Effect of the protein content of the diet on the vitamin B_{12} status in rats

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1. The effects of the protein content of the diet on the vitamin B_{12} concentration of serum and of various tissues were studied in rats by means of microbiological and radioisotopic techniques.

2. Protein starvation reduced tissue weights and nitrogen contents, serum protein concentrations and haemoglobin values.

3. The vitamin B_{12} contents of the serum and of several organs and tissues were greater in the protein-starved animals for reasons that remain unexplained.

4. The retention of radioactive cyanocobalamin per g fresh weight was significantly greater in liver, kidney, spleen and brain in the protein-depleted rats than in those given adequate protein. However, the total tissue retention was not significantly different between these groups, so that there was a greater capacity for tissue binding and for body conservation of vitamin B_{12} in the protein-starved animals.

In an earlier investigation on undernourished Indian children serum concentrations of vitamin B_{12} were found to be significantly raised (Satoskar, Kulkarni, Mehta, Sanzgiri & Bamji, 1962). This observation contrasts with reports of reduced serum concentrations of other vitamins, e.g. A, E, C, riboflavin, nicotinic acid and pteroyl-monoglutamic acid in such subjects (Moore & Sharman, 1951; Trowell, Moore & Sharman, 1954; Scrimshaw, Behar, Guzman, Viteri & Arroyave, 1955; Scrimshaw, Behar, Perez & Viteri, 1955; Bhagwan, Rao & Sreenivasan, 1961; Foy & Kondi, 1968; Viteri, Alvarado, Luthringer & Wood, 1968).

The higher concentration of vitamin B_{12} in the serum of hypoproteinaemic patients prompted this investigation of the effects of protein depletion on the vitamin B_{12} status of rats. A detailed study of the tissue distribution and excretion of this vitamin and the metabolism of injected radioactive cyanocobalamin was undertaken in animals given adequate protein and in protein-depleted animals given a protein-free diet.

EXPERIMENTAL

Animals and diet. Young rats (laboratory-bred Wistar strain), weighing about 90 g, were divided into three groups of eight each. They were fed *ad lib.* for 3 weeks on purified diets containing (g/kg): (1) 160 vitamin-free casein, (2) 160 vitamin-free gluten or (3) starch alone, the protein in diets 1 and 2 replacing starch.

The basal diet contained (g/kg): maize starch 850, sesame oil 80, succinyl sulpha-thiazole 20, vitaminized sucrose 10 and salt mixture 40. The salt mixture was prepared according to Hawk–Oser's method (Hawk & Oser, 1931). The following vitamins were added per kg diet: thiamin hydrochloride 10 mg, nicotinic acid 40 mg, choline chloride 1 g, *myo*-inositol 800 mg, biotin 0·6 mg, menaphthone 10 mg, calcium pantothenate 100 mg, pteroylmonoglutamic acid 1 mg, DL-α-tocopherol 6 mg, vitamin A acetate 0·275 mg and ergocalciferol 0·0029 mg. The fat-soluble vitamins were added to the sesame oil and the others mixed with sucrose and added to the diet. No source of vitamin B_{12} was given.

Determination of the distribution of radioactive cyanocobalamin. Tissue distribution of [^{57}Co]cyanocobalamin, administered orally as well as intraperitoneally, was studied in eight weanling rats given the casein diet and in eight protein-depleted animals. For this purpose sixteen weanling rats were divided into two groups and maintained on the diet containing 160 g devitaminized casein/kg as control or on the protein-free starch diet. The animals were force-fed with 6–8 g of the diet daily for 21 d. They were given 0·5 μCi [^{57}Co]cyanocobalamin of specific activity 11·5 μCi/μg, either intraperitoneally or orally, 24 h before being killed. The tissue radioactivity, together with a standard, was estimated in triplicate in a well-type scintillation counter (Fast Differential Count-ing System, BARC, India). The radioactivity of the tissue or organ was expressed as a percentage of the radioactivity administered.

Intestinal absorption of radioactive cyanocobalamin. Two groups of weanling rats, three in each group, were fed on the casein- or on the protein-free diet for 21 d. Intestinal absorption was measured in vitro in a loop of ileum of about 30–40 mm in length by the method of Wolff & Nebat (1962).

