Effects of vitamin B_{12} deficiency on lipid metabolism of the rat liver and nervous system

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1. Rats bred from vitamin B_{12}-depleted dams were fed on a vitamin B_{12}-deficient diet for 12–15 months and developed a severe vitamin B_{12} deficiency, as judged from methylmalonic acid excretion and tissue vitamin B_{12} levels at slaughter. Control rats were supplemented with vitamin B_{12} in the drinking-water.

2. Neurological signs were recorded after 7 months but the motor nerve conduction velocities remained normal. Neuropathological examination revealed mild changes in the peripheral nerves but no changes in the central nervous system.

3. The amounts of total lipids and phospholipids were normal, but in all examined tissues the proportions of pentadecanoate (C_{15} fatty acid) and heptadecanoate (C_{17} fatty acid) were considerably increased in vitamin B_{12} deficiency.

4. ^3H_2O was incorporated to the same extent into the fatty acids of nervous tissue from vitamin B_{12}-deficient and control rats after 48 h. Less ^3H was found in the liver fatty acids of the vitamin B_{12}-deficient rats.

5. Neurological dysfunction can be demonstrated in the vitamin B_{12}-deficient rat; the relation of the biochemical and neuropathological changes to the neurological signs needs further study.

It has been speculated that symptoms and signs in vitamin B_{12} deficiency could be due to the incorporation into myelin of odd-numbered or branched-chain fatty acids, synthesized from the excess of propionic and methylmalonic acid (MMA) resulting from the enzymic block, induced by the deficiency (Cardinale, Carty & Abeles, 1970; Barley, Sato & Abeles, 1972; Nutrition Reviews, 1974); also that the slower myelin synthesis in vitamin B_{12} deficiency does not keep pace with its physiological degradation (Fehling, Jägerstad & Arvidson, 1977). However, little has actually been proved in this field, partly because of a lack of suitable animal models. Recently, the Egyptian fruit-bat (Green, van Tonder, Dettle, Cole & Metz, 1975) and the rhesus monkey (Agamanolis, Chester, Victor, Kark, Hines & Harris, 1976) were shown to exhibit neurological signs attributable to vitamin B_{12} deficiency, provided they were kept on a vitamin B_{12}-deficient diet for 7 months and 3 years respectively. However, this information is of little help to investigators who lack access to special animal facilities. One of the best-known small animals, the white rat, proved highly resistant to vitamin B_{12} deprivation. In previous investigations, it showed neither haematological (Braunsteiner, Gisinger & Pakesch, 1953) nor neurological symptoms (Turner & Cevallos, 1968; Petersen & Vahouny, 1975; Fehling & Jägerstad, 1977).

We report here the occurrence in the rat of discrete neurological symptoms in long-term, severe vitamin B_{12} deficiency under controlled conditions. Chemical analyses and isotope experiments indicated changes in the lipid metabolism of both liver and nervous tissue, and structural aberrations were found in the peripheral nerves.

Table 1. *Concentrations (mg/kg) of essential nutrients in the vitamin B_{12}-deficient diet fed to the rats after 5 months*

Retinyl acetate	400
Ergocalciferol	23·8
α-Tocopherol	1330
Thiamine HCl	9·5
Riboflavin	11·4
Nicotinic acid	9·5
Calcium pantothenate	11·4
Pyridoxine HCl	4·7
Pteroylmonoglutamic acid	0·95
Biotin	0·19
p-aminobenzoic acid	9·5
myo-inositol	189
Choline chloride	760
L-methionine	11400*
Calcium	7900*
Potassium	2700*
Sodium	15600*
Chlorine	4700
Phosphorus	7100*
Magnesium	700*
Iron	335*
Copper	19
Manganese	74
Iodine	0·39
Zinc	32†

* This value includes the amount present in Promine D, according to the specifications of the manufacturer (Central Soya, Chicago, Ill. 60639, USA).

† This concentration was obtained by atomic absorption spectrophotometry.

EXPERIMENTAL

Animals

Adult female Wistar rats (Möllegard-Hansens Avlslaboratorier, Skensved, Denmark) fed on a vitamin B_{12}-deficient diet from weaning were divided in two groups, one of which was supplemented with cyanocobalamin (40 μg/l) in the drinking-water from the time of mating. The offspring received the same regimen as their respective dams. All animals had free access to food and water and were housed under identical conditions at 25° in stainless-steel cages with elevated wire-mesh floors, minimizing coprophagy. Ten or eleven rats were kept in one cage (560 × 310 × 200 mm). Weights were recorded monthly, and the vitamin B_{12} concentration in plasma was determined bi-monthly. Ten male rats and eleven female rats, killed after 12 and 15 months respectively, were used for the lipid analyses.

