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REQUIREMENTS OF RATS FOR VITAMIN B_{12} DURING GROWTH, REPRODUCTION AND LACTATION

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Large sectors of the human populations live on a more or less exclusively vegetarian diet. This fact induced us several years ago to initiate studies on the effect of such a type of feeding on experimental animals kept under similar conditions for many generations. Preliminary experiments showed the existence of some factor (Jaffé, '46), later identified as vitamin B_{12} (Jaffé, '48) lacking in this kind of ration. Because vegetarian diets are low in this vitamin, it seemed interesting to conduct some long-range experiments on their effect on experimental animals. Moreover, an attempt has been made to determine the minimum vitamin B_{12} requirements for growth, reproduction and lactation of the rat under conditions of uniform intake during more than one generation.

EXPERIMENTAL

The animals were descendants of the "Sprague Dawley" strain. All the rats of the experimental series were from a stock kept on a soybean oil meal-corn ration since 1948, while a control group was always fed a commercial stock diet. The animals were housed in screen bottom cages in a room without air conditioning. Large litters were always reduced to 6 within 48 hours after birth, weaned at 21 or 28 days of age and kept together in a common cage to permit brother and sister mating for the following generation. In special cases,

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the females of one litter were bred with males of another of the same experimental group. Pregnant females were weighed and put in single cages. Females were bred only once except in a few cases when they were bred a second time.

The composition of the experimental basal diet in parts by weight was as follows: soybean oil meal, 46; whole yellow corn meal, 46; USP Salt Mixture no. 2, 2; sesame oil containing 0.2% of percomorphum oil and 0.2% of wheat germ oil, 5; and the following vitamins per kilogram of diet: thiamine hydrochloride, 3.0 mg; riboflavin, 3.0 mg; pyridoxine hydrochloride, 2.0 mg; Ca-pantothenate, 2.0 mg; niacin, 20 mg; folic acid, 0.25 mg; biotin, 0.10 mg; *p*-amino benzoic acid, 250 mg; inositol, 100 mg; choline chloride, 1 gm.

This diet had only traces of vitamin B_{12} activity, as determined with *L. leichmannii.* The control diet was a commercial pelleted rat ration,[1] which according to the manufacturer, contains about half animal and half vegetable proteins. Its content of vitamin B_{12} was about 30 μg/kg as determined with *L. leichmannii.* This and the experimental rations contained about 24% of crude protein. Ad libitum feeding was used throughout.

The rats in the series fed the diets supplemented with vitamin B_{12} were descendants of animals kept for 10 or more generations on the basal experimental diet and the females were transferred to the supplemented rations when they were transferred to individual cages, one to 6 days before parturition. The litters were bred continuously on the same supplemented diet. The experiments were set up so as to avoid any carry-over or repletion of diminished reserves of the vitamin. Therefore, only animals held on the respective diets at least through the second generation are included.

The animals were weighed individually and the mean weaning weight of each litter calculated. For the tabulation, these mean weights, rather than individual weights, were used in order to avoid a possible bias due to the tendency of rats in larger litters to be lighter.

[1] Ratarina, produced locally.

For the determinations of soluble, reduced nonprotein sulfhydryl compounds in the livers, a modification (Jaffé and Budowski, '54) of the Grunert and Phillips ('51) method for glutathione was used. Vitamin B_{12} was determined with a modification of the USP method.

RESULTS

In table 1 data are presented on the reproduction of rats kept for 7 years on the soybean oil meal-corn ration. The enumeration of the generations starting with the 5th is only approximate as the groups were selected according to the date of the birth of their litters and the corresponding generation calculated only with the first and last animals of each group. Therefore, some overlapping may exist among the last 4 groups.

The litters in the first generation showed distinctly better performance than later ones with respect to the number of animals born in each litter, number of surviving animals, and weaning weights. This was to be expected, as the complete stock ration was fed during gestation. The performance in this group is virtually the same as for the animals kept on the stock ration for the whole lactation period. Starting with the second generation, a very considerable deterioration in the performance of the litters may be observed. The number of animals weaned per litter was only about half that in the first generation. As the number of young which died between the second day after birth and the weaning age was always small, it is clear that this is due mostly to the higher number of stillbirths or to deaths in the first two days of life. There was a tendency for all of the young in litters of certain mothers to die in contrast to a low death rate in the litters of others.

No difference between the first and succeeding generations could be observed with respect to the weight gain of the mothers during the 4 weeks of lactation or to the mean weight of the young at birth.

