The Effect of Vitamin B₁₂ on the Metabolism of Formate and Certain Formate Precursors by the Rat

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(Received 1 September 1959)

The ability of vitamin B₁₂ (cobalamin) to spare the dietary requirement of animals for labile methyl donors, such as choline or methionine, is now well established (for a review see Arnstein, 1958*a*). This effect appears to be due to stimulation of synthesis of methyl groups, as shown by an increased utilization of a variety of ¹⁴C-labelled precursors, including formate (Arnstein & Neuberger, 1953), methanol (Verly & Cathey, 1955), the α -carbon atom of glycine (Stekol, Weiss & Weiss, 1952; Arnstein & Neuberger, 1953) and the β -carbon atom of serine (Arnstein & Neuberger, 1953). It has also been known for some time that certain micro-organisms, notably *Escherichia coli*, require vitamin B₁₂ for the biosynthesis of the methyl group of methionine (Davis & Mingioli, 1950), and considerable progress has been made towards elucidating the biochemical function of the vitamin in this reaction (Guest, 1959; Woods, 1958). There is some doubt, however, to what extent this function of vitamin B₁₂ in the biosynthesis of labile methyl groups can account for its physiological importance, particularly in animal metabolism where methionine is an essential amino acid which can only reduce the requirement for vitamin B₁₂ but not replace it completely.

In order to investigate this problem and the specificity of the action of vitamin B₁₂ on biosynthesis of methyl groups, a comparison has now been made of the effect of vitamin B₁₂ on the conversion of formate and two formate precursors, serine and histidine, into methyl groups and on the metabolism of formate and histidine into other products such as serine. Some of the results of this work have been published in a preliminary note (Arnstein, 1958*b*).

EXPERIMENTAL

Animals and diets. Albino rats of Institute stock were used, most experiments being done with litter mates (see Table 1). All animals were bred from mothers fed on a vitamin B₁₂-low diet (diet A) during pregnancy and until weaning. Deficient animals were then fed on the diets stated in Table 1, without vitamin B₁₂, and control animals were given the same diet with vitamin B₁₂ at various times after weaning as described later.

Diet A had the composition (g./100 g. of diet): maize starch, 39; sucrose, 36; ethanol-extracted casein, 12; arachis oil, 6.4; cod-liver oil, 1.6; salt mixture (Glaxo

Laboratories Ltd. no. DL 6), 4; L-cystine, 0.35; DL-threonine (omitted after 80 g. body wt. was reached), 0.1; succinylsulphathiazole, 0.5. Vitamins were added in the amounts (mg./kg. of diet): thiamine, 10; riboflavin, 10; pyridoxine, 10; nicotinic acid, 10; *p*-aminobenzoic acid, 10; calcium D-pantothenate, 50; inositol, 100; biotin, 0.1; pteroylglutamic acid, 2; α -tocopherol, 40; vitamin K, 1; choline chloride, 2500.

These modified diets have also been used (see Table 1): Diet A/L: identical with diet A, except that lactose replaced sucrose. Diet B: identical with diet A, except that cod-liver oil was replaced by arachis oil with vitamin A (8000 i.u./kg. of diet) and vitamin D₃ (4000 i.u./kg. of diet). Diet B/A: identical with diet B, except that succinylsulphathiazole was omitted and aureomycin (200 mg./kg. of diet) was added 3 weeks after weaning. Diet C (g./100 g.): soya-bean meal, 36; sucrose, 25; starch, 26; arachis oil, 8; salt mixture, 4; DL-methionine 0.6; succinylsulphathiazole, 0.5; vitamins as in diet B.

The extracted casein was prepared by boiling vitamin-low casein (Genatosan Ltd., Loughborough) six times with ethanol (4 vol.). This procedure decreased the vitamin B₁₂ content from about 8 to less than 1 (usually 0.6) μ mg./g. of protein, as estimated by microbiological assay with *Ochromonas malhamensis* and *Lactobacillus leichmannii*, kindly carried out by Dr J. E. Ford, National Institute for Research in Dairying.

Food consumption and body weight. All animals were weighed three times a week. The food consumption of deficient rats was almost the same as that of the control animals. Details of changes in body weight and of the food consumption during the experimental feeding period are given in Table 1.