Diets

During the first 5 months, a casein-based diet was used (Altromin, Lage, West Germany), described in detail elsewhere (Fehling & Jägerstad, 1977). Because we considered its content of vitamin B_{12} was not low enough it was changed to a diet, modified after Williams, Spray, Newman & O'Brien (1969), containing less than 2 μg vitamin B_{12}/kg and composed as follows (g/kg diet): sucrose 195, lactose 105, glucose 30, soya-bean protein (Promine D; Central Soya, Chicago, Ill. 60639. USA) 571, soya-bean oil 48, vitamin and salt mixtures 55. Table 1 lists the calculated contents of the various vitamins, minerals, and other essential nutrients.

Neurological examinations

Functional tests included the animals' ability to stay on a tilted steel platform and their ability to strike the ground with their feet first after being somersaulted, as described in detail elsewhere (Fehling, Abdulla, Brun, Dictor, Schütz & Skerfving, 1975). Motor nerve conduction velocity was measured in the tail on three occasions according to Miyoshi & Goto (1973) with a few modifications (Fehling *et al.* 1975).

Preparation of tissues and analysis of vitamin B_{12}

This was done as previously described (Fehling & Jägerstad, 1977). Adipose tissue was sampled from the epididymal fat pad. Liquid nitrogen was used for immediate freezing of the solid tissues. *Euglena gracilis* was used as test organism in the assay of vitamin B_{12} according to Killander (1957) with a few modifications.

MMA determination

Urine content (24 h sample) of MMA was determined in six vitamin B_{12}-deficient male rats and three controls after 12 months. The rats were housed individually for 24 h in metabolic cages with free access to food and water. Urine was collected quantitatively and acidified. It was purified by chromatography on Dowex 50W-X4 (Bio-Rad Laboratories, Richmond, Calif. 94804, USA) and DEAE-Sephadex A 25 (Pharmacia, Uppsala, Sweden). The fractions containing MMA were extracted with diethyl ether and esterified with propanol in acetyl chloride before quantitative analysis with gas–liquid chromatography (SP 900; Perkin-Elmer, Norwalk, Connecticut, USA) (S. Colleen & E. Brante, unpublished results).

Isotope experiment

^3H_2O (10 mCi) (The Radiochemical Centre, Amersham, Bucks., UK) was administered in 1 ml 0·15 M-sodium chloride intraperitoneally to six vitamin B_{12}-deficient and five supplemented female rats 48 h before slaughter. This interval was chosen because we wished, primarily, to examine the incorporation of activity into brain lipids.

Lipid extraction and analysis

Weighed samples of the frozen tissues were homogenized for 1 min in at least 6 vol. chloroform–methanol (1:1, v/v). Plasma was extracted in the same way. The mixture was left for 1 h at room temperature and the tissue residue was removed by centrifugation or filtration followed by rinsing with chloroform–methanol (1:1, v/v). The residue from nervous tissues was extracted once more with chloroform–methanol–water (45:45:10, by vol.). To the combined extracts NaCl (10 g/l) in water was added to obtain a value for methanol-water of 1:1 (v/v). After phase separation the chloroform phase was evaporated and the lipids were determined gravimetrically. They were dissolved in chloroform–methanol (2:1, v/v) and stored at −20°.

Phosphorus was determined according to Chen, Toribara & Warner (1956). Lipids were separated by thin-layer chromatography on silica gel H (E. Merck GmbH, Darmstadt, West Germany) with light petroleum (b.p. 40°–60°)–diethyl ether–acetic acid (70:30:1, by vol.) or chloroform–methanol–acetic acid–water (65:25:4:4, by vol.) as developing solvents. The zones from the plate were either eluted with chloroform–methanol–acetic acid–water (50:39:1:10, by vol.) (Åkesson, Elovson & Arvidson, 1970) or directly scraped into liquid-scintillation vials for radioactivity determination (Sundler, Åkesson & Nilsson, 1974).

For determination of the incorporation of ^3H_2O into fatty acid and cholesterol, the total lipid extract was hydrolysed with alkaline ethanol (Kates, 1972). After acidification, lipids were extracted several times with light petroleum (b.p. 40°–60°) and then separated by thin-layer chromatography as described previously.

