Observations on the Influence of Vitamin B_{12} and Folic Acid on Protein Utilization in the Growing Rat[1]

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Since the recognition of vitamin B_{12} as a major constituent of animal protein factor, several reports have emphasized improvement in the utilization of low-quality vegetable proteins by supplementation with this vitamin (Marfatia and Sreenivasan, '51; Baliga et al., '54; Sure, '54, '57). The effectiveness of vitamin B_{12} is particularly marked at low-protein intakes (Hartman et al., '49; Luecke, '49; Emerson, '49) and may vary with different vegetable proteins and also with the animal species. (Wokes and Picard, '56). A number of workers have established the existence of relationships between vitamin B_{12}, folic acid (PGA), methionine and choline in the rat, mouse, chick and certain other species (Bennett, '50; Schaefer et al., '50; Schaefer and Knowles, '51; Stekol et al., '52; Jukes and Stokstad, '52; Sauberlich, '59). An interdependence of vitamin B_{12} and PGA may also be inferred from the common and as yet undissociable functions of these vitamins in several metabolic processes and also from observations on their mutual potentiation (Sreenivasan, '51; Girdwood, '59; Fatterpaker et al., '55; Narayanan et al., '56; Ellis et al., '59). The object of the present investigation was to assess the influence of vitamin B_{12} and PGA, in the growing rat, on protein utilization using varying levels of dietary protein.

EXPERIMENTAL

Young, male Wistar rats of approximately 50-gm weight were used in the trials with peanut protein and casein. The animals were initially depleted of their vitamin B_{12} and PGA reserves by maintenance on a deficient, iodo-casein diet consisting of (in gm per 100 gm of diet): vitamin-free casein, 10; iodinated casein,[3] 0.15; arachis oil, 6; shark liver oil, 2; sucrose, 9.85; corn starch, 68; and salt mixture (U. S. P. no. 14), 4. The sucrose provided the following vitamin levels (in mg per kg of diet): thiamine·HCl, 6; riboflavin, 10; Ca pantothenate, 20; pyridoxine·HCl, 6; biotin, 1; nicotinic acid, 30; choline chloride, 500; and inositol, 500. The arachis oil carried supplements of α-tocopherol and of 2-methyl-1, 4-naphthoquinone at levels of 50 mg and 10 mg, respectively, per kilogram of diet. The vitamin levels provided were considered adequate for the hyperthyroid condition.

At the end of 4 weeks, the animals were divided into groups of 8. One group was replaced on the original iodo-casein diet modified by the omission of iodinated casein and inclusion of succinyl sulphathiazole at a 2% level (with appropriate adjustment of the starch content). A second group received the protein (vitamin-free casein) at an 18% level in the modified ration, the extra protein addition being made at the expense of corn starch. Two similar groups were fed with defatted, hot alcohol-extracted peanut meal at 10 and 18% protein levels. There were corresponding groups in each case receiving supplements of vitamin B_{12} and PGA at levels of 150 μg and 5 mg, respectively, per kg of diet. There were 4 groups in addition to these 8, fed the casein or peanut protein

Received for publication March 2, 1960.
[1] This work was supported by a research grant from the Williams Waterman Fund Research Corporation, New York.
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[3] Iodinated casein, Protomone, Cerophyl Laboratories, Kansas City, Missouri.

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at a 10% level and receiving supplements as above either of vitamin B_{12} or PGA.

The animals were maintained in individual, suspended cages with 1/2-inch mesh screen bottoms. The average initial weight for the groups (around 86 gm) did not differ by more than 2 gm. The rats were weighed twice weekly and fed ad libitum, with food intake recorded.