Administration of labelled compounds. All labelled compounds (obtained from The Radiochemical Centre, Amersham, Bucks.) were diluted with carrier to give the following specific radioactivities, and the diluted substances were added to the diet: (a) Sodium [¹⁴C]formate, 0.56 μ C/mg. (expt. 1, 1.12 μ C/10 g. of diet), 0.78 μ C/mg. (expt. 2, 1.56 μ C/10 g. of diet), 0.74 μ C/mg. (expts. 3 and 4, 1.46 μ C/10 g. of diet); (b) L-[2 ring-¹⁴C]histidine monohydrochloride monohydrate, 0.48 μ C/mg. (0.48 μ C/10 g. of diet); (c) DL-[¹⁴C]serine, 0.816 μ C/mg. (0.816 μ C/10 g. of diet). In Expt. 5, sodium [¹⁴C]formate (0.73 μ C/mg.) was injected intraperitoneally, 5.83 μ C (8 mg.) being used for rats 63/1 and 63/2 and 2.92 μ C (4 mg.) for rats 63/4 and 63/5.

Administration of vitamin B₁₂. Cyanocobalamin (Cyta-men, Glaxo Laboratories Ltd., Greenford) was administered to control animals in the amounts shown in Table 1. In Expts. 1 and 2 it was given orally from weaning until the animals were killed, i.e. for 31 and 58 days respectively. In the other experiments it was injected intraperitoneally

twice weekly in equal doses, starting 17 days (Expts. 3 and 4), 12 days (Expt. 6, litter 54), 18 days (Expt. 6, litter 55) or 23 days (Expt. 7) before the end of the experiment. In Expt. 5, rat 2 was given 10 μ g. on the last and fifth day and rat 5 was given 10 μ g. on the last, ninth and thirteenth day before the injection of the labelled formate.

Vitamin B₁₂ content of tissues. Vitamin B₁₂ was estimated microbiologically with *Ochromonas malthamensis* as test organism, essentially by the method described by Ford (1953).

Isolation of metabolic products

Protein and amino acids. The animals were killed by dislocation of the spine and the viscera (liver, kidneys, intestine, spleen, heart and lungs) were quickly removed, washed with water and disintegrated in cold ethanol in an Ato-Mix blender. The tissue was extracted six times with boiling ethanol-ether (3:1, v/v) and finally with ether. The dry, defatted tissue was extracted three times with 20 vol. of aq. 10% NaCl at 100° for 24 hr. to remove nucleic acids. The residue was washed twice with water, ethanol and ether and will be referred to as protein.

The protein (approx. 4 g.) was hydrolysed in a sealed tube with 5N-HCl (10 vol.) at 105° for 24 hr. The cooled solutions were diluted with water, filtered and evaporated to dryness *in vacuo*, the last traces of HCl being removed in a vacuum desiccator over KOH. Amino acids were separated by chromatography, first on an anion-exchange resin (De-Acidite FF, The Permutit Co. Ltd., London) in the acetate form to separate the acidic amino acids, followed by a cation-exchange resin (Zeo-Karb 225, The Permutit Co. Ltd., London) for the neutral and basic amino acids, essentially as described by Hirs, Moore & Stein (1954), but with 1.5N- and 2.5N-HCl as eluent for the cation-exchange-resin column (Stein & Moore, 1949). About 100 g. of De-Acidite resin and 300 g. of Zeo-Karb 225 resin in columns of 2.5 cm. and 3 cm. diameter respectively were sufficient for the preliminary separation of the required amino acids.

Glutamic acid and aspartic acid were isolated from fractions containing the pure amino acids by evaporating the solution to dryness, removing excess of HCl in a vacuum desiccator, dissolving the residue in a little water and adding pyridine to pH 5.0. If necessary, ethanol was added to complete the crystallization. The amino acids were recrystallized from water by addition of ethanol. There was no change in their specific radioactivity on further recrystallization. Histidine was converted into the monohydrochloride monohydrate by a similar procedure.

The other amino acid hydrochlorides were converted into the free amino acids by adsorption on Zeo-Karb 215 cation-exchange resin (The Permutit Co. Ltd., London) and displacement with aq. 0.02N-NH₃ soln. The eluate was evaporated to dryness and the residue was crystallized from aq. ethanol. Serine was usually contaminated by threonine and was purified by conversion into the hydroxy-azobenzenesulphonate, which was recrystallized two to three times from water.

