_{10}, for haematocrit values.

Blood smears. Two smears were made from each animal, one stained with May-Grünwald-Giemsa and one with Leishman's stain.

Red- and white-cell counts. Red- and white-cell counts were made by the usual techniques. In series C_7, C_8 and C_{10} counts of cells with lobed nuclei as well as total white cells were made. Examination of the blood smears in series C_7 showed that the

leucocytes were predominantly small and large lymphocytes and polymorphonuclear neutrophil leucocytes. No basophils were seen. Eosinophils were very rare. The lobed forms are referred to as polymorphs and the non-lobed forms as lymphocytes.

Haemoglobin estimation. The alkali-haematin method of Wu (1922*a*, *b*) was used, the extinction being measured photoelectrically. The dilutions used were greater than those given by Peters & Van Slyke (1932). In series C_5 and C_7, 20 mm^3 of blood were added to 4 ml. of 0·1 N-HCl, and 1 ml. of 5% (w/v) NaOH was added when the tubes had been kept for 2 h at room temperature. In series C_8 and C_{10} the dilution was 20 mm^3 to 8 ml. 0·1 N-HCl, with 2 ml. 5% (w/v) NaOH. Even at this dilution designed to permit use of 15 ml. tubes in an EEL colorimeter (Evans Electroselenium Ltd, Harlow, Essex) the readings were reproducible. In series C_5 and C_7 haemoglobin concentrations are expressed as a percentage of that of stock rats, since the haemoglobin concentrations of the standard were not estimated. In series C_8 and C_{10} the concentrations of haemoglobin are given as g/100 ml.

Haematocrit values. Haematocrit values were obtained from heparinized blood centrifuged for 1 h in 0·8 mm tubes at 900 ***g*** (maximum value at bottom of tube).

RESULTS

Growth. Even in the series C_3 with very few animals, a graded growth effect was obtained with doses of 0, 2·5, 5·0 and 10 μg pyridoxin hydrochloride daily. This was confirmed in series C_4 and C_6. The results are briefly summarized in Table 1 without

Table 1. *Weight increases of groups of rats in preliminary tests with graded doses of pyridoxin hydrochloride for 4 weeks*

Exp. no.	Dose of pyridoxin hydrochloride (μg)	Dosing period (weeks)	No. of rats	Weight: Initial (g)	Weight: Final (g)	Mean weekly gain (g)
C_3 with no preliminary depletion	0	4	5	59	100	10
	2·5	4	5	52	100	12
	5·0	4	5	56	119	16
	10	4	5	55	127	18
C_4 with 3 weeks' depletion	0	7	9	46	70	3·5
	2·5	4	8	47	108	10
	5·0	4	8	47	116	13
	10	4	9	47	128	14
C_6 with 3 weeks' depletion	2·5	4	8	39	106	12
	5·0	4	8	41	122	15
Copping (1943) with depletion	2·5	4	30	—	—	10·3
	5·0	4	36	—	—	13·8

statistical treatment. The small differences in growth response of rats receiving 5 or 10 μg pyridoxin hydrochloride suggested that the dose of 10 μg was probably adequate as a contribution to the vitamin B-complex content of a purified diet. For comparison, mean values obtained in some earlier work on the estimation of vitamin B_6 (Copping, 1943) are included in the table, and it should be noted that our stock of rats shows a similar response to vitamin B_6 after about 10 years. In series C_4 and C_5, in which

a depletion period of 3 weeks preceded the dosing period of 4 weeks, the development of severe skin lesions was noted in rats having no dose. Since it occurred within the 7 weeks of the test, a more extensive study of the development and cure of skin lesions was made in one series, C_7, which was carried on for 11 weeks. It was clear that on the basal diet with synthetic supplements skin lesions developed rapidly and showed the characteristics described previously (Copping, 1936) in rats having a much less purified diet with a liver preparation as source of some of the B-vitamins. In series C_7 twenty-eight rats received no dose of vitamin B_6 from weaning for 7 weeks. At this stage some animals were in a precarious condition and were accordingly dosed with 10 μg pyridoxin hydrochloride. In spite of treatment three died, and altogether fourteen animals required curative dosing within the next 3 weeks.