TABLE 1

Reproduction and lactation performance of rats kept for several generations on a vegetable diet low in vitamin B_{12}

GENERATION	LITTERS BORN	LITTERS WITH NO SURVIVORS	YOUNG BORN PER LITTER	AV. WT. OF YOUNG AT BIRTH	YOUNG WEANED PER LITTER [1]	AV. WEANING WTS. (4 WKS.)	YOUNG DEAD PER LITTER BETWEEN DAY 2 AND WEANING	WT. GAIN OF MOTHER DURING LACTATION
				gm		*gm*		*gm*
1	9	0	8.4	5.7	5.3	63.1	0.3	9.0
2–3	24	8	6.0	5.8	2.1	41.4	1.0	6.2
3–4	19	6	7.1	5.6	3.0	47.9	0.1	10.5
5–7	32	11	7.7	6.0	2.3	38.9	1.1	2.1
8–12	32	8	6.9	5.6	4.2	43.1	0.8	10.3
13–15	30	9	7.6	5.3	3.6	45.7	0.6	7.6
16–18	36	10	7.0	5.3	3.1	42.9	0.7	0

[1] Larger litters were reduced to 6.

TABLE 2

Reproduction and lactation performance of rats kept on different experimental diets

GROUP NO.	DIET AND SUPPLEMENTS	LITTERS		YOUNG BORN PER LITTER	AV. WT. OF YOUNG AT BIRTH	YOUNG WEANED PER LITTER	YOUNG DEAD PER LITTER BETWEEN DAY 2 AND WEANING	AV. WEANING WTS. (4 WKS.)	WT. GAIN OF MOTHER DURING LACTATION	AV. WT. OF MOTHERS AT BIRTH OF FIRST LITTER	AV. AGE OF MOTHERS AT BIRTH OF FIRST LITTER
		BORN	DEAD								
					gm			*gm*	*gm*	*gm*	*days*
1	Basal	30	9	7.6 ± 0.37 [1]	5.4 ± 0.19	3.6	0.5	46.7 ± 3.1	9.1 ± 3.0	184 ± 3.9	147 ± 2.5
2	+ 3 μg of vit. B_{12}	15	1	8.0 ± 0.62	5.8 ± 0.13	5.3	0.1	57.7 ± 2.2	21.4 ± 3.0	197 ± 5.5	95 ± 4.4
3	+ 5 μg of vit. B_{12}	26	3	8.2 ± 0.43	5.7 ± 0.19	4.8	0.4	66.6 ± 1.0	17.1 ± 3.7	196 ± 5.5	111 ± 3.8
4	+ 30 μg of vit. B_{12} + 0.2% methionine	20	1	7.5 ± 0.50	5.5 ± 0.22	5.2	0.7	62.8 ± 3.1	7.6 ± 5.6	..	..
5	Stock	103	2	9.3 ± 0.29	6.0 ± 0.05	5.5	0.3	66.6 ± 1.0	0.8 ± 2.1	215 ± 5.6	96.5 ± 3.7

[1] Standard error of the mean.

The results of various supplements of vitamin B_{12} on reproduction and lactation performance are summarized in table 2. The performance of the animals on the basal diet was poor in comparison with the B_{12}-supplemented groups with respect to the survival of litters, number of animals weaned per total litters born, weaning weights at 4 weeks and the age of the mothers at the birth of their first litters. The results of group 2 show that the addition of 3 μg of vitamin B_{12} per kilogram of diet did not result in a similar weight gain of the young as

TABLE 3

Post weaning growth of rats bred on different experimental diets

DIET AND SUPPLEMENTS	NO. OF ANIMALS	SEX	WEIGHT AT 3 WEEKS	WEIGHT AT 7 WEEKS
			gm	*gm*
Basal	23	M	27.9 ± 0.9 [1]	119.2 ± 4.9 [1]
Basal	21	F	29.3 ± 2.1	109.2 ± 3.6
Basal + 3 μg/kg vit. B_{12}	25	M	34.5 ± 1.1	154.5 ± 3.1
Basal + 3 μg/kg vit. B_{12}	28	F	34.4 ± 1.0	123.5 ± 1.7
Basal + 5 μg/kg vit. B_{12}	30	M	41.8 ± 0.6	170.2 ± 3.3
Basal + 5 μg/kg vit. B_{12}	30	F	41.5 ± 0.7	137.0 ± 1.9
Stock	24	M	42.1 ± 0.9	163.6 ± 3.4
Stock	28	F	42.3 ± 1.8	134.4 ± 2.1

[1] Standard error of the mean.

that observed with larger supplements, although the performance was nearly the same in all other respects.

Supplements of 5 μg/kg of vitamin B_{12}, and 30 μg/kg together with 0.2% of methionine gave identical results. A comparison between the group of rats fed the complete stock diet and the two latter supplemented groups shows that there were no significant differences in all those aspects studied in our experiments with the only exception of the litter size, the birth weight, and possibly the number of litters in which all animals died before weaning time. In experiments not included in the present paper there was no consistent difference in birth weight between stock and experimental groups.