In Expts. 3 and 6b, methionine was separated from leucine by chromatography on Whatman no. 3MM paper with pentan-1-ol-pyridine-water (7:7:6, by vol.). This procedure gave pure methionine in the first case, but in Expt. 6b the two samples still contained 10 and 15% of leucine respectively (estimated by quantitative paper

chromatography). Since leucine was, however, non-radioactive, the specific radioactivity of the methionine (Table 3) has been corrected for this inactive impurity. In Expt. 1, methionine was oxidized to the sulphone with bromine-water and separated from leucine on a column of Dowex-50 cation-exchange resin (8% cross-linked, Microchemical Specialities Inc., Berkeley, Calif., U.S.A.) (1.5 cm. diam. $\times$ 55 cm.) with 1.5N-HCl as eluent. Methionine sulphone was eluted first. The appropriate fractions were evaporated to dryness and the free amino acid was obtained by displacement from Zeo-Karb 215 resin with dilute aq. NH₃ soln. as described above. Methionine sulphone was recrystallized from water by addition of ethanol.

The purity of all amino acids isolated was checked by paper chromatography, with phenol-water (5:2, v/v, with NH₃ in the tank) and pentan-1-ol-pyridine-water (7:7:6, by vol.).

Choline. Choline was isolated by the method of du Vigneaud, Cohn, Chandler, Schenk & Simmonds (1941). The conversion of choline reineckate into chloroplatinate was carried out either as described or, more conveniently, by the following procedure. Choline reineckate was dissolved in aq. 50% acetone and the solution was passed through a column of Zeo-Karb 225 (H⁺ form) which had been washed free of excess of acid. The column was washed with aq. 50% acetone until the effluent was colourless, then with a little water. Choline was eluted with 2N-HCl and the solution was evaporated to dryness. The choline chloride was converted first into the mercurichloride complex (m.p. 170°) and finally into the chloroplatinate, as previously described (Arnstein & Neuberger, 1953). The chloroplatinate was recrystallized from water by addition of ethanol, the specific radioactivity remaining unchanged. The purity of the salt was checked by Pt analysis, the found values being within 0.4 of the theoretical value in all cases.

Choline was degraded into trimethylamine by the method of du Vigneaud *et al.* (1941), the purity of the trimethylamine chloroplatinate being checked by Pt analysis (values found were within 0.4 of the theoretical value). In Expt. 7 (deficient group), however, the sample was contaminated with ammonium chloroplatinate (see Arnstein, 1952). This sample was therefore also analysed for carbon by microanalysis and its reported specific radioactivity has been corrected for the low carbon content (Found: C, 6.87. Calc. for C₆H₂₀N₃Cl₆Pt: C, 13.6%).

Oxidation and excretion of formate (Expt. 5). After injection of the labelled formate (see above) the animals were placed in desiccators, through which CO₂-free air was passed slowly. The expired CO₂ was trapped in excess of 40% NaOH and converted into BaCO₃ by addition of Ba(OH)₂. The BaCO₃ was washed with water, ethanol and ether, dried, weighed and counted. In the experiment with the first pair of rats the NaOH was changed at 2 and 4 hr., in that with the second pair at 1, 2, 3, 4, 6, 8, 10 and 12 hr. after the injection of the formate, CO₂ being collected for a further 2 and 13 hr. respectively.

Urine was collected either separately for the first 6 hr. and the subsequent 18 hr. (rats 1 and 2) or for 24 hr. (rats 4 and 5). It was stored at -20°. Carrier sodium formate (27.5 mg. to the 6-24 hr. collection, 68.7 mg. to the other samples) was added, the formate was oxidized to CO₂ as described by Weinhouse & Friedmann (1952) and counted as BaCO₃.

Radioactivity measurements

^{14}C was estimated by counting 'infinitely thick' samples on 1 or 2 cm.² polythene disks (Popják, 1950), with an end-window Geiger-Müller tube and Dekatron scaler. A 1 cm.² disk of poly- ^{14}C methyl methacrylate (The Radiochemical Centre, Amersham, Bucks.), specific radioactivity $1\mu\text{C/g.}$, was used as a reference standard. The standard error of all counts is less than 5%.

