Experimental production of vitamin B_{12} deficiency in rats and mice on a maize–groundnut-meal diet

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Difficulties in the production of vitamin B_{12} deficiency in rats and mice are encountered, and can be attributed to the high maternal reserves of the vitamin in animals bred from normal mothers, as well as to intestinal synthesis. Iodinated casein, thyroid extract or thyroxine have been frequently used in purified diets to accelerate depletion of vitamin B_{12} reserves (see, for example, Ershoff, 1947; Bosshardt, Paul, O'Doherty, Huff & Barnes, 1949; Nichol, Dietrich, Cravens & Elvehjem, 1949; Fatterpaker, Marfatia & Sreenivasan, 1955). Alternatively, various types of all-vegetable diets, usually with a large proportion of soya, and sometimes with maize in addition, have been used (Rubin & Bird, 1947; Zucker & Zucker, 1950; Ling & Chow, 1952; Register, 1954; Ericson, Harper, Williams & Elvehjem, 1956). A more severe deficiency can be induced if the animals are bred from mothers previously maintained on such diets. Specially reared animals of that type are used in some laboratories for routine biological assays of vitamin B_{12} (Zucker & Zucker, 1950; Bliss & György, 1951; Jukes & Williams, 1954).

There is a possibility that supplementation with iodinated protein or other thyroid-active material may not always cause uncomplicated vitamin B_{12} deficiency, and the work described here was therefore undertaken to ascertain: (1) the effectiveness of an all-vegetable protein diet of maize-and-groundnut meal in producing vitamin B_{12} deficiency in mice and rats; (2) the nature and severity of the deficiency in comparison with that produced by incorporating iodinated casein in the same diet, and (3) the counteraction by dietary vitamin B_{12} of the deficiencies produced.

EXPERIMENTAL

Animals and procedure

Male adult mice of the Swiss strain, weighing from 30 to 35 g, and young male Wistar rats of from 50 to 60 g weight were used. The basal diet was a modification of the maize–soya diet of Ericson *et al.* (1956) and contained 46·2% maize meal, 46·2% defatted groundnut meal, 2% salts 4 (Hegsted, Mills, Elvehjem & Hart, 1941), 5% arachis oil, 0·3% cystine, and, per kg diet, 5 mg thiamine hydrochloride, 5 mg riboflavin, 10 mg nicotinic acid, 20 mg calcium pantothenate, 2·5 mg pyridoxine hydrochloride, 0·1 mg biotin, 100 mg inositol, 0·2 mg folic acid, 1·5 g choline chloride, 10 mg menaphthone, 10 mg α-tocopherol, 5000 i.u. vitamin A, and 50 i.u. vitamin D. When used, iodinated casein was supplied as 1 g Protomone (Cerophyl Laboratories, Kansas City, Mo., U.S.A.)/kg diet and vitamin B_{12} was added at 20 μg/kg diet. In diets containing iodinated casein the vitamin additions were doubled.

The animals were divided into four similar groups of six to twelve, housed in individual cages, and fed *ad lib.* for periods of from 6 to 8 weeks on one of the diets: (*a*) basal diet, (*b*) basal diet with vitamin B_{12}, (*c*) basal diet with iodinated casein, (*d*) basal diet with vitamin B_{12} and iodinated casein.

Rats and mice were weighed once a week. At the end of the experimental period the animals were killed under ether anaesthesia. Blood was obtained from the hepatic portal vein, and a portion was immediately heparinized and the rest allowed to clot at 37° for 1 h and centrifuged at 2° to obtain the serum. Livers were quickly excised, chilled, and made into 20% homogenates with ice-cold distilled water with a Potter–Elvehjem-type homogenizer. Samples of liver homogenates from three or four mice were pooled to give quantities sufficient for all determinations. Rat-liver homogenates were analysed individually.

Analytical methods

In both species, blood-cell counts were made by routine haematological procedures, and haemoglobin was determined by the acid-haematin method. In the blood serum of mice only, total protein was determined by the biuret method of Gornall, Bardawill & David (1949); fractionation of serum proteins was carried out by electrophoresis on Whatman no. 3 paper strips with barbiturate buffer at pH 8·6 and $I = 0{\cdot}075$; the separated proteins were stained with bromophenol blue and evaluated densitometrically, as described by Mulgaonkar & Sreenivasan (1957).

