Changes in the metabolism of vitamin B_{12} and methionine in rats fed unheated soya-bean flour

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1. Young rats were fed on diets containing heated or unheated soya-bean flour.

2. Feeding unheated soya-bean flour decreased concentrations of vitamin B_{12} in liver, kidneys and blood serum, urinary excretion of sulphate and concentration of reduced glutathione in liver. Blood glutathione level and urinary excretion of methylmalonic acid and formimino-glutamic acid following loads of propionate and histidine, respectively, were increased.

3. Supplementing a diet of unheated soya-bean flour with vitamin B_{12} had no effect on the level of reduced glutathione in the liver. Adding methionine to this diet decreased excretion of methylmalonic acid and the level of glutathione in blood.

4. The activity of liver methyl-tetrahydrofolate:L-homocysteine S-methyltransferase was not affected by the nature of dietary soya-bean flour nor by supplementation with vitamin B_{12}.

5. It is concluded that heat-labile substances present in soya-bean flour induce metabolic changes which can bring about an increased requirement for vitamin B_{12} and a deficiency of sulphur-containing amino acids.

Soya beans contain a heat-labile substance or substances exerting adverse effects on young rats and chicks and possibly on other animals (see review by Mickelsen & Yang, 1966). Recently we reported (Edelstein & Guggenheim, 1969) that rats subsisting on a diet containing 18% protein derived from unheated soya-bean flour (SBF) responded to a dose of propionate by excreting increased amounts of methylmalonic acid (MMA) in their urine. Addition of vitamin B_{12} improved weight gain and decreased MMA excretion. When vitamin B_{12} was added to a diet of heated SBF, weight gain was not increased and MMA excretion was only slightly reduced. These results were taken as evidence of an increased requirement for vitamin B_{12} by rats fed unheated SBF. A coenzyme derivative of vitamin B_{12} is involved in the synthesis of methionine in various animal tissues (Weissbach & Dickerman, 1965). Methionine added to a diet of unheated SBF improves growth of young rats and chicks but has no effect, or only a small effect, with diets of heated SBF (Mickelsen & Yang, 1966). Therefore, the effect of unheated SBF on certain metabolic aspects related to vitamin B_{12} and methionine was studied in young rats.

METHODS

Young male rats of a local strain derived from Wistar rats were used. Their basic diet provided 18% protein derived from low-fat SBF, either unheated or commercially heat-processed. The SBF was supplemented with vegetable oil to give 5% lipids in the ration, 4% salts (US Pharmacopeia XIV) and maize starch up to 100%. All diets were enriched with the conventional vitamins, vitamin B_{12} being omitted

(Edelstein & Guggenheim, 1969). In some experiments 15 g DL-methionine were added per kg ration replacing 15 g maize starch. The rats were kept on their diets for 10 weeks, unless otherwise stated.

For load tests, 100 mg sodium propionate/100 g body-weight were administered to each rat. Two portions of 2·0 ml of a 2·5% propionate solution dissolved in 62·5% aqueous sucrose solution were given by stomach tube at a 6 h interval. Urine was collected during a 24 h period and MMA excretion was measured (Giorgio & Plaut, 1965).

For the study of urinary excretion of formimino-glutamic acid (FIGLU), a load of 50 mg histidine/100 g body-weight was administered in two portions, 6 h apart. Histidine was dissolved in a 62·5% sucrose solution. Urine was collected during a 24 h period and FIGLU measured by the alkaline ferricyanide–nitroprusside reaction (Tabor & Wyngarden, 1958).

The activity of liver transmethylase, (methyl-tetrahydrofolate:L-homocysteine S-methyltransferase), an enzyme requiring a cobamide coenzyme, was assayed in rats fed for 13 weeks diets of heated or unheated SBF. The diet of one-half of each group was supplemented with 0·1 mg of vitamin B_{12}/kg ration during the last 8 weeks.

Vitamin B_{12} levels in liver, kidneys and blood serum were measured microbiologically (Aronovitch & Grossowicz, 1958), reduced glutathione (GSH) in liver and blood according to Beutler, Duron & Kelly (1963), and excretion of sulphate with urine according to Berglund & Soerbo (1960). Activity of transmethylase in liver was assayed according to Kutzbach, Galloway & Stokstad (1967).

RESULTS

Table 1 presents concentration of vitamin B_{12} in the liver, kidneys and blood serum of the experimental rats. The concentrations were significantly lower in those fed unheated SBF than in those receiving heated SBF.

Table 1. *Concentrations of vitamin B_{12} in liver, kidneys and blood serum of rats fed for 10 weeks on soya-bean diets*

(Mean values with their standard errors for seven to eleven rats)

Soya-bean flour	Liver (ng/g)	Kidneys (ng/g)	Blood serum (pg/ml)
Heated	24 ± 1·5	105 ± 5·0	366 ± 28·8
Unheated	14 ± 2·1	73 ± 7·3	282 ± 23·8
P	< 0·001	< 0·001	< 0·05

The urinary excretion of FIGLU and MMA following loads of histidine and propionate, respectively, are presented in Tables 2 and 3. Unheated SBF significantly increased the excretion of these metabolites.