RESULTS

Effect of vitamin B₁₂ on the growth and food consumption of deficient rats and on the vitamin B₁₂ content of their internal organs

With all casein diets there was a high mortality of some litters within a few days of birth, but survivors grew well and the administration of large

Table 1. *Food consumption, growth and liver vitamin B₁₂ content of deficient and control rats*

Compositions of the diets and other experimental details are given in the text. Ages of the animals (column 4) and the initial body weights (column 7) refer to the beginning of the administration of labelled compounds, the increase being the gain in weight during the subsequent 7 days. In Expt. 5, however, the body weights are those before fasting and the animals were killed 24 hr. after injection of labelled formate.

Expt.	Litter and rat no.	Sex	Age (days)	Diet	Amount of vitamin B ₁₂ given	Body wt. (g.)		Food consumption (g./7 days)	Vitamin B ₁₂ content of liver (μmg./g.)
						Initial	Increase		
[¹⁴ C]Formate experiments									
1	50/1	M.	50	A	1 μg./day	144	30	102.4	29
	50/8	F.	50	A	1 μg./day	114	15	80.8	67
	50/2	M.	50	A	None	146	29	106.8	7.8
	50/4	F.	50	A	None	117	10	79.3	9.1
2a	52/1	M.	76	A/L	2.5 μg./day	224	18	97.5	138
	52/2	M.	76	A/L	2.5 μg./day	234	12	105.0	112
	52/4	M.	76	A/L	None	217	2	99.8	9.0
	52/5	M.	76	A/L	None	230	5	104.1	10
2b	52/3	F.	76	A/L	2.5 μg./day	155	2	80.6	150
	52/6	F.	76	A/L	None	163	5	80.8	52*
3a	57/5	M.	50	C	20 μg./week	129	20	106.2	102
	57/1	F.	50	C	20 μg./week	115	8	70.5	119
	57/6	M.	50	C	None	142	23	113.2	18
	57/2	F.	50	C	None	106	14	75.7	91*
3b	57/7	M.	50	B	20 μg./week	178	38	116.2	102
	57/3	F.	50	B	20 μg./week	141	18	89.2	102
	57/8	M.	50	B	None	172	33	110.0	6.0
	57/4	F.	50	B	None	123	11	71.3	6.8
4	59/3	M.	47	B	20 μg./week	146	35	102.7	75
	59/4	M.	47	B	20 μg./week	142	30	94.4	58
	59/6	M.	47	B	20 μg./week	156	36	113.1	97
	59/5	M.	47	B	None	149	29	96.4	9.1
	59/7	M.	47	B	None	142	34	109.3	10.0
	59/8	M.	47	B	None	137	33	100.8	5.0
5	63/2	M.	49	B/A	20 μg.	129	—	—	45
	63/1	M.	49	B/A	None	135	—	—	8.3
	63/5	M.	57	B/A	30 μg.	176	—	—	115
	63/4	M.	57	B/A	None	176	—	—	21
L-[2 ring- ¹⁴ C]Histidine experiments									
6a	54/4	M.	48	B	20 μg./week	156	35	105.8	68
	55/2	M.	48	B	20 μg./week	138	20	81.8	105
	54/5	M.	48	B	None	158	40	114.9	12
	55/3	M.	48	B	None	160	29	99.2	18
6b	54/2	F.	48	B	20 μg./week	130	13	70.9	110
	54/1	F.	48	B	None	134	15	74.3	24
DL-[3- ¹⁴ C]Serine experiment									
7	60/3	M.	47	B	20 μg./week	142	25	110.2	125
	60/5	M.	47	B	20 μg./week	145	28	113.2	136
	60/7	M.	47	B	20 μg./week	151	30	115.4	150
	60/4	M.	47	B	None	134	14	80.7	8.8
	60/6	M.	47	B	None	145	30	108.4	2.7
	60/8	M.	47	B	None	141	31	113.1	6.8

* Unusually high values, possibly resulting from intestinal biosynthesis of vitamin B₁₂.