Portions of liver homogenates from mice and rats were hydrolysed at 37° for 12 h under toluene with 25 mg papain (British Drug Houses Ltd)/g fresh tissue in 0·1 M-acetate buffer at pH 4·6. The samples were then analysed for vitamin B_{12} with *Euglena gracilis* as the test organism by the method of Hoff-Jørgensen (1954). Separate portions were autolysed in 0·1 M-phosphate buffer at pH 7·2 under toluene at 37° for 12 h and assayed with *Streptococcus faecalis* R for folic acid (PGA), and with *Leuconostoc citrovorum* for citrovorum factor (CF) as described by Mitbander & Sreenivasan (1954).

In mouse liver, total nitrogen was determined, after micro-Kjeldahl digestion of the tissue, by direct nesslerization (Umbreit, 1946). Soluble sulphydryl in liver was determined by the method of Grunert & Phillips (1951).

Methionine was estimated in hydrolysed samples of rat liver by the method of Horn, Jones & Blum (1946). Choline was assayed microbiologically by the method of Horowitz & Beadle (1943), after liberation from the tissue by a modification (Rao, Tamhane & Sreenivasan, 1958) of the method of Luecke & Pearson (1944). The conjugation of *p*-aminobenzoic acid (PABA) and glycine in vitro was studied in fresh rat-liver homogenates by a modification (Fatterpaker *et al.* 1955) of the procedures of Borsook & Dubnoff (1947) and Chantrenne (1951).

RESULTS

Growth, blood-cell count and haemoglobin content of blood

The results for rats and mice are given in Table 1. In both species, growth was adversely affected by the absence of vitamin B_{12} from the diet, the effect being more pronounced when the diet contained iodinated casein, and the improvement brought about by the addition of vitamin B_{12} to the diet was less pronounced when iodinated casein was also added.

Table 1. *Growth, blood-cell count and haemoglobin content of mice and rats on a vitamin B_{12}-free diet, alone or with vitamin B_{12} or iodinated casein or both*

(Mean values with their standard errors)

Addition to basal diet	No. of animals	Total weight gain in 6 weeks (g)	Erythrocytes (10^{-6}/mm^3)	Leucocytes (10^{-3}/mm^3)	Haemoglobin (g/100 ml)
		Mice			
None	12	9·0 ± 0·6	10·6 ± 1·1	8·6 ± 0·3	12·4 ± 0·3
Vitamin B_{12} (2 μg/100 g)	10	12·1 ± 0·8	12·5 ± 0·7	10·8 ± 0·2	13·9 ± 0·3
Iodinated casein (0·1 %)	11	5·5 ± 1·0	7·1 ± 0·9	9·5 ± 0·7	11·2 ± 1·1
Vitamin B_{12} and iodinated casein	9	10·0 ± 1·5	9·7 ± 1·4	12·1 ± 0·6	14·0 ± 0·3
		Rats			
None	8	66·0 ± 5·5	5·1 ± 0·4	8·7 ± 0·5	13·0 ± 0·4
Vitamin B_{12} (2 μg/100 g)	6	99·0 ± 8·0	8·3 ± 1·2	9·3 ± 0·2	14·3 ± 0·3
Iodinated casein (0·1 %)	8	54·0 ± 4·5	4·6 ± 0·8	9·1 ± 0·4	11·5 ± 0·6
Vitamin B_{12} and iodinated casein	7	73·5 ± 6·5	6·9 ± 0·9	10·0 ± 0·7	14·8 ± 0·4

The haemoglobin content of the blood and the erythrocyte count were appreciably lower when the diet contained no vitamin B_{12} and a more pronounced lowering occurred when iodinated casein was given.

Serum proteins

The proteins of mouse serum were resolved by electrophoresis into eight identifiable fractions. Occasionally the α_3-globulin band appeared as two separate components but, since such patterns were not always reproducible, the two were combined as α_3-globulin. The results are summarized in Table 2. The serum concentration of total protein was slightly lower, with lower values for albumin and α_3-globulin, in the animals on the basal diet alone than in those on the diet with vitamin B_{12}. The addition of iodinated casein resulted in still lower values for total protein, albumin and α_3-globulin and a lower value for γ-globulin, but in a higher value for α_1-globulin. These effects were produced by iodinated casein even when vitamin B_{12} was also given.