It can be seen from Table 4 that transmethylase activity was similar in the four groups examined, regardless of whether the diet contained heated or unheated SBF and whether it was supplemented with vitamin B_{12} or not.

Table 2. *Urinary excretion of formimino-glutamic acid (FIGLU) by rats fed for* 10 *weeks on soya-bean diets*

(Mean values with their standard errors for thirteen to fifteen rats)

Soya-bean-flour	Excretion of FIGLU over a period of 24 h	
	μmoles	μmoles/100 g body-weight
Heated	38 ± 6·8	13 ± 2·4
Unheated	210 ± 24·0	133 ± 19·5
P	< 0·001	< 0·001

Table 3. *Effect of supplementation of soya-bean diets with methionine on weight increase and urinary excretion of methylmalonic acid (MMA) in rats*

(Mean values with their standard errors for five to six rats)

Group no.	Soya-bean flour	Supplement	Weight increase during 7 weeks (g)	Excretion of MMA during 24 h	
				mg	mg/100 g body-weight
1	Heated	—	200 ± 7·6	24·4 ± 5·0	10·3 ± 2·2
2	Heated	Methionine*	192 ± 4·4	24·7 ± 5·0	10·6 ± 2·0
3	Unheated	—	124 ± 4·2	93·6 ± 17·7	59·2 ± 12·3
4	Unheated	Methionine*	144 ± 4·9	44·2 ± 5·2	24·5 ± 2·8
P	3 *v.* 4	—	0·01	0·02	0·02

* 15 g DL-methionine/kg ration.

Table 4. *Transmethylase activity in livers of rats fed on soya-bean diets*

(Mean values with their standard errors for five to six rats)

Soya-bean flour	Vitamin B_{12} added	Transmethylase activity (n-moles methionine synthesized/min mg protein)
Heated	−	0·015 ± 0·0013
Heated	+	0·019 ± 0·0026
Unheated	−	0·012 ± 0·0076
Unheated	+	0·018 ± 0·0023

Table 5. *Urinary excretion of sulphate by rats fed for* 10 *weeks on soya-bean diets*

(Mean values with their standard errors for seven rats)

Soya-bean flour	Excretion of sulphate during 24 h	
	μmoles	μmoles/100 g body-weight
Heated	232 ± 15·4	76 ± 9·7
Unheated	67 ± 15·0	36 ± 8·3
P	< 0·001	< 0·01

Dietary SBF also influenced urinary excretion of sulphate (Table 5) and GSH levels in blood and liver (Table 6). Feeding unheated SBF decreased excretion of sulphate and GSH concentration in liver and increased blood GSH level. Supplementation of the diet with 0·1 mg vitamin B_{12}/kg ration had no effect on the GSH level of blood or liver. However, adding methionine to the unheated SBF diet significantly decreased blood GSH level and increased concentration of this metabolite in liver.

Table 6. *Effect of supplementation of soya-bean diets with methionine or vitamin B_{12} on concentration of reduced glutathione (GSH) in blood and liver of rats*

(Mean values with their standard errors for five to eight rats)

Group no.	Soya-bean flour	Supplement	Reduced glutathione (GSH) mg/100 ml blood	Reduced glutathione (GSH) mg/100 g liver
1	Heated	—	38 ± 1·0	158 ± 9·6
2	Heated	Methionine*	39 ± 1·8	152 ± 8·8
3	Unheated	—	68 ± 3·7	96 ± 8·0
4	Unheated	Methionine*	43 ± 1·7	200 ± 6·2
5	Heated	—	43 ± 2·2	156 ± 6·3
6	Heated	Vitamin B_{12}†	46 ± 1·4	152 ± 5·6
7	Unheated	—	72 ± 5·9	99 ± 5·2
8	Unheated	Vitamin B_{12}†	68 ± 3·0	83 ± 4·5
P	3 *v.* 4	—	< 0·001	< 0·001
	5 *v.* 7	—	< 0·001	< 0·001
	6 *v.* 8	—	< 0·001	< 0·001

* Rats were fed on the soya-bean diet for 13 weeks. During the last 3 weeks 15 g DL-methionine were added per kg ration.

† Rats were kept for 13 weeks on the soya-bean diet. During the last 8 weeks 100 μg vitamin B_{12} were added per kg ration.

DISCUSSION

Our findings on vitamin B_{12} content in liver, kidneys and blood serum confirm previous conclusions, that feeding rats on unheated SBF increases their requirement for vitamin B_{12} and precipitates deficiency of this vitamin if it is lacking in the diet. The cause of this phenomenon is still unknown and is at present under investigation in this laboratory.