doses of vitamin B₁₂ (1–3 µg. of cyanocobalamin/day) had little effect on growth and food consumption (Table 1). Nevertheless, the concentration of vitamin B₁₂ in the liver of deficient animals was much lower than normal (Table 1), except in two cases (indicated by an asterisk) where synthesis of vitamin B₁₂ by intestinal micro-organisms may have occurred. Other tissues (spleen, kidneys, heart, lung and brain) of deficient rats apparently also contained less vitamin B₁₂ than those of rats fed on the same diet and given vitamin B₁₂ or kept on a stock diet, but, except for brain and spleen, tissues of only one deficient animal and one animal on stock diet were examined. The values for brain and spleen (see Table 1 for details of diets and animals) were (µmg./g. of fresh tissue): deficient spleen, 5.2 (rat 63/1), 9.0 (rat 63/4), 16.1 (rat 50/3, a male litter-mate of the animals used for Expt. 1;

body wt. 183 g.); normal spleen, 19.5 (rat 63/2), 55.6 (rat 63/5), 19.3 (male rat on stock diet, body wt. 178 g.); deficient brain, 2.8 (rat 63/1), 5.3 (rat 53/4), 2.6 (rat 50/3); normal brain, 13.1 (rat 63/2), 23.0 (rat 63/5); 22.3 (rat on stock diet).

Incorporation of labelled compounds into protein

The incorporation of labelled serine or histidine into the protein of mixed viscera was not increased significantly by vitamin B₁₂ (Table 2). These results were confirmed by the observation that the specific radioactivity of histidine, which was isolated from the protein, was actually slightly decreased (Table 3). On the other hand, the incorporation of formate into protein was almost doubled by vitamin B₁₂ (Table 2). The specific radioactivities of both serine and methionine isolated from the protein were increased to a similar

Table 2. *Incorporation of labelled serine, histidine and formate into protein*

Labelled compounds were fed for 7 days; where more than one animal was used, the average amount of isotope per rat is given. For other experimental details see text and Table 1.

Expt.	Litter and rat no.	Precursor fed		Vitamin B ₁₂ given	Specific radioactivity of protein (mµc/g.)
		Compound	µc/rat		
7	60/3, 5, 7	DL-[3- ¹⁴ C]Serine	4.58	+	40.9
	60/4, 6, 8	DL-[3- ¹⁴ C]Serine	4.11	–	42.5
6a	54/4	L-[2 ring- ¹⁴ C]Histidine	5.08	+	110.8
	54/5	L-[2 ring- ¹⁴ C]Histidine	5.52	–	116.4
	55/2	L-[2 ring- ¹⁴ C]Histidine	3.93	+	116.0
	55/3	L-[2 ring- ¹⁴ C]Histidine	4.76	–	108.3
6b	54/2	L-[2 ring- ¹⁴ C]Histidine	3.40	+	112.3
	54/1	L-[2 ring- ¹⁴ C]Histidine	3.47	–	118.3
1	50/1, 8	Sodium [¹⁴ C]formate	10.3	+	72.0
	50/2, 4	Sodium [¹⁴ C]formate	10.4	–	42.4
3b	57/3, 7	Sodium [¹⁴ C]formate	15.0	+	51.6
	57/4, 8	Sodium [¹⁴ C]formate	13.4	–	26.3

Table 3. *Distribution of radioactivity in some amino acids isolated from the protein of animals fed with labelled formate or histidine*

The amino acids were isolated from the proteins of the mixed viscera (Table 2) as described in the text.

Expt.	Labelled precursor	Amino acid isolated	Specific radioactivity (µc/mole)		Ratio of specific radioactivities (a/b)
			Vitamin B ₁₂ present (a)	Vitamin B ₁₂ absent (b)	
1	Sodium [¹⁴ C]Formate	Serine*	76.0	42.3	1.80
		Methionine†	84.0	52.5	1.60
		Aspartic acid	1.73	1.36	1.28
		Glutamic acid	1.91	1.60	1.19
3b	Sodium [¹⁴ C]Formate	Serine*	62.7	23.3	2.70
		Methionine	59.0	24.7	2.40
6b	L-[2 ring- ¹⁴ C]Histidine	Histidine‡	757	818	0.93
		Serine*	10.9	9.59	1.13
		Methionine	3.30	3.76	0.88

* Counted as hydroxyazobenzenesulphonate.

