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The Effect of Vitamin B_{12} Deprivation on the Enzymes of Fatty Acid Synthesis*

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SUMMARY

The enzymes of fatty acid synthesis from liver and brain in normal and B_{12}-deprived rats were studied. Both total and specific activities of fatty acid synthetase and acetyl coenzyme A carboxylase were 2- to 5-fold greater in B_{12} deprivation than in the normal state. The presence of excess activators in the B_{12}-deprived rat or an excess inhibitor in the normal rat was not found by serial admixtures of the respective cytosol preparations or by partial purification of the enzymes. Since B_{12} deficiency is associated with an increase in the tissue concentrations of propionic and methylmalonic acid, the effect of the coenzyme A derivatives of these acids on fatty acid synthetase and acetyl-CoA carboxylase activity was studied. Propionyl-CoA was a substrate for fatty acid synthetase, while methylmalonyl-CoA markedly inhibited synthetase activity. Methylmalonyl-CoA markedly inhibited acetyl-CoA carboxylase activity. Propionyl-CoA was shown to be a substrate for acetyl-CoA carboxylase, competing with acetyl-CoA, and the product synthesized was methylmalonyl-CoA. Thus, in vitamin B_{12} deprivation propionyl-CoA competes with acetyl-CoA as substrate providing a mechanism for odd chain fatty acid production, and its product, methylmalonyl-CoA, may function as an inhibitor of the enzymes of fatty acid synthesis.

Vitamin B_{12} is known to participate as a coenzyme in two biochemical reactions in man, *i.e.* (*a*) the methyltransferase-catalyzed reaction of homocysteine metabolism (5-methyltetrahydrofolic acid + homocysteine $\xrightarrow[\text{coenzyme } B_{12}]{\text{methyltransferase}}$ methionine + tetrahydrofolic acid) (*S*-adenosylmethionine:L-homocysteine *S*-methyltransferase (EC 2.1.1.10)); and (*b*) the mutase-catalyzed reaction of methylmalonyl coenzyme A metabolism (methylmalonyl coenzyme A $\xrightarrow[\text{coenzyme } B_{12}]{\text{mutase}}$ succinyl coenzyme A) (methylmalonyl-CoA:CoA-carbonylmutase (EC 5.4.99.2)). Functional integrity of hematopoietic and mucosal cells depends upon the first pathway, thus explaining the requirement for vitamin B_{12} in these tissues. Since folic acid can be reduced to tetrahydrofolic acid by other tissue reductases, the resultant coenzymatically active folate forms can circumvent the requirement for vitamin B_{12} in these tissues. Such is not true for the second reaction, and in vitamin B_{12} deficiency methylmalonyl coenzyme A and its precursor propionyl coenzyme A might be expected to accumulate.

It is now well documented that B_{12} deprivation results in altered fatty acid metabolism in man (2, 3), animals (4, 5), and glial cells grown in tissue culture (6). Previous studies in peripheral nerve biopsies from patients with pernicious anemia have demonstrated decreased fatty acid synthesis from radiolabeled propionate (the metabolism of which proceeds by way of the pathway containing the mutase-catalyzed reaction noted above) and the presence of odd chain fatty acids not found in normal nerves (7). Accumulation of odd chain fatty acids has also been demonstrated by Abeles and co-workers (4) in pigs deprived of vitamin B_{12}. Finally, rat glial cells in culture have been shown to produce two odd chain fatty acids during growth in a B_{12}-deficient media (6). In view of these findings, we decided to study the enzymes of fatty acid synthesis in vitamin B_{12} deficiency.

METHODS

Animals and Their Maintenance—Weanling albino rats of the Sprague-Dawley strain (obtained from Pel-Freez Bio-Animals, Inc., Rogers, Ark.) weighing 40 to 50 g were maintained in stainless steel metabolic cages with open grid floors to minimize coprophagy. Animals were placed on one of three diet programs: (*a*) "routine rat chow" (obtained from Feeders Supply, Co., Dallas, Texas) subsequently referred to as "normal diet"; (*b*) vitamin B_{12}-deficient rat diet (obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio) subsequently referred to as the "B_{12}-deprived diet," or (*c*) vitamin B_{12}-deficient rat diet supplemented by intramuscular injections of 100 μg of vitamin B_{12} (cyanocobalamin obtained from Calbiochem, San Diego, Calif.) as indicated in the respective studies and hereafter referred to as the "B_{12}-supplemented diet." The respective diet and water were supplied to the animals *ad libitum*.