Fatty acid composition of individual lipids or total lipids was determined after transesterification in 0·38 M-sulphuric acid in methanol–toluene (1:1, v/v), as described previously (Åkesson et al. 1970). Fatty acid methyl esters were separated by gas–liquid chromatography (F and M instrument model 402; Hewlett-Packard, Avondale, USA) (Åkesson et al. 1970). As stationary phases ethyleneglycol succinate polyester, silicone oil W-98 (a methyl-vinyl silicone; Hewlett-Packard), or EGSS-Y (ethyleneglycol succinate methyl silicone copolymer; Applied Science, State College, USA) were used. In several experiments, the identity of the fatty acid methyl esters was established by comparison with the retention times of standard compounds (Nu Chek Prep, Elysian, USA) using several stationary phases. The fatty acids are designated in the following way: number of carbon atoms: number of double bonds; for $(n-x)$, n is the total number of C atoms and x is the number of C atoms between the methyl group and the closest double bond (Journal of Biological Chemistry, 1967).

Neuropathological examination

In eight controls and nineteen vitamin B_{12}-deficient animals, the nervous system was studied with regard to pathological alterations according to the technique used previously (Fehling et al. 1975). The study comprised a blind investigation of teased fibres from whole fascicles of sciatic and sural nerves as well as 1 μm epon-embedded and osmium-stained transverse sections, multiple paraffin sections from the above-mentioned peripheral nerves, brain and spinal cord, stained for myelin (Luxol fast blue), axons (Naoumenko), cellular details (haematoxylin and eosine and Cresyl violet), connective tissue (van Gieson) and in a number of animals also frozen sections stained for fat (Scarlet red).

Statistical methods

Student's t test was used for the calculation of the significances of differences in weights and in motor nerve conduction velocities. The results of the neurological tests were compared by the Fisher exact probability test and the Mann–Whitney U test (Siegel, 1956). The latter was also used one-tailed for the biochemical measurements because of the small numbers of rats involved.

RESULTS

Weights

The control rats showed a normal growth curve, indicating that both diets were adequate for growth when supplemented with vitamin B_{12}, as previously reported (Williams et al. 1969; Fehling & Jägerstad, 1977). Fig. 1 demonstrates the growth curves of the male rats. The vitamin B_{12}-deficient animals weighed less than the controls on all occasions, but they gained weight at the same rate as the controls for 5 months; they then began to lose weight. The female rats followed a similar pattern.

Vitamin B_{12} status of the animals

The vitamin B_{12}-deprived rats had low plasma vitamin B_{12} levels already at 6 weeks. The values of the females were somewhat lower than those of the males until 6 months; thereafter they were similar. Fig. 2 shows extremely low plasma levels during most of the animals' lifetime. No control plasma values below 500 pg/ml were recorded.
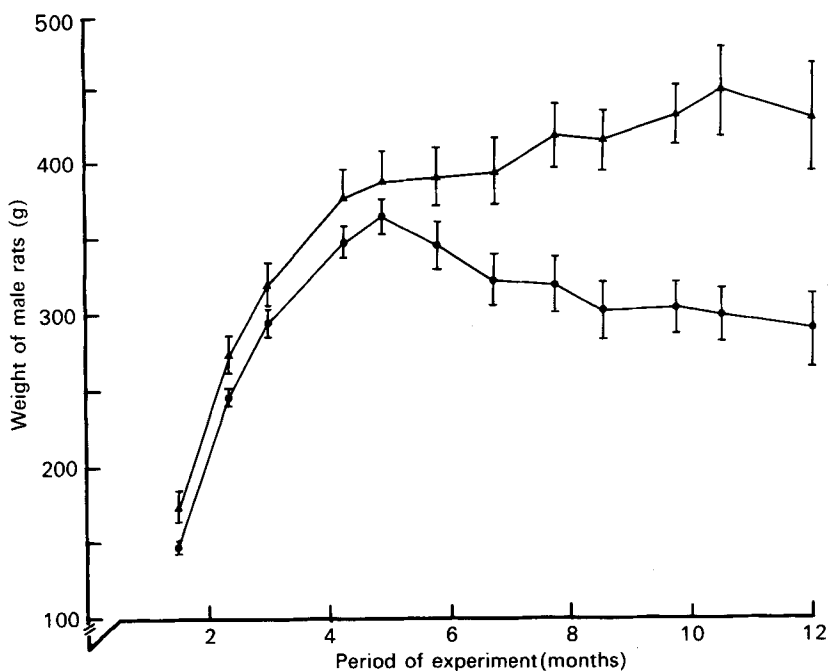
Fig. 1. Growth curves of vitamin B$_{12}$-deficient (●) male rats and controls (▲). Vertical bars represent 2 SE. For details of animals and diets, see p. 502.
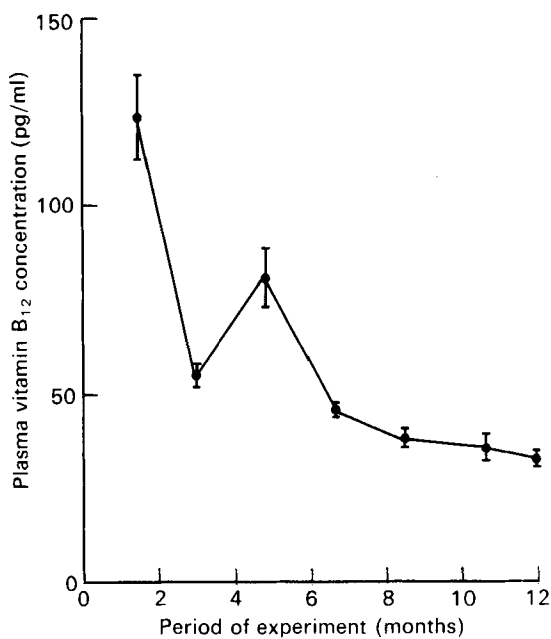