† Counted as sulphone.

‡ Counted as monohydrochloride monohydrate.

Table 4. *Incorporation of sodium [^{14}C]formate, DL-[3- ^{14}C]serine and L-[2 ring- ^{14}C]histidine into choline and choline methyl groups by vitamin B_{12} -deficient and control rats*

Choline was isolated and degraded to trimethylamine as described in the text. A dash signifies that the choline sample was not degraded.

Expt.	Labelled precursor	Vitamin B_{12} given	Specific radioactivity ($\mu\text{mc/g.}$)	
			Choline chloroplatinate	Trimethylamine chloroplatinate
1	Formate	+	401	—
		—	214	—
2a	Formate	+	317	—
		—	144	—
2b	Formate	+	384	—
		—	348	—
3a	Formate	+	211	—
		—	153	—
3b	Formate	+	412	317
		—	117	112
6a	Histidine	+	40.1	34.6
		—	36.6	35.6
6b	Histidine	+	44.4	40.8
		—	42.8	51.0
7	Serine	+	171	66.3
		—	131	21.1

Table 5. *Effect of vitamin B_{12} on the incorporation of sodium [^{14}C]formate into the choline of liver, intestine and other internal organs*

In Expt. 4, labelled formate was fed with the diet for 7 days to groups of three rats; in Expt. 5 labelled formate was injected intraperitoneally (see text and Table 1). Choline was isolated from the various tissues as described in the text.

Expt.	Tissue	Vitamin B_{12} given	Specific radioactivity of choline chloroplatinate ($\mu\text{mc/g.}$)
4	Liver	+	642
		—	307
	Intestine	+	448
		—	199
	Other internal organs (mixed)	+	284
		—	157
5	Liver	+	608
		—	292

extent, whereas the specific radioactivity of aspartic acid and glutamic acid was much less affected (Table 3).

Conversion of labelled formate, serine and histidine into choline

The conversion of formate into choline was markedly decreased by the absence of vitamin B_{12} (Table 4), provided that the animals had sufficiently low amounts of vitamin B_{12} in their livers. In Expts. 2b and 3a the difference in the specific

radioactivities of the choline from the deficient and control animals was relatively small; in both cases the liver of one of the rats in the deficient group was found to contain an exceptionally high level of vitamin B_{12} (rats 52/6 and 57/2, Table 1).

The specific radioactivities of choline isolated separately from liver, intestine and the remaining internal organs were affected to about the same extent by vitamin B_{12} deficiency (Table 5) and the effect of vitamin B_{12} on the radioactivity of liver choline was the same whether the labelled formate was injected (Expt. 5) or fed with the diet (Expt. 4).

Degradation of the choline (Table 4) showed that with labelled formate or serine as precursor vitamin B_{12} increased the labelling of choline methyl groups approximately threefold. The radioactivity of the ethanolamine moiety of choline (obtained by difference between the radioactivities of choline and trimethylamine chloroplatinates) was also greatly increased by vitamin B_{12} when labelled formate was the precursor but not when labelled serine was used.

Table 4 also shows that vitamin B_{12} deficiency appeared to have little or no effect on the utilization of labelled histidine for the biosynthesis of either total choline or choline methyl groups.

The relative importance of serine, histidine and formate as precursors of choline and choline methyl groups is shown in Table 6, in which previous results have been expressed in terms of standardized radioactivities. Serine is the best precursor of choline, which is partly due to its extensive conversion into the ethanolamine moiety.

Table 6. *Effect of vitamin B₁₂ on the relative utilization of serine, histidine and formate for the biosynthesis of choline*

The results given in Table 4 have been standardized for the administration of 100 μ C/100 g. body wt. (average weight during the 7-day-feeding period). With serine, allowance has been made for the fact that D-serine is not converted into choline (Arnstein, 1951). The radioactivity of the ethanolamine moiety is obtained by difference and the error of the result is calculated on the assumption of 5% counting errors for choline and trimethylamine chloroplatinates. The choline of Expt. 1 was not degraded.