In the trials with corn-peanut meal, male weanling rats weighing from 40 to 50 gm were used, receiving the rations described in table 4. By analysis the protein content of the basal ration was 30% and with appropriate additions of starch, where necessary, this level was maintained in all other rations. The basal ration was similar in composition to that developed in this laboratory for the production of vitamin B_{12} deficiency in rats and mice (Fatterpaker et al., '59), and included succinyl sulphathiazole. Its percentage composition was as follows: defatted, alcohol-extracted peanut meal, 46; corn meal, 44; vitaminized arachis oil, 5; vitaminized sucrose, 1; salts mixture (no. 4, Hegsted et al., '41), 2; and succinyl sulphathiazole, 2. The vitamin levels provided by sucrose and arachis oil corresponded to those used in the iodinated-casein diet in the previous experiment. Each rat was given orally at the beginning of the experiment and each week thereafter two drops of shark liver oil to provide 300 I.U. of vitamin A and 4 I.U. of vitamin D. During the experimental 6-week period the animals had free access to food and water. At three weeks of feeding, nitrogen retention studies with 4 animals per group were made during three equally spaced 4-day periods in the 4th, 5th and final week. Individual, round metabolism cages were used, urine and feces being collected before the morning feeding. Urine was stored with the addition of a few drops of sulphuric acid and toluene. Feces were dried at 80°C for 24 hours and weighed. The nitrogen content of the excreta pooled for each 4-day period was determined by the Kjeldahl method. Growth rate and food intake were recorded throughout the entire period.

The rats were dissected under ether anesthesia. Livers were quickly excised, chilled and made into 20% homogenates with ice-cold distilled water. Total liver nitrogen was determined by the micro-Kjeldahl procedure of Umbreit ('46). Total liver fat determination was made according to the method of Sperry ('54). Protein-free metaphosphoric acid extracts of the tissue were analyzed for non-protein sulphydryl by the nitroprusside method of Grunert and Phillips ('51) standardized against glutathione. In the trials with casein and peanut protein, livers were additionally analyzed for vitamin B_{12} and PGA, and blood was also obtained in this experiment for determination of serum vitamin B_{12}. Blood was drawn from the hepatic portal vein prior to excision of the liver and was allowed to clot at 37°C for an hour and later centrifuged in cold to separate the serum. Total vitamin B_{12} in the latter was determined by the method of Ross ('52) using the *bacillaris* strain of *Euglena gracilis*. Portions of liver homogenates were incubated at 37° for 12 hours under toluene with papain (25 mg/gm of fresh liver) in 0.1 M acetate buffer of pH 4.6. The samples were then analyzed for vitamin B_{12} using *E. gracilis*, according to the method of Hoff-Jorgensen ('54). Separate portions of the homogenates were autolyzed in 0.1 M phosphate buffer of pH 7.2 under toluene at 37° for 12 hours and assayed with *Streptococcus faecalis R* for PGA as described by Mitbander and Sreenivasan ('54).

RESULTS

Administration of vitamin B_{12}, and especially of PGA, or of the two simultaneously was associated with a marked increase in food intake in both peanut protein- and casein-fed animals (table 1). The efficiency ratio with peanut protein was lower than with casein at both low- (0.54, $P < 0.01$) and high- (0.47, $P < 0.01$) protein intakes. The 10% protein diet with vitamin B_{12} or PGA resulted in better utilization of peanut protein, with marked improvement when both vitamins were fed simultaneously, irrespective of the amount of protein consumed. The gains in body weight followed a similar trend although PGA was somewhat more effective than vitamin B_{12} in reducing the difference in the growth rate due to protein quality.

With low-protein diets the liver nitrogen value was higher with casein (23.6 mg/

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TABLE 1

Summary of individual and combined effects of dietary vitamin B_{12} and PGA on the efficiency of peanut protein and casein utilization by the rat[1]