Tables 2 and 3 record the growth of the animals on which blood studies were made. In series C_8 the regression coefficients were calculated for the mean weight increases plotted against the logarithm of the dose and gave the following values:

for bucks, $b = 60{\cdot}63 \pm 0{\cdot}75$; $P < 0{\cdot}01$,
for does, $b = 36{\cdot}13 \pm 10{\cdot}93$; $P < 0{\cdot}01$.

These were highly significant.

Table 2. *Mean values with their standard errors for weight increases, red-cell count, haemoglobin percentage and mean corpuscular haemoglobin for rats in series C_5, C_7 and C_8*

Exp. no.*	Dose of pyridoxin hydrochloride (μg)	Dosing period (weeks)	No. of rats	Weight: Initial (g)	Weight: Final (g)	Weight: Main weekly gain (g)	Red blood cells (10^6/mm^3)		Haemoglobin as percentage of that in stock rats		Mean corpuscular haemoglobin ($\mu\mu$g)	
C_5	0	7	8	46	90	6·2 ± 0·45	8·31	± 0·23	97	± 3·2	—	
	2·5	4	4	44	111	12·4 ± 0·36	7·29		95		—	
		7	4	49	120	10·0 ± 1·08						
	5·0	4	3	45	131	15·5 ± 0·90	6·93		95		—	
		7	4	50	138	12·6 ± 0·88						
	10	4	4	45	135	16·6 ± 2·09	6·77		98		—	
		7	4	49	158	15·8 ± 0·69						
	Stock diet	—	12	—	202	—	6·54		104		—	
C_7	0	10–11	9	43	82	3·6 ± 0·34	8·57	± 0·15	63	± 3·2	—	
	10	11	6	40	167	11·5 ± 0·79	7·37		97		—	
		2–4	8	38	113	15·1 ± 1·31	7·08		86		.....	
									g/100 ml.†			
C_8	0	7	6	45	76	4·6 ± 0·67	7·90	± 0·15	11·6	± 0·21	14·8	± 0·35
	2·5	7	8	43	123	11·9 ± 0·66	7·34		11·8		16·2	
	5·0	7	8	45	145	14·9 ± 0·99	6·99		12·3		17·7	
	10	7	8	42	150	16·0 ± 0·96	6·66		12·2		18·4	
	Stock diet	7	8	43	217	26·3 ± 1·89	6·48		12·8		19·8	

* For details see p. 50. † See p. 51.

Red-cell counts. Tables 2 and 3 show that the average red-cell count obtained for each group increased with the extent of the vitamin B_6 deficiency. With the logarithm of the dose and the logarithm of the red-cell count for each dose, 2·5, 5·0 and 10 μg, it was possible to fit a regression curve to these values which proved to be a straight

line in series C_5 and C_8. However, when the regression coefficient was calculated it was found that the scatter of the observed red-cell counts was such that the slope of the line was significant only in series C_8.

In series C_5, $b = -0{\cdot}0546 \pm 0{\cdot}0310$; $P < 0{\cdot}1$.
In series C_8, $b = -0{\cdot}0714 \pm 0{\cdot}0241$; $P < 0{\cdot}01$.

In series C_7 only three groups of animals were studied, one group receiving no pyridoxin hydrochloride, a second receiving 10 μg daily from weaning and a third given a curative dose after a long deprivation period. The animals were on the experimental regimen for 11 weeks, which was 4 weeks longer than for other series. In the

Table 3. *Weight increases and blood values for rats in series C_{10}*

Group no.	No. of rats	Mean weekly weight increase (g)	Haemoglobin (g/100 ml.)	Red blood cells (10^6/mm^3)	Mean corpuscular haemoglobin ($\mu\mu$g)	Haematocrit (percentage of R.B.C.)	Mean corpuscular volume (μ^3)	Mean corpuscular haemoglobin concentration (%)
1	7	4·9	11·63	7·57	15·4	37·0	49·2	31·4
2	8	13·6	13·12	6·88	19·1	39·7	58·0	33·1
3	8	13·9	12·89	6·70	19·3	38·7	58·0	33·4
4	8	15·7	13·36	6·78	19·8	40·0	59·1	33·5
5	8	15·1	12·89	6·61	19·5	38·7	58·7	33·4
6	8	23·3	13·12	6·30	20·9	38·9	62·0	33·8
Standard error of the mean		1·0	0·21	0·14	0·4	0·6	1·0	0·4