Fig. 2. Plasma concentration of vitamin B$_{12}$ (pg/ml) in male rats fed on a vitamin B$_{12}$-deficient diet. Vertical bars represent 2 SE. For details of animals and diets, see p. 502.

Table 2. *Tissue vitamin B_{12} concentrations and 24 h urinary excretion of methylmalonic acid (MMA) of individual male and female rats deprived of or supplemented with vitamin B_{12} for 12 and 15 months respectively**

Tissue	Vitamin B_{12}-deprived						Vitamin B_{12}-supplemented					
Male												
Plasma (pg/ml)	33	35	42	29	28	31	755	980	725	—	—	—
Cerebrospinal fluid (pg/ml)	—	—	<3†	—	—	—	—	174†	—	—	—	—
Liver (ng/g wet wt)	1	2	5	2	2	1	72	79	91	—	—	—
Spinal cord (ng/g wet wt)	2	1	3	2	1	1	23	22	31	—	—	—
Brain (ng/g wet wt)	3	1	7	3	1	1	45	42	56	—	—	—
Urine (mg MMA/24 h)	10·8	79·3	15·3	112·7	144·5	43·1	0·12	0·13	0·11	—	—	—
Female												
Plasma (pg/ml)	63	49	101	46	63	63	725	580	725	525	580	
Liver (ng/g wet wt)	12	15	13	8	9	8	51	70	71	75	70	
Spinal cord (ng/g wet wt)	3	5	4	4	4	4	22	18	22	22	32	
Brain (ng/g wet wt)	5	7	4	5	3	3	30	32	38	36	45	

* For details of diet, see p. 502.
† Pooled samples.

Table 3. *Total lipid (mg/g wet tissue) and phospholipid ($\mu mol/g$ wet tissue) concentrations in livers from female rats deprived of or supplemented with vitamin B_{12} for 15 months**

(Mean values with their standard errors; no. of animals in parentheses)

	Vitamin B_{12}-deprived (6)		Vitamin B_{12}-supplemented (5)	
	Mean	SE	Mean	SE
Total lipid	49·4	1·3	51·6	1·3
Phospholipid	40·4	1·2	38·6	1·1

* For details of diet, see p. 502.

Table 2 shows unequivocal evidence of severe tissue depletion of vitamin B_{12} in both male and female rats. The tissue levels were reduced to 5–20 % of the control values. There was a more than 100-fold increase of methylmalonate excretion.

Neurological examinations

The tilting-plane test showed significant differences between the groups of rats when first performed after 7·5 months ($P < 0.01$) and thereafter. The largest difference ($P < 0.0003$) was recorded at 9·5 months. The somersault test was significant for the female rats after 9·5 months only, but the control rats showed a clear trend to perform better on all occasions.

The motor nerve conduction velocities were the same in the vitamin B_{12}-deprived group and the controls on all occasions. They increased somewhat with age. At 12 months, the mean ($\pm$ SE) velocities (m/s) for the vitamin B_{12}-deficient males were 43.4 ± 0.92 ($n11$) and those for the controls, 43.0 ± 0.56 ($n4$). The corresponding values for the females were 46.2 ± 1.10 ($n9$) and 46.9 ± 1.13 ($n10$).