Increased excretion of FIGLU following a histidine load has been described in deficiency of folic acid (Stokstad & Koch, 1967) in rats (Silverman & Pitney, 1958; Gardiner & Silverman, 1960) and hamsters (Cohen, Reyes, Typpo & Briggs, 1967), in chickens on vitamin B_{12}-deficient diets (Fox, Ludwig & Baroody, 1961; Fox & Ludwig, 1961) and in some patients with pernicious anaemia (Zalusky & Herbert, 1961). The reason for the increased excretion of this metabolite of histidine is a block in the transfer of the formimino group of FIGLU to tetrahydrofolic acid to synthesize 5-formimino-tetrahydrofolic acid. In vitamin B_{12} deficiency, the conversion of homocysteine into methionine is blocked since the enzyme catalysing this reaction requires a vitamin B_{12} coenzyme (see review of Weissbach & Dickerman, 1965). The methyl donor of this reaction is 5-methyl-tetrahydrofolic acid which is converted into tetrahydrofolic acid. It is believed that not enough tetrahydrofolic acid is generated in vitamin B_{12} deficiency to serve as acceptor of the formimino group (Weissbach &

Dickerman, 1965; Herbert & Zalusky, 1962; Stokstad, Webb & Shah, 1966). Our finding (Table 4) that the activity of the cobamide-containing liver transmethylase was neither decreased in rats fed unheated SBF nor increased by supplementation of the diet with vitamin B_{12} does not necessarily contradict the conclusion that feeding unheated SBF leads to a deficiency of vitamin B_{12}; it can be explained by assuming that the vitamin B_{12} deficiency is not severe enough to affect the activity of this enzyme. Methylmalonyl-CoA mutase, a cobamide-containing enzyme catalysing the isomerization of methylmalonyl CoA to succinyl CoA, which is considerably affected under these conditions (Table 3), appears to be more sensitive to lack of vitamin B_{12}.

If the activity of liver transmethylase remained intact in the rats of our study, another explanation has to be sought for the increased excretion of FIGLU. Baker, Frank, Gellene & Levy (1964) suggested, and Vitale & Hegsted (1967) recently claimed, that formimino-transferase, the enzyme involved in the conversion of FIGLU into glutamate, requires vitamin B_{12}. This would mean that the cobamide-containing transmethylase is not necessarily the limiting factor which prevents increased excretion of FIGLU in vitamin B_{12} deficiency.

The finding of an increased excretion of MMA by rats fed unheated SBF confirms a previous report from the laboratory (Edelstein & Guggenheim, 1969). However, the effect of methionine on MMA excretion is difficult to explain. Since feeding unheated SBF induces a deficiency of sulphur-containing amino acids (see below) it is possible that methionine is needed for synthesis or activity, or both, of methylmalonyl-CoA mutase. Lack of this amino acid would, therefore, limit the activity of this enzyme. An analogous effect of methionine on 5,10-methylenetetrahydrofolate reductase has been postulated by Dickerman & Weissbach (1964). Another possibility is that methionine influences the intestinal flora so that more vitamin B_{12} is made available and absorbed by the intestine.

No apparent explanation of the higher GSH level in blood of rats fed unheated SBF offers itself. However, the lower concentration in liver and its increase by methionine supplementation, as well as the decreased urinary excretion of sulphate can be regarded as a result of depletion of sulphur-containing amino acids. While it is true that both diets contain similar amounts of methionine, the methionine in the unheated SBF diet is less available for synthesis of body protein and, possibly, of sulphur-containing peptides. The growth-promoting effect of methionine in rats and chicks subsisting on diets with unheated SBF has often been reported (see review by Mickelsen & Yang, 1966) and been confirmed by us here (Table 3). The reason is probably an increased excretion of sulphur-containing amino acids by the enlarged pancreas (Goldberg & Guggenheim, 1964; Khayambashi & Lyman, 1966) which are largely lost with the faeces (Evans & McGinnis, 1948). Kakade, Arnold, Liener & Waibel (1969) have recently shown that the cystine present in navy bean trypsin inhibitor is not available, probably owing to an interference in the extent to which it can be digested. Although the trypsin inhibitor represents only 2·5% of navy bean protein it contains 40% of its cystine. Feeding trypsin inhibitor accentuates, therefore, a deficiency of cystine produced by endogenous losses of this amino acid with pancreatic juice.

Reduced glutathione or non-protein sulphhydryl has been reported to be decreased in red blood cells (Ling & Chow, 1953; Register, 1954; Hsu, Chow & Okuda, 1959; Kasbekar, Lavate, Rege & Sreenivasan, 1959) and livers (Ling & Chow, 1953; Register, 1954; Kasbekar *et al.* 1959; O'Dell, Erickson, Newberne & Flynn, 1961) of vitamin B_{12}-deficient rats. O'Dell *et al.* (1961), working with severely deficient animals, concluded that the vitamin is concerned with the maintenance of sulphhydryl compounds in the reduced state. This observation was confirmed in a recent study (Biswas & Johnson, 1964) in which glutathione reductase activity was found to be lowered in vitamin B_{12}-deficient chickens. However, Cohen *et al.* (1967), working with golden hamsters, reported an increased rather than a decreased serum level of GSH in vitamin B_{12}-deficient animals.

It seems, therefore, that heat-labile substances present in SBF lead to significant metabolic changes resulting from an increased requirement for vitamin B_{12} and a lack of sulphur-containing amino acids.

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