Expt.	Precursor	Vitamin B ₁₂ given	Standard specific radioactivity (μ C/mole)		
			Choline	Trimethylamine	Ethanolamine
7	L-[3- ¹⁴ C]Serine	+	3680	1220	2460 $\pm$ 245
		-	2990	413	2577 $\pm$ 170
6a	L-[2 ring- ¹⁴ C]Histidine	+	869	598	171 $\pm$ 73
		-	771	572	199 $\pm$ 67
6b	L-[2 ring- ¹⁴ C]Histidine	+	1104	876	228 $\pm$ 99
		-	1078	1003	75 $\pm$ 104
1	Sodium [¹⁴ C]formate	+	1639	—	—
		-	641	—	—
3b	Sodium [¹⁴ C]formate	+	1473	969	504 $\pm$ 121
		-	425	347	78 $\pm$ 38

Fig. 1. Oxidation of sodium [¹⁴C]formate to respiratory carbon dioxide by vitamin B₁₂-deficient and control rats. The cumulative amount of ¹⁴C expired in the respiratory CO₂ during 6 and 24 hr. respectively, after injection of labelled formate, is given as the percentage of the dose. For experimental details see text.

In the presence of vitamin B₁₂, the relative efficiency of these compounds for methyl biosynthesis was serine > formate > histidine, but in vitamin B₁₂-deficient animals it was histidine > serine > formate.

Both histidine and formate are poorer precursors of the ethanolamine moiety of choline than is

serine. The utilization of formate for this reaction is reduced by vitamin B₁₂ deficiency, as would be expected since incorporation into serine, which is the immediate precursor of ethanolamine (Arnstein, 1951), must be an obligatory step. It should be noted, however, that the radioactivity of the ethanolamine moiety is obtained by difference and 5% errors in the counting of choline and trimethylamine could markedly affect the magnitude of the difference between the deficient and control rats. For this reason the reduction in the conversion of histidine into the ethanolamine in Expt. 6b may not be significant.

Oxidation and urinary excretion of formate

The effect of vitamin B₁₂ deficiency on the oxidation of formate is shown in Fig. 1. The initial rate of oxidation was less in the deficient rats, but after about 2 hr. the deficient animals expired somewhat more ¹⁴CO₂ than the controls. The total oxidized in 6 or 25 hr. by the deficient animals was only slightly less than that of the controls.

The amounts of formate excreted in the urine were: rat 63/1 (deficient), 0-6 hr. 0.70 μ C (12%), 6-25 hr. < 0.001 μ C; rat 63/3 (control), 0-6 hr. 0.504 μ C (8.6%), 6-25 hr. < 0.001 μ C; rat 63/4 (deficient), 0-25 hr. 0.685 μ C (23.5%); rat 63/5 (control), 0-24 hr. 0.144 μ C (5.0%). Formate excretion was thus essentially complete within 6 hr. of the injection of the labelled formate and the deficient animals excreted a greater proportion of the dose than did the controls.

When these results are combined, the total amount of formate excreted and oxidized to CO₂ was: rat 63/1, 3.02 μ C (51.8%); rat 63/2 (control), 2.93 μ C (50.3%); rat 63/4 (deficient), 2.27 μ C

(78.0 %); rat 63/5 (control), 1.89 μ C (65.0 %). The differences between the deficient animals and the corresponding controls are thus less than 20 %.

DISCUSSION

The observed stimulation of the biosynthesis of choline methyl groups from serine and formate by vitamin B₁₂ is qualitatively similar to that previously obtained with rats fed on amino acid diets deficient in methionine (Arnstein & Neuberger, 1953). The greater difference in the utilization of formate by deficient and control rats in the present work is not surprising in view of the likelihood that the deficient animals were more thoroughly depleted of vitamin B₁₂ since they had been specially bred from mothers also kept on a deficient diet. This interpretation is supported by the apparent correlation between the conversion of formate into choline and the vitamin B₁₂ content of the liver, which must be less than about 25 μ mg./g. before effects on formate metabolism can be demonstrated. The normal growth and food consumption of deficient animals suggests that the changes in formate metabolism which have been observed in this work can occur in deficiency states which are still relatively mild. These metabolic effects may thus be an early consequence of vitamin B₁₂ deficiency.