Group 1: basal diet alone. Group 2: basal diet with 10 μg pyridoxin hydrochloride. Group 3: basal diet with 10 μg pyridoxin hydrochloride and vitamin E. Group 4: basal diet with 10 μg pyridoxin hydrochloride, vitamin E and vitamin B_{12}. Group 5: basal diet with 10 μg pyridoxin hydrochloride and vitamin B_{12}. Group 6: stock diet.

group receiving no vitamin B_6 the red-cell counts were the highest obtained in any experiment. However, in the rats recovering after deprivation the counts appeared to drop below those of the group that had received 10 μg daily throughout the experiment, although the difference between the counts for the two groups was not significant ($P > 0{\cdot}1$). In series C_{10} there was a significant increase in the red-cell count in the group receiving no vitamin B_6, but no differences were shown between the counts of groups receiving vitamin B_6 alone or with addition of vitamin E or vitamin B_{12} or both.

Haemoglobin. In series C_5 and C_8 there were no significant differences between haemoglobin values. In series C_7 significant differences were found between all groups. Rats receiving no vitamin B_6 had haemoglobin values lower than those of animals dosed after deprivation ($P < 0{\cdot}01$), and the values of the latter were lower than those of animals that received 10 μg pyridoxin hydrochloride for 11 weeks. Therefore, with the longer deprivation period a fall in haemoglobin concentration occurred. In series C_{10} the animals deprived of vitamin B_6 gave significantly lower values than those in any other group.

In series C_8 and C_{10} the mean corpuscular haemoglobin (M.C.H.) was calculated (see Tables 2 and 3). In both series the M.C.H. of animals deprived of vitamin B_6

was significantly lower than that in any other group. In series C_8 it was possible to fit a regression curve to the logarithm of the M.C.H. and that of the dose, 2·5, 5·0 or 10 μg. It was a straight line with the regression coefficient $b = 0{\cdot}0919 \pm 0{\cdot}0184$; $P < 0{\cdot}01$.

Haematocrit values. These were obtained in series C_{10} only (Table 3). In animals deprived of vitamin B_6 the packed-cell volume was significantly lower than that of all other groups. Calculation of the mean corpuscular volume (M.C.V.) and mean corpuscular haemoglobin concentration (M.C.H.C.) showed that the red blood cells of the deprived rats were microcytic and hypochromic.

Table 4. *Mean values with their standard errors for white-cell counts of rats in series C_5, C_7, C_8 and C_{10}*

(Occasionally blood samples could not be obtained, and figures in parentheses give numbers of rats where these differ from those in column 3)

			Heart blood			Tail blood		
Exp. no.	Dose of pyridoxin hydrochloride (μg)	No. of rats	Total (10^3/mm^3)	Lymphocytes (10^3/mm^3)	Polymorphs (10^3/mm^3)	Total (10^3/mm^3)	Lymphocytes (10^3/mm^3)	Polymorphs (10^3/mm^3)
C_5	0	8	3·7	—	—	6·6	—	—
	2·5	8	5·4	—	—	8·4 (6)	—	—
	5·0	7	4·8 ±0·76	—	—	8·1 ±1·22	—	—
	10	8	4·9	—	—	11·1	—	—
	Stock diet	10	5·4	—	—	10·0 (9)	—	—
C_7	0	8	2·4	1·4	1·0	4·2	2·5	1·7
	10	6	5·0 ±0·31	3·9 ±0·21	1·1 ±0·14	6·7 ±0·51	5·3 ±0·37	1·4 ±0·26
	10 after 7–9 weeks depletion	8	2·6	2·0	0·6	4·6 (7)	3·4	1·2
C_8	0	6	1·9	1·0	0·9	3·8 (4)	2·0	1·8
	2·5	8	3·2	2·0	1·2	5·0	3·2	1·8
	5·0	8	3·5 ±0·46	2·5 ±0·31	1·0 ±0·23	6·0 ±0·64	4·1 ±0·48	1·9 ±0·33
	10	8	3·5	2·7	0·8	5·8	4·3	1·5
	Stock diet	8	6·2	4·9	1·3	9·4	7·2	2·2
C_{10}	0	7	2·9	1·6	1·3	5·6	3·3	2·3
	10	8	5·9	4·6	1·3	9·3	7·3	2·0
	10+ vitamin E	8	4·8	3·4	1·5	8·4	5·9	2·4
	10+ vitamin E+ vitamin B_{12}	8	6·3 ±0·65	5·0 ±0·58	1·3 ±0·22	10·6 ±1·22	8·6 ±1·07	2·0 ±0·35
	10+ vitamin B_{12}	8	4·6	3·3	1·4	8·2	5·8	2·5
	Stock diet	8	7·9	6·4	1·5	14·0	11·7	2·4