Other determinations. Haemoglobin and serum proteins were determined by standard techniques (Wintrobe, 1956; Gornall, Bardawill & David, 1949). Serum vitamin B_{12} was determined by the method of Ross (1952) with *Euglena gracilis* var. *bacillaris* as the test organism. Tissue vitamin B_{12} was measured in a sample of homogenate after papain digestion by the same method. The total vitamin B_{12} content was estimated after sterilizing the assay medium at 100° for 15 min. The samples were incubated at 28° for 8 d. After appropriate dilution, growth was measured turbidimetrically at 660 nm in a Klett Summerson photoelectric colorimeter. The vitamin B_{12} concentration was estimated from a standard curve (range 0–50 pg/ml). Vitamin B_{12} activity of serum and tissue was alkali-labile and the recovery of cyanocobalamin added to the serums and tissue was found to be 92·0 % (SE 1·51).

RESULTS

Body-weights and organ weights. Table 1 shows the body-weights and organ weights of the groups fed on the different diets for 3 weeks. The mean weight gain in casein-fed animals was 30·3 g, whereas the rats receiving the starch diet lost, on average, 11·9 g. The relative weight of the spleen expressed as mg/g body-weight decreased signi-ficantly in the animals on the starch diet compared with those given the casein diet and the relative weight of the brain increased. No significant difference was observed

Table 1. *Effect of dietary protein content on the body-weights, tissue weights, serum protein and haemoglobin concentrations of rats given diets containing casein, gluten or starch*

(Mean values with their standard errors for groups of eight rats)

	Diet					
	Casein		Gluten		Starch	
	Mean	SE	Mean	SE	Mean	SE
Body-wt (g):						
Initial	94·0	11·3	92·0	11·2	105·0	12·9
Final	124·0	15·2	98·0	11·9	93·0	11·5
Change	+30·3	5·0	+6·1**	5·5	−11·9***	4·2
Tissue wt (mg/g body-wt):						
Liver	38·1	1·7	45·2*	2·9	32·4	2·9
Kidney	6·7	0·2	7·4	0·3	6·6	0·5
Spleen	6·7	0·7	4·7*	0·2	3·1***	0·2
Brain	11·1	0·4	13·2*	0·6	13·9***	0·4
Heart	3·5	0·1	3·5	0·1	3·9	0·2
Serum protein (g/l)	57·4	1·5	44·9***	2·5	39·9***	1·7
Haemoglobin (g/l)	134·0	12·1	130·0	12·2	93·0**	8·4

Values significantly different from control values (casein diet): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Other values were not significantly different from control values.

Table 2. *Effect of dietary protein content on the vitamin B$_{12}$ concentrations in the serum and tissues of rats given diets containing casein, gluten or starch*

(Mean values with their standard errors for groups of eight rats)

	Diet					
	Casein		Gluten		Starch	
	Mean	SE	Mean	SE	Mean	SE
Serum (pg/ml)	364	71·9	690*	107·1	1967***	317·1
Liver: ng/g fresh wt	65·9	6·0	76·8	7·5	91·7*	9·9
Total (ng)	309·8	32·5	326·2	29·8	265·6	21·5
Kidney: ng/g fresh wt	128·5	17·0	196·3*	19·5	341·5***	35·9
Total (ng)	107·3	16·8	141·3	15·3	200·3***	22·2
Spleen: ng/g fresh wt	32·3	1·4	61·7***	3·8	68·6**	9·1
Total (ng)	26·6	2·7	27·8	4·6	19·7	3·4
Brain: ng/g fresh wt	28·7	2·4	47·2*	7·4	55·4*	9·5
Total (ng)	39·5	3·9	60·6	10·0	67·8*	11·8
Heart: ng/g fresh wt	61·4	3·4	90·4**	7·0	82·2*	8·8
Total (ng)	26·4	1·5	30·2	2·4	28·6	3·0
Muscle (ng/g fresh wt)	14·2	1·7	46·5***	7·5	29·8	7·3

Values significantly different from control values (casein diet): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Other values were not significantly different from control values.