Lipid composition

Liver. There was no quantitative difference in total lipid or phospholipid between livers from vitamin B_{12}-deficient rats and controls (Table 3). Analysis of the relative amounts of individual fatty acids in triacylglycerol and phospholipid revealed at least threefold increases in the odd-numbered fatty acids, pentadecanoate and heptadecanoate (Table 4).

Nervous tissue. Table 5 shows that there was no difference in total lipid concentration of the brain or spinal cord between the vitamin B_{12}-deficient and the control animals, nor was there any difference in the brain phospholipid level. The fatty acids of the brain showed the same increase in heptadecanoate in vitamin B_{12} deficiency as was found in the liver (Table 6). The proportions of other fatty acids were similar in the two groups of rats, both in liver and in brain. Pentadecanoate was not analysed in this experiment.

Analysis of brain samples from the male rats (Table 7) demonstrated that also pentadecanoate was clearly increased in vitamin B_{12} deficiency. Docosapentaenoate ($22:5$ ($n-6$)) was decreased in the brain of the vitamin B_{12}-deficient females (Table 6) but no clear relations were observed in nervous tissues from males (Table 7).

Other tissues. In all analysed tissues, the proportions of pentadecanoate and heptadecanoate were increased in vitamin B_{12} deficiency. The increase was most pronounced for brain and liver, and less for spinal cord and adipose tissue. The proportions of unsaturated fatty acids tended to be larger in vitamin B_{12} deficiency, especially in plasma lipids.

Table 4. *Fatty acid composition (% total fatty acid weight) in liver lipids from individual male rats deprived of or supplemented with vitamin B_{12} for 12 months**

Fatty acid	Vitamin B_{12}-deprived			Vitamin B_{12}-supplemented	
	Triacylglycerols				
14:0	0·5	0·5	0·4	0·5	0·4
15:0	1·1	0·7	0·9	0·3	0·3
16:0	26·1	27·2	25·9	29·9	28·2
16:1	1·7	1·4	1·4	2·0	1·8
17:0	0·7	0·6	0·8	0·2	0·1
18:0	3·8	3·8	4·9	2·9	2·5
18:1 + 18:2	57·1	57·6	58·4	58·6	60·8
20:4	8·8	8·1	6·9	4·2	5·8
Others	0·2	0·1	0·4	1·4	0·1
	Phospholipids				
14:0	—	0·1	0·1	0·1	0·1
15:0	0·5	0·4	0·5	0·1	0·1
16:0	15·4	15·8	17·1	17·0	18·8
16:1	0·4	0·4	0·4	0·7	0·5
17:0	1·2	1·2	1·3	0·3	0·4
18:0	25·2	23·6	23·1	24·8	24·1
18:1 + 18:2	21·5	21·2	18·8	22·1	20·8
20:4	30·1	31·4	30·4	30·6	30·9
Others	5·3	5·9	8·3	4·3	4·3

* For details of diet, see p. 502.

Table 5. *Total lipid (mg/g wet tissue) and phospholipid (μmol/g wet tissue) concentrations in nervous tissue from rats deprived of or supplemented with vitamin B_{12}**

(Mean values with their standard errors; no. of animals in parentheses)

	Vitamin B_{12}-deprived		Vitamin B_{12}-supplemented	
	Mean	SE	Mean	SE
Brain lipids†	126·3	8·0 (6)	119·6	1·2 (4)
Brain phospholipids†	62·4	0·7 (5)	62·7	1·2 (4)
Spinal cord lipids‡	224·4	12·2 (4)	220·8	4·3 (3)

* For details of diet, see p. 502.
† These values are from female rats, killed after 15 months.
‡ These values are from male rats, killed after 12 months.

Lipid biosynthesis

^3H_2O was used to estimate the synthesis of fatty acid and cholesterol in brain and liver. Substantial amounts of radioactivity were recovered from the brain, but no differences in total lipids, fatty acids or cholesterol could be found between vitamin B_{12}-deficient and control rats. In the liver, however, significantly less ^3H was found in the fatty acids of the vitamin B_{12}-deficient animals than in the controls. The opposite was true for the cholesterol fraction (Table 8). The distribution (%) of ^3H among different liver lipids in the vitamin B_{12}-deficient rats was (values from control rats in parentheses): neutral lipids 23·9 (24.3), ethanolamine phosphoglyceride 20·8 (18·5), choline phosphoglyceride 52·6 (48·2), and sphingomyelin plus lysophosphatidylcholine 2·7 (8·9); all results are mean values from three rats.