Diet description		Peanut protein-fed rats			Casein-fed rats[2]		
Protein content	Supplements	Protein consumed	Weight gained	Protein efficiency ratio[3]	Protein consumed	Weight gained	Protein efficiency ratio[3]
%		*gm*	*gm*		*gm*	*gm*	
10	None	20.1 ± 1.2[4]	13.7 ± 2.0	0.68 ± 0.03	18.0 ± 0.8	22.0 ± 2.4++	1.22 ± 0.11++
10	Vitamin B_{12}	25.0 ± 1.2	26.5 ± 2.1	1.06 ± 0.09	26.5 ± 1.4	39.5 ± 3.3++	1.49 ± 0.10++
10	PGA	31.4 ± 1.3	34.8 ± 3.1	1.11 ± 0.08	29.0 ± 1.3	43.8 ± 3.0+	1.51 ± 0.10++
10	Vitamin B_{12} + PGA	31.8 ± 1.8	53.4 ± 3.4	1.68 ± 0.12	33.5 ± 1.8	61.0 ± 2.8	1.82 ± 0.07
18	None	41.6 ± 2.1	27.9 ± 2.8	0.67 ± 0.08	37.4 ± 1.6	42.6 ± 3.8++	1.14 ± 0.08++
18	Vitamin B_{12} + PGA	54.3 ± 3.2	83.1 ± 4.6	1.53 ± 0.14	58.6 ± 3.4	100.2 ± 6.2+	1.71 ± 0.09

[1] Data obtained over a 5-week period of maintenance of vitamin B_{12}- and folic acid-deficient animals on deficient, peanut protein and casein diets with and without supplements of vitamin B_{12} (B_{12}, 150 μg/kg of diet) and folic acid (PGA, 5 mg/kg of diet).
[2] Statistically significant differences from corresponding values of peanut protein-fed animals are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.
[3] Grams gain in body weight per gram of protein consumed.
[4] Standard error of the mean.

TABLE 2

Effects of vitamin B_{12} and PGA on liver composition with peanut protein and casein diets[1]

Diet description		Liver analysis of peanut protein-fed rats			Liver analysis of casein-fed rats[2]		
Protein content	Supplements[3]	Total nitrogen	Total fat	Non-protein sulphydryl	Total nitrogen	Total fat	Non-protein sulphydryl
%		*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*
10	None	18.8 ± 0.5	43.4 ± 1.8	0.61 ± 0.07	23.6 ± 0.7++	34.2 ± 2.1++	1.01 ± 0.06++
10	Vitamin B_{12}	24.2 ± 1.2	32.2 ± 2.7	0.84 ± 0.06	28.2 ± 0.9+	25.9 ± 1.0++	1.37 ± 0.06++
10	PGA	20.8 ± 1.4	35.4 ± 1.9	0.65 ± 0.09	27.3 ± 0.8++	28.6 ± 2.6++	1.02 ± 0.05++
10	Vitamin B_{12} + PGA	27.1 ± 1.4	28.2 ± 2.1	0.95 ± 0.04	29.4 ± 1.1	22.7 ± 2.0++	1.52 ± 0.04++
18	None	19.4 ± 0.8	35.7 ± 1.8	0.88 ± 0.07	26.2 ± 0.4++	25.8 ± 2.9++	1.51 ± 0.05++
18	Vitamin B_{12} + PGA	27.3 ± 1.0	23.5 ± 1.6	1.88 ± 0.08	31.2 ± 0.7++	21.8 ± 1.4	1.85 ± 0.07++

[1] Values are on wet basis and include the standard error of the mean.
[2] Statistically significant differences from corresponding values of the peanut protein-fed are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.
[3] B_{12} = vitamin B_{12}, 150 μg/kg of diet; PGA = folic acid, 5 mg/kg of diet.

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gm) than with peanut protein (18.8 mg/gm) (table 2). Significant reduction of this difference was not obtained with vitamin B_{12} or PGA alone, but only with their simultaneous provision. With increased intake of protein there was a significant gain in liver nitrogen in the casein-fed animals (2.6 mg/gm, $P < 0.05$) but not in the peanut protein-fed group (0.6 mg/gm, $P > 0.1$). As a result, using the high-protein diets the difference in nitrogen values between the two groups was magnified (6.8 mg/gm, $P < 0.01$) and although with vitamin B_{12} and PGA this was considerably rectified, a significant difference still prevailed (3.9 mg/gm, $P < 0.01$). Livers of the doubly deficient animals showed marked fatty infiltration especially from those from rats fed low-protein diets and with peanut protein. Vitamin B_{12} or PGA brought about appreciable reductions in liver fat, the two vitamins together causing still further depression. These reductions were in every instance attended by a significant diminution of the effect due to protein quality. With a high-protein regimen the lipotropic effects of the vitamins extended to bringing about a near equalization of the liver fat contents in the casein and peanut protein groups. The liver content of non-protein sulphydryl was appreciably lower in the peanut protein-fed rats than in those receiving casein. Administration of vitamin B_{12} alone caused a significant improvement, whereas PGA was effective only in presence of vitamin B_{12}, especially in low-protein rations.