Observations on post-weaning growth of young from different groups are included in table 3. These animals were weaned at three weeks. It can be seen from the results that growth during the 4-week period was about 75% of the normal

TABLE 4

Reduced liver glutathione in adult male rats bred on different diets

DIET AND SUPPLEMENT	NO. OF ANIMALS	LIVER GLUTATHIONE
		mg/100 gm
Basal	17	211 ± 6 [1]
Basal + 3 μg/kg of vitamin B_{12}	11	222 ± 5
Basal + 5 μg/kg of vitamin B_{12}	6	214 ± 5
Basal + 30 μg/kg of vitamin B_{12}	12	202 ± 5
Basal + 30 μg/kg of vitamin B_{12} and 0.2% of methionine	6	245 ± 11
Stock	20	269 ± 6

[1] Standard error of the mean.

TABLE 5

Content of vitamin B_{12} in the livers and kidneys of adult male rats kept on different diets

DIET	AVERAGE VITAMIN B_{12}	
	Liver	Kidney
	μg/kg	*μg/kg*
Basal	0.013 ± 0.0013 [1]	0.101 ± 0.031
Basal + 5 μg B_{12} per kg	0.033 ± 0.0057	0.181 ± 0.028
Stock	0.108 ± 0.0060	2.25 ± 0.25

[1] Standard error of the mean.

rate in the vitamin B_{12}-deficient group kept on the basal diet, and that there were no significant differences in this respect between the groups receiving supplements of 3 or 5 μg/kg or the stock ration. No post-weaning death was observed in this experiment.

The results of determinations of soluble, reduced liver non-protein sulfhydryl compounds are presented in table 4. There were no significant differences between the unsupplemented and vitamin B_{12}-supplemented groups; the stock animals however, had higher levels. Hemoglobin, hematocrit, and urea determinations as well as red and differential white blood cell counts were made on groups of adult male rats from groups 1, 3, and 5. All the values found were within the range accepted as normal. Vitamin B_{12}-deficient animals had slightly higher values of blood urea than stock animals (0.27 $\pm$ 0.017 gm/l vs. 0.24 $\pm$ 0.016 mg/l).

In table 5, the values for vitamin B_{12} in livers and kidneys of adult male rats, kept on one of three different diets, are presented. It can be seen that the diet containing 5 µg/kg of vitamin B_{12} did not cause as high tissue levels of this vitamin as the stock diets although the values were higher than they were with the basal ration.

DISCUSSION

In confirmation of Dryden et al. ('52), the data in tables 1 and 2 show that in mother rats fed a diet low in vitamin B_{12} there is a tendency for the entire litters of certain mothers to die; this is in contrast to a relatively low mortality rate for the litters produced by other individuals. This suggests that by applying the principles of selective breeding it might be possible to develop a strain of rats that is relatively resistant to vitamin B_{12} deficiency. We used mostly brother and sister matings in order to accentuate any such tendency.

Nevertheless, the differences observed between the different deficient groups in subsequent generations are small and of doubtful significance. The first deficient group (second to third generation) showed the poorest overall performance, but the observed differences are not impressive in view of the fluctuations between the following experimental groups. Between the third to 4th and 16th to 18th generations, no such selective trend was observable.

The lack of a significant difference between the first groups and the last, which were observed after 6 to 7 years of almost continued brother-sister breeding on the vitamin B_{12}-low diet, is contrary to what we had expected to find. It may be related possibly to the fact that the performance of our rats on the soybean-corn ration was much better than that described by several other investigators (Schultze, '53; Perdue and Phillips, '54, and Sherman, Schilt and Schaeffer, '55). These authors have reported results showing poor performance and especially a very high post-weaning mortality rate in rats on a diet low in vitamin B_{12} even after supplementation with this vitamin. We were unable to make similar observations. Usually, the mortality was near zero after weaning. There were some instances in the course of these experiments when the young of several litters in the group fed the B_{12}-deficient diet died one to 6 weeks after weaning and in one case, several litters of the stock animals died at the same time under very similar circumstances. In all cases, vitamin B_{12}-supplemented litters showed the same mortality as those reared on the unsupplemented ration. There were usually no gross abnormalities observable at autopsy. These cases were very infrequent, the total not reaching even 5% of those weaned. No gross congenital malformations have been observed in the course of the present experiments. It seems possible that the high mortality observed by other authors was due to infection or to changes in the intestinal flora. As our colony has been kept very isolated — no new animals have been brought in from outside since the start of these experiments — it may be in an unusally healthy condition. The lack of growth-stimulating action of aureomycin in our vitamin B_{12}-low animals (Jaffé, '51) may possibly be interpreted in a similar manner.

As Schultze ('53) has shown, there may be differences between lots of soybean protein with respect to their nutritional value for rats. As the soybean oil meal used during the years of experimentation came from several sources, there may have been differences which could have obscured the outcome of our experiment. There are other deficiency symptoms such as sterility, which were not studied in the present experiments and which may have had some influence on the results.