The animals were weighed weekly, and urine was collected

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twice weekly for determination of propionic acid (8) and methylmalonic acid (9). Blood was collected before sacrifice for routine hematologic studies, and the animals were then decapitated. Serum was obtained for vitamin B_{12} assay (10, 11), and liver and brain were promptly excised, rinsed in cold 0.9% NaCl solution, blotted, and weighed. Aliquots of liver and brain were utilized for determination of B_{12} content (12) and $^{14}CO_2$ production rates from [^{14}C]propionate. Tissues are subsequently named by the dietary programs of the animal (as normal, B_{12}-deprived, and B_{12}-supplemented).

Of the three diets employed, it was established that only the B_{12}-deprived diet resulted in decreased growth of weanling animals; this altered growth rate was corrected by the administration of vitamin B_{12}. To achieve quantitative and qualitative dietary consistency in the B_{12}-deprived and the B_{12}-supplemented animals, the B_{12}-supplemented member of each pair was limited in his food intake (after B_{12} supplementation was begun) to equal that of the B_{12}-deprived mate.

Assay of Propionate Catabolism—Propionate oxidation, a B_{12} coenzyme-dependent pathway, was measured with a modification of the method of Rosenberg *et al.* (13). This procedure consisted of incubating tissue slices in Krebs bicarbonate buffer, pH 7.2, containing 25 μmoles of propionate and 200,000 cpm of [^{14}C]propionate (obtained from New England Nuclear Corp.; specific activity, 47 mCi per mmole). The concentration of propionate added was determined by us to be sufficiently high so that further increase in propionate concentration did not result in increased $^{14}CO_2$ production in liver or brain from animals on normal diet. Incubation was for 2 hours and $^{14}CO_2$ was trapped and counted as described by Rosenberg *et al.* (13).

Fatty Acid Synthetase Activity—From a 20% homogenate of tissue a 104,000 × *g* supernatant solution was prepared by the method of Easter and Dils (14) and assayed for fatty acid synthetase activity as described by Carey and Dils (15). The reaction mixture consisted of 200 mM potassium phosphate buffer (pH 6.3), 1 mM dithiothreitol, 1 mM EDTA, 0.24 mM TPNH (NADPH), 30 μM acetyl-CoA, and 44 μM malonyl-CoA to which was added 100 μl of the 104,000 × *g* supernatant solution providing a total reaction volume of 1.0 ml. The supernatant fraction was diluted so that 100 μl would result in an absorbance change of 0.005 to 0.020 per min. The reaction was carried out at room temperature and the oxidation of TPNH was followed spectrophotometrically (Gilford recording spectrophotometer 240) at 340 nm. The substrate conditions for maximum activity for normal liver were found to be in agreement with those previously reported (14, 15), so that increasing the acetyl-CoA or malonyl-CoA concentrations did not result in an increased activity in the normal or B_{12}-deprived tissues. The CoA derivatives (acetyl-CoA, propionyl-CoA, and malonyl-CoA) were obtained from P-L Biochemicals, Milwaukee, Wisc. The spectrophotometric assay was corroborated by measuring the incorporation of [^{14}C]malonyl-CoA into fatty acids (16). Both assays yielded similar rates of fatty acid synthetase activity. Protein concentration of the supernatant fraction was determined by the method of Lowry (17).

Partial purification of the fatty acid synthetase was accomplished by ammonium sulfate fractionation (18). Assay conditions for the fatty acid-synthesizing enzyme were 100 mM potassium phosphate buffer, pH 6.8, 3 mM EDTA, 1 mM dithiothreitol, 5 mM mercaptoethanol, 0.3 mM TPNH (NADPH), 12.5 μM acetyl-CoA, and 50 μM malonyl-CoA.

Acetyl Coenzyme A Carboxylase Activity—Preparation of the 104,000 × *g* supernatant fraction was carried out by the method of Lane and co-workers (19). An aliquot of the supernatant solution (0.5 to 1.0 ml) was further stabilized by gel filtration on Sephadex G-25 (medium) (19). The effluent was collected in 2-ml fractions, and 90% of the enzyme activity was recovered in the void volume (20). Assay of acetyl-CoA carboxylase was performed by the method of Lane and co-workers (19) with a reaction volume of 0.5 ml for the citrate preincubation and 1.0 ml for the incubation.