White-cell count. Table 4 shows the average white-cell count for each group. Since the white-cell counts of tail blood were consistently higher than those of heart blood, both counts are given. The reasons for the difference are being investigated. In series C_5 and C_8 the average white-cell count of animals on the stock diet was the same. In series C_5 the white-cell count of animals receiving 10 μg pyridoxin hydrochloride was close to that of rats having stock diet, being slightly lower in heart blood and slightly higher in tail blood, but the differences were not significant. In series C_8, however, the white-cell counts of animals receiving vitamin B_6 were lower than those of animals having the corresponding doses in series C_5. Low values were found also for rats

having 10 μg pyridoxin hydrochloride in series C_7. The white-cell counts of animals having synthetic diets in the later series C_7 and C_8 were thus lower than those in the earlier series C_5, whereas the white-cell counts of rats on the stock diet did not change. The only change in the synthetic diet of which we are aware was the omission of tocopherol from series C_7 and C_8. It was omitted because it is generally accepted that tocopherol does not affect the early growth of rats (Olcott & Mattill, 1937; Mason, 1944), and there was no reason to expect that it would have any effect on the blood picture. In series C_{10} tocopherol was added to the synthetic diet in some groups, but it caused no change in the blood picture. The addition of vitamin B_{12}, with or without tocopherol, also had no effect.

In series C_5 there were no significant differences between the white-cell counts for the groups. In series C_7, C_8 and C_{10}, in which lymphocytes and polymorphs were counted separately while the total count was being made, the total white-cell count was lower in the group having no dose than in other groups. The polymorphs showed no change, the fall in total count being entirely due to the fall in lymphocyte count.

In series C_7 the longer duration of the test produced more marked differences between the white-cell count of rats receiving no dose and of those receiving 10 μg pyridoxin hydrochloride. After 3–4 weeks' recovery period on a dose of 10 μg pyridoxin hydrochloride rats that had previously been deprived showed no significant increase in lymphocyte or total white-cell count above values found for rats deprived for the whole 11-week period, that is, the lymphocytopenia showed no improvement. In this respect the white-cell picture differed from the red-cell.

DISCUSSION

We have seen no previous reference to the effects of graded doses of vitamin B_6 on the blood picture in rats. We used no inanition controls, but Agnew (1949) found no difference in red-cell count, haemoglobin or colour index between rats fed *ad lib.* and those pair-fed with animals deprived of vitamin B_6. Our results on red-cell counts in complete deprivation of vitamin B_6 agree with those of Agnew (1949), Carpenter & Kodicek (1948–9, 1952) and Hawkins, Lechow & Evans (1952).

We found that the haemoglobin fell in series C_7, in which the animals were maintained on the deficient diet for 11 weeks, and in series C_{10} where they were deprived for only 7 weeks. Fouts & Lepkovsky (1942) found a slight fall in haemoglobin in vitamin B_6-deficient rats, as did Agnew (1949), but Hawkins *et al.* (1952) and Carpenter & Kodicek (1948–9, 1952) found no change in haemoglobin. Their animals, however, were older at the beginning of the deprivation period, and the long survival times would seem to indicate that they had not reached such a late stage of deficiency as our animals, of which a number actually died during the experiments, as mentioned above.