Observations on serum and liver vitamin B_{12} and liver PGA are summarized in table 3. The depletion of the liver reserves of the vitamins in the doubly deficient animal was greater in the peanut protein than in the casein-fed animals. A similar, though less marked trend, was seen in the serum concentration of vitamin B_{12}. However, in the replete groups the serum level of vitamin B_{12} was considerably higher in the peanut protein than in the casein-fed rats although the liver levels of the vitamins were again lower in the former. Such reductions of liver vitamin B_{12} and PGA levels, with elevation of serum vitamin B_{12} concentration, also accompanied low-protein intakes. In general, the administration of either vitamin B_{12} or of PGA to the doubly deficient animal raised the liver concentration of the other vitamin (also serum concentration of vitamin B_{12} when PGA was fed).

The effects of partial replacement of peanut protein by egg albumen in the basal corn-peanut meal on the response to vitamin B_{12} and PGA are summarized in tables 4 and 5. Although the rats were fed unrestrictedly, no significant differences in food intakes were observed as a result of vitamins or egg albumen supplementation. Either supplement effected higher nitrogen retention and improved the growth rate. The effectiveness of the vitamins was markedly reduced in the egg albumen-containing ration. This trend was supported by the liver analyses (table 5). With supplements of egg albumen or vitamin B_{12} and PGA there resulted significant ($P < 0.01$) increases in liver nitrogen and non-protein sulphydryl and a decrease ($P < 0.01$) in liver fat. With the incorporation of egg albumen in the diet, vitamin B_{12} and PGA supplements effected only a small, although significant ($P < 0.05$), increase in non-protein sulphydryl, nitrogen and fat contents remaining unchanged.

DISCUSSION

According to Baernstein ('37) and Randoin and Boisselot ('43), the proteins of peanut are approximately equal to casein in promoting the growth of rats when fed at the 20% level. Peanut protein is deficient in sulphur-containing amino acids, particularly methionine (Grau, '46). This, while accounting for the low liver non-protein sulphydryl using peanut protein diets, would suggest that the growth-promoting effect observed with vitamin B_{12} and PGA is due, at least in part, to the known methionine-sparing effect of the vitamins. Both from the growth data as well as the liver analysis this effect would appear to be an additive one. Particularly it was seen that the liver nitrogen content of peanut protein-fed rats improved only when both vitamins were supplied. The lipotropic effect of the vitamins also appeared to be additive and unrelated to increased food intake or growth. The observation that this effect is not well-marked with casein to the same extent that it is with peanut protein under identical con-

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TABLE 3

Serum and liver vitamin B_{12} and liver PGA[1]