Identification of Products Formed from Acetyl-CoA and Propionyl-CoA in Acetyl-CoA Carboxylase Reaction—Acetyl-CoA and propionyl-CoA were used as substrates for the acetyl-CoA carboxylase reaction. Following the assay the thioesters produced were hydrolyzed in alkali, and the acids identified in the following manner (21). The pH of the reaction mixture, after assay of acetyl-CoA carboxylase activity (approximately 80% of the total volume), was adjusted to pH 12 with saturated NaOH and allowed to stand for 1 hour at room temperature. The mixture was then acidified to pH 2 by addition of concentrated HCl and extracted 5 times with 4 ml of diethyl ether. The ether phases were pooled and dried under N_2 and the residue dissolved in 0.5 ml of acetone. A 0.1-ml aliquot of the acetone solution was then counted in Bray's solution in a liquid scintillation spectrometer (7). A similar aliquot was analyzed by thin layer chromatography, and radioactivity of the free acid products was determined (21). Thin layer separation was performed on Silica Gel G (E. Merck, Darmstadt, Germany) prepared 0.25 mm thick on glass plates (20 × 20 cm). The plates were developed for approximately 1 hour in water-saturated diethyl ether-88% formic acid (15:1) (22). Malonic, methylmalonic, and succinic acid standards (Calbiochem, San Diego, Calif.) were utilized, and spots were detected by spraying the standards with a 1:1 mixture of 0.1% methyl red and 0.3% bromphenol blue (22). The adjacent sample spots with R_F values similar to those of the standards were scraped and counted in Bray's solution.

Preparation of Methylmalonyl Coenzyme A—Methylmalonyl-CoA was synthesized by the method of Trams and Brady (23), and it was purified by DEAE-Sephadex A-25 column chromatography (24). Four criteria of purity of the product were employed: (*a*) the absorbance at 260 nm following thioester cleavage (24); (*b*) the liberated sulfhydryl groups were determined by 5,5′-dithiobis(2-nitrobenzoic acid) reaction after cleavage (25); (*c*) the concentration of thioesters by hydroxylamine assay before cleavage (26); and (*d*) thin layer chromatography of the acids after cleavage (22). By all these criteria the synthesized product was approximately 95% pure.

Immunochemical Titration of Fatty Acid Synthetase Preparations—Antibody to fatty acid synthetase[1] reacted with the enzyme preparations from the livers of normal, B_{12}-deprived, and B_{12}-supplemented animals and yielded a single connecting precipitin band by the Ouchterlony double diffusion technique.

The fatty acid synthetase for the immunochemical titrations was simultaneously prepared from each set of livers (from B_{12}-supplemented and B_{12}-deprived animals). The homogenization and preparation of the supernatant fraction of liver was by the method of Hsu *et al.* (27), the purification of the 104,000 × *g* supernatant fraction was carried out by DEAE-cellulose chromatography as described by Burton *et al.* (28), and the final dialysis was by the method of Collins *et al.* (29). Quantitative

[1] Antibody to fatty acid synthetase was generously provided by John W. Porter, Ph.D., Chief of Lipid Metabolism Laboratory, Madison Veterans Administration Hospital, Madison, Wisconsin.

precipitin reactions were as described by Craig and co-workers (30).

RESULTS

Characterization of B_{12}-deprived Animals—Animals were killed after evidence of propionic and methylmalonic aciduria occurred in the animals on the B_{12}-deprived diet. The degree of propionic and methylmalonic aciduria was quite variable. Since the appearance of both these intermediates is known to depend upon multiple factors in the rat (31–34), characterization of the B_{12}-deprived state was defined by multiple parameters. Table I delineates the difference in serum, liver, and brain B_{12} levels and propionate catabolism in animals fed the normal, B_{12}-deprived, and B_{12}-supplemented diets. The animals were considered to have B_{12} deprivation only when their serum and tissue B_{12} levels and propionate catabolism were in the range shown in Table I. The term B_{12} deprived was used, rather than deficient, since neither clinical nor histologic evidence of tissue alteration was seen in hematopoietic, gastrointestinal, or neural sites. Vitamin B_{12} administration to the above defined B_{12}-deprived group corrected the serum and tissue B_{12} values and the altered propionate catabolism.

Fatty Acid Synthetase Activity—Fatty acid synthetase activity in liver and brain of the animals on the normal and B_{12}-deprived diets is shown in Table II. In the complete system (both acetyl-CoA and malonyl-CoA) the B_{12}-deprived animals demonstrated a 4- to 5-fold greater specific activity of fatty acid synthetase than the normal animals. Smaller effects were seen in neural tissue, although again the B_{12}-deprived animals had greater activity than the normal animals.