Table 3. *Effect of dietary protein content on the distribution (expressed as percentage retention of the dose) of intraperitoneally injected [^{57}Co]cyanocobalamin in the tissues of rats given diets containing casein or starch*

(Mean values with their standard errors for groups of eight rats)

| | Diet | | | |
| | Casein | | Starch | |
Tissue	Mean	SE	Mean	SE
Liver: /g fresh wt	0·9	0·06	2·3***	0·17
Total organ	4·4	0·44	6·7**	0·44
Kidney: /g fresh wt	7·7	0·39	16·8***	1·67
Total organ	9·2	0·41	12·2*	1·21
Spleen: /g fresh wt	2·8	0·11	4·2***	0·27
Total organ	1·0	0·09	0·8	0·09
Brain: /g fresh wt	0·8	0·08	1·3*	0·20
Total organ	1·1	0·03	1·7**	0·18
Heart: /g fresh wt	1·9	0·07	2·2*	0·10
Total organ	1·0	0·40	0·8	0·06
Muscle (/g fresh wt)	0·5	0·03	0·7**	0·07

Values significantly different from control values (casein diet): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Other values were not significantly different from control values.

between the relative weights of liver and kidney in these two groups. However, when absolute weights of the tissues were compared, the loss in tissue weights was marked. Thus, the mean values with their standard errors for liver, spleen and kidneys in casein-fed rats were 4·68 g ± 0·2, 0·81 g ± 0·08 and 0·82 g ± 0·07, respectively, compared with 2·97 g ± 0·18, 0·29 g ± 0·02 and 0·59 g ± 0·03 in starch-fed animals. These results indicate that the reduction in weights of liver and kidneys in animals fed on the starch diet was proportional to the reduction in body-weight and therefore no marked differences were observed when these weights were expressed as mg/g body-weight. In the animals fed on the gluten diet, only the spleen showed a significant decrease in relative weight compared with that of the group fed on the casein diet ($P < 0.05$) and the relative weights of the liver and brain increased.

Serum protein concentrations and haemoglobin content. There was a marked decrease in serum protein concentration in gluten-fed as well as in starch-fed animals compared with casein-fed animals. The haemoglobin content was significantly reduced in starch-fed rats.

Vitamin B$_{12}$ concentrations in serum and tissue. The mean vitamin B$_{12}$ concentrations in serum and tissues are presented in Table 2. There was a significant rise in serum vitamin B$_{12}$ concentration in the animals fed on the starch and gluten diets as compared with animals fed on the casein diet. Vitamin B$_{12}$ concentrations of kidneys, spleen, brain and heart expressed as ng/g fresh weight were significantly greater in gluten- and starch-fed animals than the corresponding values observed in casein-fed rats. Compared with casein-fed animals, the total vitamin B$_{12}$ content of kidneys and brain

Table 4. *Effect of dietary protein content on the distribution (expressed as percentage retention of the dose) of orally administered [^{57}Co]cyanocobalamin in the tissues of rats given diets containing casein or starch*

(Mean values with their standard errors for groups of eight rats)

	Diet			
	Casein		Starch	
Tissue	Mean	SE	Mean	SE
Liver: /g fresh wt	1·2	0·06	2·0***	0·14
Total organ	2·1	0·19	3·1**	0·25
Kidney: /g fresh wt	8·2	0·78	13·2***	0·91
Total organ	3·6	0·31	4·2	0·28
Spleen: /g fresh wt	4·4	0·47	6·7**	0·45
Total organ	0·4	0·05	0·4	0·16
Eviscerated carcass: /g fresh wt	0·37	0·04	0·44	0·02
Total organ	29·3	3·11	23·6	1·58
Large intestine: /g fresh wt	4·7	0·89	7·0*	0·48
Total organ	0·7	0·03	0·8	0·07
Small intestine: /g fresh wt	3·0	0·29	4·6***	0·26
Total organ	2·5	0·27	2·6	0·20
Blood (/ml)	0·08	0·01	0·10	0·02
Urine (24 h total)	0·12	0·02	0·33*	0·08
Faeces (total)†	56·4	3·41	60·5	2·58

† Includes the amount collected during 24 h and that obtained by washing of large intestines at the end of 24 h.

Values significantly different from control values (casein diet): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Other values were not significantly different from control values.

was significantly more in the animals fed on the starch diet, but none of the tissues of the gluten-fed animals showed significant changes in total vitamin B$_{12}$.

These results were confirmed by subsequent experiments in which changes in vitamin B$_{12}$ concentration in serum and tissues from animals fed on protein-free or casein diets were compared with the concentrations observed in a comparable group of six animals killed at the beginning of the test period. In casein-fed animals there was no significant increase in concentration of vitamin B$_{12}$ in serum or tissues at the end of the experimental period compared with the initial value. In the protein-starved animals, however, the serum values for vitamin B$_{12}$ increased significantly ($P < 0.05$). Further, in these animals the vitamin B$_{12}$ concentrations in the liver, spleen, kidneys and muscle tissue showed a highly significant increase ($P < 0.001$) compared with the initial values.