Table 2. *Protein content (g/100 ml) of serum of mice on a vitamin B_{12}-free diet, alone or with vitamin B_{12} or iodinated casein or both*

(Mean values for eight animals in each group with their standard errors)

Addition to basal diet	Total protein	Albumin	α_1-Globulin	α_2-Globulin	α_3-Globulin	α_4-Globulin	β_1-Globulin	β_2-Globulin	γ-Globulin
None	4·61 ± 0·03	1·74 ± 0·07	0·35 ± 0·04	0·26 ± 0·05	0·47 ± 0·04	0·37 ± 0·02	0·23 ± 0·03	0·65 ± 0·03	0·53 ± 0·03
Vitamin B_{12} (2 μg/100 g)	4·75 ± 0·02	1·95 ± 0·05	0·30 ± 0·03	0·20 ± 0·01	0·63 ± 0·06	0·31 ± 0·04	0·19 ± 0·02	0·65 ± 0·05	0·51 ± 0·04
Iodinated casein (0·1 %)	4·18 ± 0·03	1·62 ± 0·07	0·43 ± 0·02	0·24 ± 0·04	0·32 ± 0·05	0·34 ± 0·03	0·24 ± 0·05	0·63 ± 0·05	0·35 ± 0·02
Vitamin B_{12} and iodinated casein	4·22 ± 0·10	1·59 ± 0·07	0·40 ± 0·03	0·21 ± 0·02	0·37 ± 0·05	0·35 ± 0·06	0·17 ± 0·04	0·74 ± 0·04	0·39 ± 0·05

Table 3. *Vitamin B_{12}, pteroylglutamic acid (PGA) and citrovorum factor (CF) in the livers (per g wet weight) of rats and mice on a vitamin B_{12}-free diet, alone or with vitamin B_{12} or iodinated casein or both*

(The number of animals in each group is shown in Table 1)

	Mice*			Rats†		
Addition to basal diet	Vitamin B_{12} (mμg/g)	PGA (mg/g)	CF (mg/g)	Vitamin B_{12} (mμg/g)	PGA (mg/g)	CF (mg/g)
None	54	2·50	2·05	33 ± 4	2·90 ± 0·10	2·48 ± 0·03
Vitamin B_{12} (2 μg/100 g)	216	2·48	2·50	162 ± 8	3·00 ± 0·12	3·10 ± 0·12
Iodinated casein (0·1 %)	31	1·12	1·02	24 ± 4	1·71 ± 0·25	1·35 ± 0·08
Vitamin B_{12} and iodinated casein	189	1·12	0·98	108 ± 6	1·90 ± 0·10	1·42 ± 0·06

* Values are means for three sets of pooled livers from three or four animals each.
† Values are means with their standard errors for all liver samples.

Liver constituents

Table 3 gives the values for vitamin B_{12}, PGA and CF contents of the livers of both species. The livers of the mice and rats fed on the basal diet alone were considerably depleted of vitamin B_{12} and somewhat depleted of CF, but the PGA content was unaffected. The addition of iodinated casein to the diet resulted in a greater depletion of vitamin B_{12} and CF and also in a lower PGA content. The addition of vitamin B_{12} to the diet containing iodinated casein partly restored the vitamin B_{12} content of the liver, but had no significant effect on the content of PGA or CF.

Table 4. *Content of nitrogen and soluble sulphydryl (mg/g wet weight) of livers of mice, and of methionine and choline (mg/g wet weight) of livers of rats, on a vitamin B_{12}-free diet, alone or with vitamin B_{12} or iodinated casein or both*

	Mice*		Rats†	
Addition to basal diet	Nitrogen	Soluble sulphydryl	Choline	Methionine
None	22·5	2·14	4·95 ± 0·08	1·66 ± 0·02
Vitamin B_{12} (2 μg/100 g)	31·7	2·14	5·60 ± 0·12	1·67 ± 0·05
Iodinated casein (0·1 %)	19·5	1·08	4·20 ± 0·11	1·64 ± 0·03
Vitamin B_{12} and iodinated casein	32·5	1·40	5·45 ± 0·03	1·64 ± 0·02

* Values are means for three sets of pooled livers from three or four animals each.
† Mean values with their standard errors for six rats in each series.