Although vitamin B₁₂ increased the oxidation of formate to respiratory carbon dioxide and decreased its excretion in the urine, the combined effect of these metabolic changes appears to be too small to account quantitatively for the observed increases in methyl-group biosynthesis. This conclusion is supported by the observation that vitamin B₁₂ actually decreased the incorporation of formate into nucleic acid adenine and guanine (Arnstein, 1958b, 1959), since the radioactivity of these metabolic products should be changed similarly to that of choline if vitamin B₁₂ affected mainly the oxidation and excretion of formate. It is not unlikely, moreover, that changes in the oxidation and excretion of formate could be due to a primary effect of vitamin B₁₂ on formate biosynthesis from precursors such as serine, which would affect the size of the metabolic pool and hence its catabolism. That the oxidation of the hydroxymethyl group of serine to the level of formate may require vitamin B₁₂ is suggested by an increased conversion of [3-¹⁴C]serine into nucleic acid adenine and guanine by the vitamin (Arnstein, 1959; Arnstein & White, unpublished results). The present work shows that vitamin B₁₂ also increases the reverse of this reaction, namely the reductive conversion of formate into serine. This result is noteworthy because it demonstrates that the metabolic function of vitamin B₁₂ on C₁

metabolism by the rat is not restricted to methyl-group biosynthesis.

Since vitamin B₁₂, unlike folic acid, is apparently not required for the transfer of C₁ compounds at the same oxidation level, as has been shown for transmethylation (Mistry, Vadopalait, Chang, Firth & Johnson, 1955), for the glycine-serine interconversion (Arnstein & Neuberger, 1953; Chang & Johnson, 1955; Arnstein & Stanković, 1956) and for purine synthesis from formate (Arnstein, 1958b), it is suggested that it functions at their interconversion by oxidation-reduction reactions. Whether the mechanism by which vitamin B₁₂ stimulates the incorporation of formate into serine and methionine is similar to that involved in methionine biosynthesis from serine by *Escherichia coli* (Guest, 1959; Woods, 1958) remains to be elucidated.

It is of interest that the experiments with labelled histidine indicate that vitamin B₁₂ may not be required for the utilization of this formate precursor for the biosynthesis of serine, methionine and choline. It is possible, however, that more severely deficient animals would show a requirement for these reactions. In any case, the physiological importance of vitamin B₁₂ in C₁ metabolism may be due to the availability of serine, a non-essential amino acid, as a metabolic source of formate in the presence of the vitamin, whereas in its absence histidine, an essential amino acid, has to be used. One result of the relatively greater importance of histidine as a formate precursor in the vitamin B₁₂-deficient animal could be a decrease in the availability of this amino acid for other metabolic reactions, including protein synthesis.

SUMMARY

1. The metabolism of sodium [¹⁴C]formate, DL-[3-¹⁴C]serine and L-[2 ring-¹⁴C]histidine has been studied *in vivo* in young rats depleted of vitamin B₁₂ by breeding from deficient mothers which were fed on a vitamin B₁₂-low casein diet.

2. The livers of rats fed on the deficient diet contained much less vitamin B₁₂ than those of control animals and other internal organs were also depleted.

3. The incorporation of serine and histidine into the mixed visceral proteins was not reduced by lack of vitamin B₁₂.

4. Administration of vitamin B₁₂ to deficient rats increased the oxidation of formate to respiratory carbon dioxide and decreased the urinary excretion of formate.

5. The conversion of formate into both serine and methionine and that of serine and formate into choline methyl groups were markedly increased by vitamin B₁₂.

6. The utilization of histidine for the biosynthesis of serine, methionine and choline was not affected by the vitamin B₁₂ deficiency.

7. It is suggested that vitamin B₁₂ is required for the interconversion of C₁ compounds by oxidation-reduction, but not for their transfer at the same oxidation level. The possible physiological significance of this function is discussed.

I should like to thank Dr J. E. Ford for the microbiological assays of casein samples and of the livers of the first experiment and for helpful advice on the estimation of vitamin B₁₂ with *Ochromonas malhamensis*. I am also grateful to Dr A. M. White for the isolation of choline in the first experiment and for advice on the use of ion-exchange chromatography in the isolation of choline and to Mrs B. Higginson for expert technical assistance throughout this work.

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