Distribution of [^{57}Co]cyanocobalamin in different organs. Table 3 summarizes the results for the retention in different organs of intraperitoneally administered [^{57}Co]-cyanocobalamin. The percentage retention/g fresh weight was significantly more in liver, kidneys, spleen, brain, heart and muscle in the rats fed on the starch diet than in those given the casein diet. Even on a whole-organ basis the picture was not changed except in spleen and heart where the difference was not significant.

Table 4 shows the percentage distribution in different organs of orally administered

[^{57}Co]cyanocobalamin. These results indicate a similar absorption and distribution of cyanocobalamin within 24 h, between the rats fed on the casein and starch diets. In all the organs examined the protein-deprived animals retained greater quantities of cyanocobalamin expressed as percentage retention/g fresh weight. Even the eviscerated carcass and washed intestines contained higher amounts of radioactivity in these animals. The mean faecal loss in 24 h was 60·5 % in rats fed on the starch diet compared with 56·4 % in those on the casein diet, but this difference was not significant. Further, the 24 h urine samples contained significantly more radioactivity in the group fed on the starch diet. Thus, the mean total tissue retentions of the radioactivity in the rats given the starch and casein diets did not differ significantly even though there was a marked body-weight loss in the former animals.

Absorption of [^{57}Co]*cyanocobalamin in the ileum.* In in vitro studies the mean intestinal absorptions of [^{57}Co]cyanocobalamin with their standard errors, expressed as percentage of dose, were 7·4 ± 0·9 and 18·6 ± 4·7/g ileum in groups of three rats fed on casein and starch diets respectively.

DISCUSSION

As a combined effect of protein starvation and inanition, the animals given the starch diet developed symptoms similar to those of marasmus rather than kwashiorkor; there was a marked loss in body-weight and the viscera and organs were smaller. Histopathological examination, however, revealed no characteristic difference in the tissues of the animals from the various groups. There was no significant structural abnormality in the liver and ileum.

As in hypoproteinaemic children (Satoskar *et al.* 1962), the serum concentration of vitamin B_{12} was significantly higher in the protein-starved rats than in the animals fed on the casein diet. In hypoproteinaemic Indian children this increase in serum vitamin B_{12} content was correlated with fatty and fibrotic changes in the liver (Satoskar *et al.* 1962). It has been suggested that protein depletion results in a lowering of the vitamin B_{12}-binding capacity of the body tissues, resulting in release of the free vitamin into the serum (Rachmilewitz, Aronovitch & Grossowicz, 1956; Mackay, Cowling & Gray, 1957). However, our results showed considerably higher concentrations of this vitamin in the tissues of protein-starved animals. The reduction in size of the organs of the protein-depleted rats was not associated with any decrease in their total vitamin B_{12} content, which suggests that there was no decrease in the vitamin B_{12}-binding capacity of the tissues. This was confirmed by a comparison of the percentage distribution of both intraperitoneally and orally administered [^{57}Co]cyanocobalamin in the organs of the protein-starved and control animals. Rosenthal (1961) has reported similar accumulations of injected radioactive cyanocobalamin in the tissues of rabbits starved for 10 d compared with control animals.

It is possible that the material responsible for the retention or binding of vitamin B_{12} in the tissues is of a non-protein nature, the relative concentration of which may increase during early protein depletion. In this connexion the observations of Bonomo, Cova, Cremonuni & Prete (1960) and Miller, Hansen & Raney (1963) that a glycoprotein or RNA complex is involved in the binding of this vitamin may be recalled.

The results of preliminary in vitro studies on the relative intestinal absorption of cyanocobalamin in the two groups suggest better absorption of this vitamin by the protein-depleted animals. Although even this observation is difficult to explain in the light of damage known to occur to intestinal epithelium during protein depletion, it again raises the question of whether the transport of cyanocobalamin involves non-protein carriers.

The results of experiments with animals given the gluten diet generally showed that their tissue concentrations were intermediate between those of the animals given the starch diet and those of the animals given the casein diet. Since gluten is nutritionally inferior to casein, these results were probably the result of suboptimal protein nutrition.

A deficiency of vitamin B_{12} in rats was intensified by increasing the protein content of the diet (Sivakumar, Nath & Nath, 1969; Dryden & Hartman, 1971), indicating that vitamin B_{12} is involved in the metabolism of proteins, and the results of the present experiments suggest that the reverse may also be true, namely that a decrease in dietary protein content increases the vitamin B_{12} status of the rat.

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