Table 5. *Synthesis of* p-*aminobenzoylglycine by rat liver in vitro*

(Mean values for the pooled livers of four rats)

Addition to basal diet	Endogenous (mg/g fresh tissue)	Net (mg/g fresh tissue)	Total (mg/g fresh tissue)
None	0·00	0·06	0·06
Vitamin B_{12} (2 μg/100 g)	0·10	0·23	0·33
Iodinated casein (0·1 %)	0·00	0·06	0·06
Vitamin B_{12} and iodinated casein	0·19	0·14	0·33

Results for liver levels of total nitrogen and total soluble sulphydryl for mice, and of choline and methionine for rats, are shown in Table 4. In mice on the basal diet alone, there was an appreciable reduction of liver nitrogen but the sulphydryl content was unaffected. Iodinated casein caused little further lowering of tissue nitrogen, but the sulphydryl level was adversely affected and was restored only partly when vitamin B_{12} was added. On the other hand, addition of vitamin B_{12} completely restored the concentration of tissue nitrogen in the mice given iodinated casein.

There was a depletion of liver choline in the rats fed on the basal diet alone and it was increased by the inclusion of iodinated casein in the diet. With and without iodinated casein, vitamin B_{12} completely restored the choline content. Liver levels of methionine were about the same in all four groups. Table 5 shows that there was no endogenous, and very little net, synthesis of *p*-aminobenzoylglycine (PABG) by homogenates of livers from rats fed on the basal diet alone. When vitamin B_{12} was added to the diet the net synthesis was greater and there was a significant endogenous synthesis. The

addition of iodinated casein to the basal diet had no effect on the net synthesis, but when it was added together with vitamin B_{12} the net synthesis was lower than when only vitamin B_{12} was added.

DISCUSSION

The decrease in the growth rate of mice when vitamin B_{12} was omitted from the diet was relatively small. In comparison, growth was less in both rats and mice when iodinated casein was given, and the effect was only partly counteracted by giving vitamin B_{12}. The results with rats are in good agreement with those obtained by Ericson *et al.* (1956) with a maize–soya diet. The higher growth rate which they recorded could be due to the difference in the nutritive value of soya-bean and groundnut proteins.

The growth rates were reflected in the liver levels of vitamin B_{12}. The results do not confirm in this respect those of Ericson *et al.*, who reported higher values for liver vitamin B_{12} on diets containing iodinated casein than on those simply deficient in vitamin B_{12}.

Iodinated casein does not interfere with the intestinal absorption of the vitamin by the rat (Kasbekar, Lavate, Rege & Sreenivasan, 1959*a*) as it is reported to do in the chick (Kano, Anderson, Hougham & Charkey, 1954). It is probable, therefore, that it interferes more directly with the retention of the vitamin by the tissues, a possibility which is now being studied.

The blood picture of the animals was again compatible with their vitamin B_{12} status. The relatively minor decrease in leucocyte count when vitamin B_{12} was omitted is in accordance with earlier observations (Mulgaonkar & Sreenivasan, 1958).

The changes observed in the proteins of mouse serum in the simple deficiency agree well with those reported by Mulgaonkar & Sreenivasan (1958), who found that, in rats depleted of vitamin B_{12} by maintenance on a purified diet with 10% casein and containing succinylsulphathiazole, total serum protein, serum albumin and α_1-globulin were reduced. The differences between these two studies, particularly in the α-globulin component affected and the absolute values obtained, are attributable to a species difference as well as to differences in the quality and level of dietary protein and in the degree of deficiency produced.

The serum-protein pattern was modified by the incorporation of iodinated casein in the basal diet, which brought about further reductions in total protein, albumin and α_3-globulin, with a drop in γ-globulin and an increase in α_1-globulin. Addition of vitamin B_{12} failed to reverse these changes. However, in a recent study, Gershoff, Vitale, Antonowicz, Nakamura & Hellerstein (1958) observed that the administration of thyroxine to rats on a vitamin B_{12}-free diet with 20% casein resulted in a lowering of total serum protein and of α_1- and γ-globulins, and an elevation of α_2-globulin; administration of vitamin B_{12} simultaneously with thyroxine partly reversed the effects of thyroxine. Hypoalbuminaemia in our animals, however, is evidence that the deficiency was of a comparatively severe nature. It is conceivable that the efficacy of vitamin B_{12} in reversing the serum-protein changes would be limited by the severity of the deficiency. When the state is severe, as it was in our study, the changes may even

become irreversible. It is probable also that the manner in which an α-globulin fraction would react might be determined by species individuality; furthermore, in our study, the changes in the serum-protein fractions might be related to their known binding of thyroxine (Robbins & Rall, 1955; Robbins, 1956).