Diet description		Peanut protein-fed rats			Casein-fed rats[2]		
Protein content	Supplements[3]	Serum vitamin B_{12}	Liver vitamin B_{12}	Liver PGA	Serum vitamin B_{12}	Liver vitamin B_{12}	Liver PGA
%		*μμg/ml*	*mμg/gm*	*μg/gm*	*μμg/ml*	*mμg/gm*	*μg/gm*
10	None	82 ± 20	9.0 ± 1.8	1.01 ± 0.32	134 ± 22++	25.9 ± 6.7++	2.16 ± 0.21++
10	Vitamin B_{12}	722 ± 28	54.6 ± 9.8	1.84 ± 0.63	636 ± 28++	74.2 ± 7.9+	2.82 ± 0.19++
10	PGA	145 ± 16	15.7 ± 4.2	3.72 ± 0.41	163 ± 14	38.6 ± 4.2++	4.98 ± 0.31++
10	Vitamin B_{12} + PGA	1320 ± 42	62.4 ± 6.6	4.31 ± 0.20	850 ± 46++	92.6 ± 7.4++	5.34 ± 0.26++
18	None	40 ± 8	14.5 ± 7.9	2.10 ± 0.38	84 ± 18++	35.9 ± 6.2++	2.84 ± 0.32+
18	Vitamin B_{12} + PGA	976 ± 36	80.7 ± 8.4	5.32 ± 0.22	625 ± 34++	121.8 ± 9.8++	6.30 ± 0.29++

[1] Mean values and their standard errors; liver composition is on wet basis.
[2] Statistically significant differences from corresponding values of peanut protein-fed are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.
[3] Vitamin B_{12}, 150 μg/kg of diet; folic acid (PGA), 5 mg/kg of diet.

TABLE 4

Nitrogen balance and growth rate of rats fed the basal corn-peanut meal ration with and without supplements of egg albumen and vitamin B_{12} and PGA[1]

Supplements to basal diet[2]	Nitrogen retention[3]		Total food intake in 6 weeks	Weight gained in 6 weeks
	Food intake	Nitrogen balance		
	gm/day	*mg/day*	*gm*	*gm*
None	8.66 ± 0.18	176 ± 11.3	348 ± 15	55.8 ± 4.1
Vitamin B_{12} + PGA	9.01 ± 0.24	215 ± 8.8++	357 ± 7	88.9 ± 3.5++
Egg albumen[4]	8.62 ± 0.11	221 ± 9.7	353 ± 14	81.0 ± 4.5
Egg albumen + B_{12} + PGA	8.78 ± 0.13	240 ± 6.9+	365 ± 12	101.2 ± 5.6++

[1] Mean values and their standard errors; statistically significant effects due to vitamin B_{12} and PGA supplementation are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$. There were 6 animals per series.
[2] $B_{12} = 150$ μg of vitamin B_{12}/kg of diet; PGA = 5 mg of folic acid/kg of diet; where used, egg albumen was at 3% level in diet and replaced an equal amount of peanut protein; protein content of all diets was by analysis 30%.
[3] Summary of data obtained with 4 animals/group over three separate periods of 4 days each.
[4] Egg albumen, Nutritional Biochemicals Corporation, Cleveland.

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TABLE 5

Liver nitrogen, fat and non-protein sulphydryl in rats on the basal corn-peanut meal ration with and without supplements of egg albumen and vitamin B_{12} and PGA[1]

Supplements to basal diet[2]	Total nitrogen	Total fat	Non-protein sulphydryl
	mg/gm	*mg/gm*	*mg/gm*
None	23.9 ± 0.5	31.6 ± 1.3	0.96 ± 0.07
Vitamin B_{12} + PGA	29.5 ± 0.6++	25.2 ± 1.6++	1.28 ± 0.04++
Egg albumen	29.7 ± 0.5	23.8 ± 2.1	1.34 ± 0.06
Egg albumen + vitamin B_{12} + PGA	31.1 ± 0.7	20.9 ± 1.8	1.54 ± 0.03+

[1] Values are on wet basis and include the standard error of the mean; statistically significant differences due to vitamin B_{12} and PGA supplements are indicated: + indicates $P = < 0.05 > 0.01$; ++ indicates $P = < 0.01$.

[2] See footnote 2, table 4.

ditions of protein intake, but presumably with a higher methionine intake, would suggest that the critical factor was the role of vitamin B_{12} and PGA in the synthesis and transfer of labile methyl groups.

Examination of the data reveals an inverse relationship between liver fat content and the level of the vitamins in this tissue. The possibility that the degree of fat accumulation in liver may influence its retention of the vitamins may, therefore, be considered. In the event of a free supply of the vitamins in the diet, greater concentrations of these may be found in plasma and in urine when the diet is based on peanut protein than when based on casein.