Table 6. *Fatty acid composition (% total fatty acid weight) in total brain lipids from female rats, deprived of or supplemented with vitamin B$_{12}$ for 15 months**

(Mean values with their standard errors; no. of animals in parentheses)

Fatty acid	Vitamin B$_{12}$-deprived (5) Mean	SE	Vitamin B$_{12}$-supplemented (4) Mean	SE
16:0	20·7	0·3	21·2	0·3
16:1	3·4	0·2	2·6	0·5
17:0	1·1	0·2	0·1	0·0
18:0	18·6	0·2	19·2	0·4
18:1	27·3	0·3	26·7	0·4
18:2	0·7	0·0	0·5	0·1
20:0	0·3	0·1	0·3	0·1
20:1	3·2	0·3	3·8	0·1
20:4 (n−6)	8·7	0·3	8·8	0·2
22:4 (n−6)	2·2	0·1	2·6	0·1
22:5 (n−6)	0·6	0·1	1·0	0·1
22:5 (n−3)	1·4	0·1	1·3	0·2
22:6 (n−3)	11·8	0·5	12·0	0·5
Unsaturated	59·3	0·4	59·2	0·4

* For details of diet, see p. 502.

Table 7. *Fatty acid composition (% total fatty acid weight) in different tissues from male rats deprived of or supplemented with vitamin B$_{12}$ for 12 months**

(Values represent pooled samples from male rats; no. of animals in parentheses)

Fatty acid	Vitamin B$_{12}$-deprived (6) Liver	Adipose tissue	Plasma	Spinal cord	Brain	Vitamin B$_{12}$-supplemented (3) Liver	Adipose tissue	Plasma	Spinal cord	Brain
14:0	0·1	0·6	0·1	0·1	0·1	0·2	0·8	0·1	0·1	0·2
15:0	0·6	0·7	0·5	0·3	0·5	0·1	0·2	0·1	0·2	0·0
16:0	25·0	18·3	17·6	11·9	18·6	30·1	23·4	20·9	11·9	20·7
16:1	1·3	3·4	0·4	0·2	0·6	1·0	5·3	1·2	—	0·9
17:0	1·8	0·6	1·0	0·5	1·5	0·4	0·2	0·2	0·1	0·1
18:0	19·5	2·6	14·8	16·7	20·0	17·4	2·2	12·6	16·9	20·6
18:1	8·0	40·3	7·6	36·7	24·5	8·2	36·3	10·7	36·1	24·6
18:2	15·1	33·0	16·3	0·7	0·7	18·4	31·3	23·8	0·5	0·5
20:1	—	—	—	18·2	2·3	—	—	—	16·7	2·5
20:3	—	—	—	0·7	0·2	—	—	—	0·5	—
20:4	22·6	0·5	38·6	4·2	9·9	21·7	0·3	28·5	4·2	12·6
22:3	—	—	—	0·6	0·4	—	—	—	0·6	0·2
22:4	—	—	—	2·0	2·9	—	—	—	1·9	2·9
22:5	—	—	—	4·5	4·6	—	—	—	8·0	2·7
22:6	6·1	—	3·1	3·1	13·1	2·5	—	1·8	2·4	11·5
Unsaturated	53·1	77·2	66·0	70·9	61·2	51·6	73·2	56·0	70·9	56·4

* For details of diet, see p. 502.

Neuropathology

Altogether 2565 teased fibres were studied, averaging ninety-five fibres per animal. The observed changes were classified according to Dyck (1975). Of these only stadiums D, E and F occurred with a frequency that allowed an assessment of the condition of the nerve fibre. The changes consisted of myelin ovoids or digestion chambers, para- or internodal de-myelination and pronounced variation (> 50 %) of the thickness of the internodal myelin

Table 8. *Distribution of ^3H (counts/min ($\times 10^{-3}$)/g tissue wet weight) in total lipids, fatty acids and cholesterol in liver and brain of female rats deprived of or supplemented with vitamin B_{12} for 15 months* and given 10 mCi ^3H_2O 48 h before death*

(Mean values with their standard errors; no. of animals in parentheses)

	Vitamin B_{12}-deprived (6)			Vitamin B_{12}-supplemented (5)	
	Mean	SE	P†	Mean	SE
Liver					
Total lipids	54·1	3·2	—	58·1	6·0
Fatty acids	19·5	2·8	0·041	28·1	4·0
Cholesterol	7·3	0·3	0·015	5·6	0·5
Brain					
Total lipids	25·6	0·6	—	23·0	1·3
Fatty acids	14·2	0·8	—	13·0	1·0
Cholesterol	1·4	0·1	—	1·6	0·2

* For details of diet, see p. 502.
† Statistical significance of differences between the vitamin B_{12}-deprived and supplemented animals (Mann–Whitney U test).

sheath. These changes usually occurred in only a few teased fibres from each specimen. The blind study showed the previously-mentioned detailed changes in 62 % of the controls (five of eight animals) and 79 % of the vitamin B_{12}-deficient animals (fifteen of nineteen animals).