Although we were unable to detect the operation of a genetic selection of animals with better performance on a diet low in vitamin B_{12}, the results presented in table 1 show that it is possible to breed rats for at least 18 and probably many more generations on a fortified soybean-corn ration not supplemented with vitamin B_{12}.

The lack of vitamin B_{12} in the diet caused a high mortality rate of the litters, a low birth weight and weaning weight, and the deficient females were older when they gave birth to their first litters. The first two deficiency symptoms were at least partially, and the latter two completely overcome by a supplement of only 5 μg/kg of vitamin B_{12}, while the dose of 3 μg/kg was insufficient to secure normal weaning weight. As there was no difference observable between the effect of supplements of 5 or 30 μg/kg, the minimum dose must be somewhere between 3 and 5 μg/kg of vitamin B_{12} in a soybean-corn diet under the present conditions.

The tendency of vitamin B_{12} supplements to cause greater birth weights in rats, observed by other authors (Daniel et al., '53; Dryden et al., '51) can also be detected in our results, although the young born of mothers fed the stock ration were still heavier at birth notwithstanding the larger litter sizes in this group. The replacement of soybean oil meal by full-fat soy flour causes the differences in litter size between the experimental and control groups to disappear (Jaffé, '55). They are therefore probably not related to vitamin B_{12}.

The females on the deficient diet were significantly older when their first litters were born than those on the diet supplemented with vitamin B_{12}. This is in accordance with observations of Dryden et al. ('54) on sexual maturation, which was found to be delayed in vitamin B_{12}-deficient animals.

It can be concluded from the data of table 3, that the difference in growth, for the 4 weeks after weaning, between rats receiving vitamin B_{12} supplements of 3 or 5 μg/kg of diet respectively, was small in both males and females (8.4 gm for males and 6.4 gm for females) as compared with the differences in weaning weights (difference of three-weeks and 7-weeks weights of groups receiving the 3 μg/kg supplements of vitamin B_{12} subtracted from corresponding difference of groups receiving 5 μg/kg of the vitamin).

This and the fact that females kept on the diet supplemented with 3 μg/kg of vitamin B_{12} gave birth to their first litters at practically the same age as the rats receiving the larger amount of this vitamin, and that they reached the same weight at this time (table 2) can be taken as evidence that the former dosage is nearly sufficient for growth.

A difference with respect to the level of reduced glutathione of livers between vitamin B_{12}-deficient and supplemented animals as described by Register ('54) could not be detected in the present experiments (table 4) in agreement with work on mice (Jaffé, '54). The higher levels in animals fed a diet supplemented with methionine are probably to be explained on the basis of observations of Leaf and Neuberger ('47) on the influence of sulfur-containing amino acids on tissue glutathione.

The levels of vitamin B_{12} found in the livers in our animals are in reasonably good agreement with values reported by Moinuddin and Bentley ('55), considering the different experimental conditions used, and show that tissue saturation has not been achieved with the addition of 5 μg/kg of this vitamin to the basal diet. The kidney values are higher than those reported by the aforementioned authors.

The present results may be interpreted as indicating that the need of the rat for vitamin B_{12} is greater for lactation than it is for growth and reproduction. Inasmuch as supplements of 5 or 30 μg/kg of diet gave identical results, the former dose may be considered sufficient for the whole reproductive cycle of the rat under the present conditions.

The present results should not be applied to other diets than that used during this study. Experiments now under way with diets in which the carbohydrate source is sucrose are giving results which would indicate a somewhat higher requirement for vitamin B_{12}.

The observations on rats presented in this paper are very similar to those made earlier on mice (Jaffé, '54), with the difference that in the latter species a supplement of 3 μg/kg of vitamin B_{12} to the corn-soybean oil ration was as effective as the higher doses.

SUMMARY

A rat colony was kept on a fortified soybean oil meal-corn ration, low in vitamin B_{12}, for 18 generations, using mostly brother and sister matings.

Litters starting with the second generation showed high mortality, low birth weights, low weaning weights, slow post weaning growth, and low liver and kidney vitamin B_{12} levels. Females of this group were older, when giving birth to their first litters, then the controls. Blood characteristics and liver glutathione levels were normal.

No significant difference between succeeding generations could be detected and therefore no indication for a genetic selection toward resistence to vitamin B_{12} deficiency could be found.

The addition of 3 μg of vitamin B_{12} per kilogram of diet eliminated most of the deficiency symptoms, but did not result in optimal weaning weights and post-weaning growth, while supplements of 5 μg of vitamin B_{12} per kilogram of diet, or 30 μg of this vitamin together with 0.2% of methionine, gave identical results in overcoming these deficiency signs. All of the animals used had been kept for at least one generation on the respective experimental diets previous to the experiments presented.

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