It is of interest to recall the observations of Fox and his associates who found that raising the fat level of a corn-soybean meal diet from 3 to 22% increased the severity of vitamin B_{12} deficiency (as assessed by growth and mortality rates) in non-depleted chicks (Fox et al., '54) and elevated the vitamin B_{12} requirement (Fox et al., '56); this high vitamin requirement could be eliminated by supplemental methionine (Fox et al., '57, '59). Although in these experiments the high level of dietary fat, apparently, did not cause depletion of liver store of vitamin B_{12}, or increase the liver fat content, it lowered the ability of the tissue to retain any administered vitamin B_{12} (Fox et al., '56, '59). In this laboratory it has been observed that with rats fed 18% casein or wheat gluten diets providing minimal or optimal levels of B vitamins, increasing the dietary level of fat from 8 to 15% increased the concentration of liver lipids by about 20 to 34%.[4]

The retention of vitamin B_{12}, PGA and, probably, also other B factors in the liver tissue could, therefore, be considered as being influenced, directly or otherwise, by the degree of fat deposition in the tissue. Such an effect may also explain the highly depleted state of the doubly-deficient animals fed peanut protein diets.

The observation that the administration of either vitamin B_{12} or PGA to the doubly-deficient animal serves to raise the liver concentration of the other vitamin, substantiates earlier reports (*vide infra*) on their mutual potentiation.

The absence of any significant effect of vitamin B_{12} and PGA on food intake in experiments with corn-peanut meal may be due to the high-protein content of the diet (30%). The data in this experiment are indicative of a greater retention of dietary nitrogen, improved growth rate and liver nitrogen and non-protein sulphydryl contents as a result of partial replacement of dietary protein by egg albumen. Since similar improvements could also be brought about with supplements of vitamin B_{12} and PGA, presumably through a sparing action on methionine, it would seem that the primary deficiency in the basal diet was that of sulphur-amino acids; the liver non-protein sulphydryl content was low with this diet. Further, the effectiveness of vitamin B_{12} and PGA was reduced with the increase in the average quality of dietary protein brought about by egg albumen supplementation. Comparative studies of protein quality and utilization have been reported by Fatterpaker et al. ('59) and Marfatia and Sreenivasan ('60).

In rats deficient in vitamin B_{12} the growth-promoting effect of the vitamin has

[4] T. Balakrishna Rao, U. Marfatia and A. Sreenivasan, unpublished data.

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been attributed to increased deposition of fat rather than to synthesis of new protein (Arnstein, '55). Henry and Kon ('56) further suggest that the effect of vitamin B_{12} on protein efficiency may be discounted since the method hinges on the composition of weight gain in relation to protein intake. A reappraisal of these ideas may be necessitated if observations reported recently (Wagle et al., '58) on the role of vitamin B_{12} in protein synthesis are corroborated.

SUMMARY

Young rats depleted of their vitamin B_{12} and folic acid reserves were used to study the influence of these vitamins on the efficiency of protein utilization from peanut meal and casein diets at two protein levels.

The low efficiency of peanut protein in comparison with casein was improved with supplements either of vitamin B_{12} or folic acid. For optimal effects supplementation with both vitamins was essential.

Livers of the doubly-deficient animals showed marked fatty infiltration, with low nitrogen and non-protein sulphydryl content, especially with low-protein diets and with peanut protein.

Comparable groups of peanut protein- and casein-fed animals exhibited differences in liver composition with respect to nitrogen, fat and non-protein sulphydryl.

In separate experiments with weanling rats fed a vitamin B_{12}- and PGA-deficient, corn-peanut meal diet (30% protein) for 6 weeks of unrestricted feeding, replacing 3% of peanut protein with egg albumen, increased nitrogen retention, improved the growth rate and liver content of nitrogen and non-protein sulphydryl and effected a decrease in liver fat. Similar improvements using other diets are discussed.

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