Light microscopy of paraffin sections revealed 'foamy' or 'blown-up' areas of the myelin sheaths which were more pronounced in the vitamin B_{12}-deficient animals than among controls and only the former showed digestion chambers. Fifteen of the nineteen vitamin B_{12}-deficient animals also revealed an increase in collagen fibrils in the endoneurium not seen in the control group. There was no concomitant axon alteration or cellular infiltration in the nerves.

The study of the spinal cord and brain revealed no differences between the two animal groups. There were no clear-cut pictures of a subacute combined degeneration.

DISCUSSION

Vitamin B_{12} status of the animals. Plasma vitamin B_{12} concentrations were low during the entire lifetime of the animals. Because depots of vitamin B_{12} are known to be small in pups from vitamin B_{12}-deficient dams (Williams *et al.* 1969), it is reasonable to suppose that tissue levels too were low after the first few months. The much increased urinary excretion of MMA indicates metabolic impairment from the low tissue vitamin levels (Cox & White, 1962; Barness, Young & Nocho, 1963). Tissue vitamin B_{12} concentrations (Table 2) were extremely low, even compared with our own previous results (Fehling & Jägerstad, 1977). Others have reported values of liver vitamin B_{12} concentration ranging from 10 µg/g (Turner & Cevallos, 1968) to 20 µg/g (Frenkel, Kitchens, Hersh & Frenkel, 1974) in vitamin B_{12}-deficient rats. The levels in cerebrospinal fluid were lower than those in plasma, which agrees with findings in man (Worm-Petersen, 1962).

Neurological examinations. Although clearly significant differences between the groups of rats were recorded specifically in the tilting-plane test, it should be emphasized that the functional disturbances were subtle. No animal displayed gross ataxia or paraparesis. It was thus impossible, from inspection of the locomotor pattern only, to pick out one vitamin B_{12}-deficient animal from a group of controls; it was necessary to perform the

neurological tests. Even so, there was considerable overlapping in performance. Because the soya-bean-based diet contained less vitamin B_{12} than the diet used during the first 5 months, it is possible that more dramatic symptoms would be produced if the soya-bean diet were used throughout.

The present tests could not indicate which part of the nervous system had been damaged. The electrophysiological studies showed that the peripheral motor nerves were not involved. The failure to observe neurological symptoms in earlier experiments could be due to the shorter period of observation (Fehling & Jägerstad, 1977). Because neurological testing was not done by others, subtle neurological signs could well have been overlooked in previous experiments with long periods of observation (Turner & Cevallos, 1968; Petersen & Vahouny, 1975). Moreover, the effects of dietary components other than vitamin B_{12} on the induction of neurological signs are not known. The amount of propionate precursors, such as valine, threonine and isoleucine, or the content of substances involved in C_1 metabolism could be important.

Lipid metabolism. Vitamin B_{12} acts in its deoxyadenosyl-cobalamin form as a coenzyme in the conversion of MMA-CoA to succinyl-CoA (Gurnani, Mistry & Johnson, 1960). In vitamin B_{12} deficiency and in other conditions where this reaction is impaired, MMA-CoA concentrations are increased in the tissues (Frenkel *et al.* 1974). Because the carboxylation of propionyl-CoA to MMA-CoA is a reversible reaction (Tietz & Ochoa, 1959), propionate too will accumulate in the tissues (Ando, Rasmussen, Nyhan, Donnell & Barnes, 1971). Fatty acids are normally synthesized from actyl-CoA and malonyl-CoA, condensing on fatty acid synthetase to yield even-numbered C chains. If MMA-CoA is substituted for malonyl-CoA and propionyl-CoA for acetyl-CoA (as could well occur if the former compounds are present in excessive amounts) the result will be the formation of branched-chain and odd-numbered fatty acids (Kishimoto, Williams, Moser, Hignite & Biemann, 1973). In propionic acidaemia (Hommes, Kuipers, Elema, Jansen & Jonxis, 1968) pentadecanoate and heptadecanoate accumulate in the liver. Propionic acid injected in normal rats becomes incorporated into odd-numbered fatty acids in nervous tissue (Hajra & Radin, 1962). In methylmalonic aciduria, branched-chain fatty acids too have been demonstrated in the nervous system (Kishimoto *et al.* 1973). In the rare disease of fatty acid metabolism, heredopathia atactica polyneuritiformis (Refsum, 1946), tetramethylhexadecanoic acid accumulates in amounts of approximately 10 % of total fatty acids in the nervous system. This probably gives rise to the neurological symptoms (Eldjarn, Stokke & Try, 1976).