It has been reported that hyperthyroid rats have low blood levels of vitamin B_{12}, PGA and CF and that the injection of vitamin B_{12} leads to an increase in the level of CF, as well as of vitamin B_{12} but not of PGA (Pfander, Dietrich, Monson, Harper & Elvehjem, 1952).

In liver homogenates from vitamin B_{12}-deficient hens, the synthesis of CF from added PGA was less than from added vitamin B_{12} (Doctor, Elam, Sparks, Lyman & Couch, 1954). The findings in our work are in general agreement with those reported, but there are two further points of interest that may be noted. First, in the simple deficiency as against that induced by iodinated casein, there was no decrease in the liver PGA, although its conversion into CF seemed to be affected. Secondly, administration of vitamin B_{12} to the animals given iodinated casein, contrary to the observations of Pfander *et al.* (1952), did not lead to any significant improvement in the capacity of the liver to convert PGA into CF. Apparently, iodinated casein caused disturbances beyond those of vitamin B_{12} deficiency, as is evidenced also by the decreased ability of the liver to retain vitamin B_{12} and its failure to carry out its normal synthesis of albumin when vitamin B_{12} was administered.

Determination of soluble sulphydryl in liver was carried out because of the known influence of vitamin B_{12} on sulphydryl metabolism (Ling & Chow, 1953; Register, 1954). Since the vitamin is implicated also in the neogenesis of labile methyl groups of choline and methionine (see Arnstein, 1955) the liver levels of these substances were studied as aids to the assessment of the degree of deficiency. The absence of any significant change in liver sulphydryl in animals fed on the basal diet despite marked reduction in the tissue level of vitamin B_{12} may have been due to the high cystine content of the diet. On the other hand, the failure to observe any significant alteration in liver methionine may have been related to its essential nature preventing an early depletion from the body under stress conditions. Thus, recent work (Kasbekar *et al.* 1959*b*) has demonstrated that administration of carbon tetrachloride to rats, though causing marked depletion of the glycogen, protein and ribonucleic acid of the liver with simultaneous loss of vitamin B_{12}, did not alter its methionine content.

The synthesis in vitro of PABG was studied as typical of energy-requiring systems. The absence of any appreciable endogenous or net synthesis by the livers of the deficient animals was perhaps to be expected since vitamin B_{12} is known to be required for glycine synthesis and in its absence no endogenous glycine would be available for conjugation with PABA. It should be noted that a substantial amount of glycine for conjugation comes from the free, non-protein glycine of the liver (Simkin & White, 1957). The observation of a suppressed synthesis of PABG, when iodinated casein was included in the diet, parallels observations from this laboratory on the behaviour of other conjugating systems in the hyperthyroid rat (Fatterpaker *et al.* 1955).

Although the severity of vitamin B_{12} deficiency produced by the maize–groundnut diet may, to a certain extent, seem to be intensified by incorporation of iodinated

casein in this diet, the intensification is, apparently, accompanied by the simultaneous development of unknown metabolic complications. In addition to the findings in the studies already mentioned, and the present observations, administration of thyroid-active preparations to rats is known to influence profoundly the activity of several enzyme systems (Barker, 1951) and to uncouple oxidative phosphorylation (DuToit, 1952). Their practical use is therefore limited, and would seem to be precluded in studies on the metabolic significance of the vitamin.

SUMMARY

1. Vitamin B_{12} deficiency was produced experimentally in rats and mice by feeding them on a maize–groundnut-meal diet.

2. Comparison of the severity and nature of the deficiency with that caused by addition of iodinated casein to the same diet was made by studying the changes in growth and in certain liver and blood constituents.

3. The extent to which the deficiency produced in the two ways could be counteracted by giving vitamin B_{12} was also studied.

4. It is concluded that vitamin B_{12} deficiency induced by the maize–groundnut diet is substantially free from other metabolic complications and is, therefore, more suitable for studies concerning the metabolic functions of vitamin B_{12} than the one induced by iodinated casein.

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