Another point that may be of importance in relation to anaemia is that vitamin B_{12} was not included in the diets at first, since previous experiments (Copping, unpublished) have given no evidence of a growth requirement for vitamin B_{12} in rats having an otherwise fully supplemented synthetic diet. This was the finding also of Lih, King, Higgins, Baumann & Strong (1951), who observed that addition of vitamin B_{12} to

their basal diet did not significantly alter the growth response of rats to graded doses of pantothenic acid. In series C_{10} the groups receiving vitamin B_{12} showed no increase in haemoglobin values over the groups not having the vitamin.

Since in series C_5 and C_8 the red-cell count increased while the haemoglobin stayed constant, and in series C_7 the red-cell count rose while the haemoglobin fell, it seemed probable that a microcytosis had developed. This was confirmed in series C_{10}, and is in agreement with the results of Carpenter & Kodicek (1948–9, 1952), Agnew (1949) and Hawkins *et al.* (1952).

Our investigations into the effect of vitamin B_6 deficiency on the white cells show a fall in the total white-cell count caused by a lymphocytopenia, the polymorphs remaining constant. This picture agrees with the findings of Hawkins & Evans (1952), though they worked with older rats and continued the experiment for a longer period of time. Carpenter & Kodicek (1948–9, 1952) found that the total count remained within normal limits, although the percentage of lymphocytes fell in deficient animals compared with pair-fed controls and those having unrestricted food intake. Agnew & Cook (1948–9) found that the total white-cell counts and lymphocyte counts of vitamin B_6-deficient rats did not differ significantly from those of pair-fed controls although both these groups appeared to have lower total white-cell and lymphocyte counts than the control group with unrestricted food intake. No statistical analysis was given.

SUMMARY

1. Rats receiving graded doses of pyridoxin, in addition to a highly purified diet containing all other essential vitamins, gave a graded growth response.

2. The blood picture showed changes with progressive vitamin B_6 deprivation.

3. The red-cell count was increased, but there was no decrease in haemoglobin in mild deficiency. In rats showing severe signs of deprivation a decrease in haemoglobin did occur. The mean corpuscular haemoglobin was lower in deprived animals.

4. In deprived rats the red blood cells were microcytic and hypochromic.

5. In rats deprived of vitamin B_6 a decrease in total white blood-cell count was due to a lymphocytopenia.

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The Effect of Antibiotics on the Metabolism of Nicotinic Acid, Biotin and Folic Acid in Rats

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It has previously been shown (Guggenheim, Halevy, Hartmann & Zamir, 1953) that oral administration of penicillin, aureomycin (chlortetracycline), streptomycin or oxytetracycline (terramycin) causes a marked stimulation of growth in rats fed on diets low in riboflavin or in pantothenic acid. A similar effect was observed with penicillin and oxytetracycline added to a diet low in thiamine, whereas the inclusion of these antibiotics in a completely vitamin-supplemented diet had no growth-promoting effect. Growth stimulation was generally associated with a higher liver level and increased urinary excretion of these vitamins. Since subcutaneous injections of antibiotics had no effect on growth or on the accumulation of these vitamins in the liver, it was concluded that the observed sparing effect of the antibiotics on the requirement of young rats for certain B-vitamins is mainly due to alterations in the intestinal flora.

The vitamins studied in our previous work are not synthesized in the rat intestine in sufficient amounts and they must, therefore, be provided in the food. This paper presents the results obtained with nicotinic acid, biotin and folic acid, which are synthesized abundantly by the rat's intestinal flora.

METHODS

Male rats weighing 40–50 g were placed on the 18% casein diet described previously (Guggenheim *et al.* 1953). In the experiments with nicotinic acid the level of casein in the diet was reduced, half of it being replaced by maize starch. The diet of the rats that received 2% succinylsulphathiazole in the diet was fortified with 19 mg menaphthone, 40 mg *p*-aminobenzoic acid and 200 mg inositol/kg. Within each experiment the food intake of treated animals was equalized with that of their controls. The antibiotics aureomycin, penicillin, oxytetracycline and streptomycin were each fed to a different group, being incorporated in the ration at a level of 50 mg/kg diet.

At the end of the 3rd and 5th weeks of the experiment urine was collected from each rat for 3 days, and the amounts of *N'*-methylnicotinamide, biotin, folic acid and