The evidence described here constituted the rationale for the present experiment. We found increased amounts of odd-numbered fatty acids in all examined tissues. Odd-numbered fatty acids are probably formed in all tissues, although transport of fatty acid between tissues also could contribute. In view of the general nature of the changes, it is reasonable to suppose that they were also present in myelin, although this fraction was not isolated. The most conspicuous differences were found in the brain, as was also observed in methylmalonic aciduria (Kishimoto *et al.* 1973). The differences were more pronounced than those recently observed in vitamin B_{12}-deficient baboons (Garton, Scaife, Smith & Siddons, 1975). A previous experiment, where the deficiency was less pronounced and protracted and neurological signs were lacking, found no changes in the fatty acid patterns despite changes in the amount of total liver lipids (Fehling *et al.* 1977).

The unchanged proportion of unsaturated fatty acids in the brain tissue in our animals (Table 6) is at variance with the findings of Dayan & Ramsay (1974) in one child with intracellular deficiency of different cobalamin coenzymes. On the contrary, the proportion of unsaturated fatty acids tended to increase in the vitamin B_{12}-deficient rats, at least in extraneural tissue (Table 7). The reason for this was not apparent. Docosapentaenoate ($22:5$ ($n-6$)) was observed in significant amounts in nervous tissue only; although differ-

ences were found, these were not consistent. Thus we can neither confirm nor deny the findings of Dayan & Ramsay (1974), which indicated high concentrations of dosapenta-enoate in white matter ethanolamine phospholipid in vitamin B_{12} dysfunction.

Because fatty acid synthesis in vitro is inhibited by MMA-CoA (Frenkel, Kitchens & Johnston, 1973), we interpreted our previous finding of decreased amounts of odd-chain fatty acids in livers of vitamin B_{12}-deficient rats after propionate load as indicative of slowing of fatty acid synthesis (Fehling et al. 1977). Our study should be compared with previous studies indicating that enzymes involved in liver fatty acid synthesis increase in amount during vitamin B_{12} deprivation (Frenkel et al. 1973). Although the rate of fatty acid synthesis normally reflects the amounts of the relevant enzymes (Volpe & Vagelos, 1973), this might not apply to vitamin B_{12} deficiency, where inhibitors such as MMA-CoA are perhaps important. The preliminary results on fatty acid synthesis from ^3H_2O in human nervous tissue (Frenkel, 1973) indicated that this pathway was depressed in pernicious anaemia. ^3H_2O has been shown to be a good marker of fatty acid synthesis (Jungas, 1968; Nilsson, Sundler & Åkesson, 1973). The lower activity of ^3H in liver fatty acids in vitamin B_{12} deficiency (Table 8) is therefore probably an expression of a slower fatty acid turnover in this condition. The higher incorporation of ^3H_2O into liver cholesterol agrees with the increased incorporation of ^{14}C-acetate found by Armstead, Hsu & Chow (1971), who also found unchanged cholesterol synthesis in brain in vitamin B_{12} deficiency (Table 8).

The neuropathological study showed alterations which differed in intensity and frequency between animal groups only within the peripheral nerves. Of the methods used the teasing technique appears to be the most sensitive but it is supplemented by the paraffin-section technique. On the basis of the combined findings vitamin B_{12} deficiency in the experimental model used here appears to result in a mild neuropathy expressed as limited myelin break-down and fibrosis. However, these changes were also seen in the control rats, although to a smaller extent. This necessitates caution in the interpretation of the results and points to the importance of an adequate control material in studies within this field.

In conclusion, neurological symptoms can thus be demonstrated after 7 months in vitamin B_{12}-deprived Wistar rats on a soya-bean-based diet. Although no neuropathological changes were seen, reminiscent of the subacute combined degeneration in humans, this model seems to be a promising tool in future investigations of the pathophysiology of the neurological manifestations of vitamin B_{12} deficiency. The role of changes in lipid composition and turnover will need further study in this context.

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