The dietary status of animals is known to critically affect the activity of the enzymes of fatty acid synthesis (35, 36). Since the net caloric intake and weights of the animals on the normal diet were greater than that of the B_{12}-deprived group, the relationship of these dietary differences to enzyme activity was evaluated by doing serial weekly fatty acid synthetase assays during the deprivation period. During early B_{12} deprivation a decline in serum and tissue B_{12} values occurred, but fatty acid synthetase activity in the B_{12}-deprived and B_{12}-supplemented animals was the same. Only after evidence of altered propionate catabolism (see Table I) and associated propionic and methylmalonic aciduria appeared did the differences in enzyme activity in the two groups become apparent.

To test whether or not dietary differences were responsible for changes in fatty acid synthesis the enzyme was determined in the livers from pair-fed groups of animals, one on the B_{12}-deprived diet and the second on the B_{12}-supplemented diet. As shown in Table III the specific activity of fatty acid synthetase was approximately 3-fold greater in the liver of the B_{12}-deprived animals when compared to activity in the B_{12}-supplemented group. In addition, propionyl-CoA was able to replace acetyl-CoA as substrate in both the B_{12}-deprived and B_{12}-supplemented group. Furthermore, propionyl-CoA (with the standard acetyl- and malonyl-CoA substrates) resulted in increased fatty acid synthetase activity, but only in the B_{12}-deprived animals.

The increased fatty acid synthetase activity in the B_{12}-deprived animals was further evaluated by partial purification of the enzyme by ammonium sulfate precipitation. In an attempt to determine if the change in fatty acid synthetase activity was the result of an inhibitor in the B_{12}-supplemented or conversely an activator in the B_{12}-deprived animals we partially purified the enzymes from the livers. Table III compares the specific and

TABLE I

Comparative values in normal and B_{12}-deprived rats

	Serum B_{12}	Tissue B_{12}		Propionate catabolism	
		Liver	Brain	Liver	Brain
	pg/ml	*ng/g*[c]		*nmoles $^{14}CO_2$/100 mg*	
Normal (112)[a]	1106 ± 265[b]	172 ± 45	50 ± 7	575 ± 81	37 ± 9
B_{12}-deprived diet (96)[a]	142 ± 52	45 ± 9	28 ± 6	273 ± 48	25 ± 4
B_{12}-deprived diet supplemented with cyanocobalamin (61)[a]	1386 ± 310	185 ± 27	66 ± 12	618 ± 52	30 ± 9

[a] The number in parentheses indicates the number of animals.
[b] ±2 S.D.
[c] Wet weight of tissue.

TABLE II

Fatty acid synthetase activity in liver and brain of normal and B_{12}-deprived rats

Assay was performed on the 104,000 × *g* supernatant obtained from tissues from 21 animals in each group. The reaction mixture consisted of 200 mM potassium phosphate buffer (pH 6.3), 1 mM dithiothreitol, 1 mM EDTA, 0.24 mM TPNH, and substrate as indicated to which 100 μl of the supernatant was added yielding a total reaction volume of 1.0 ml. Spectrophotometric disappearance of TPNH was measured at room temperature.

Substrate conditions	Liver		Brain	
	Normal diet	B_{12}-deprived diet	Normal diet	B_{12}-deprived diet
nmoles	*nmoles/min/mg protein*			
30 Acetyl-CoA	0.46 ± 0.04[a]	0.13 ± 0.06	1.0 ± 0.05	1.5 ± 0.09
44 Malonyl-CoA	0.47 ± 0.06	0.63 ± 0.04	1.1 ± 0.08	1.5 ± 0.15
30 Acetyl-CoA + 44 malonyl-CoA	1.81 ± 0.05	8.35 ± 0.19	1.2 ± 0.05	1.8 ± 0.15

[a] S.E.

TABLE III

Specific and total fatty acid synthetase activity in liver of B_{12}-supplemented and B_{12}-deprived rats

Representative values from the isolation, partial purification, and fatty acid synthetase assay from liver of B_{12}-deprived and B_{12}-supplemented animals. Partial purification of the enzyme was carried out by ammonium sulfate precipitation (18). Assay conditions for the solubilized pellet were 100 mM potassium phosphate buffer, pH 6.8, 3 mM EDTA, 1 mM dithiothreitol, 5 mM mercaptoethanol, 0.3 mM TPNH, and substrate concentrations as noted. The center group (acetyl- and malonyl-CoA) represents the standard assay substrate conditions.

Substrate conditions	Specific activity				Total activity			
	104,000 × *g* supernatant		$(NH_4)_2SO_4$ pellet		104,000 × *g* supernatant		$(NH_4)_2SO_4$ pellet	
	B_{12}-supplemented diet	B_{12}-deprived diet	B_{12}-supplemented diet	B_{12}-deprived diet	B_{12}-supplemented diet	B_{12}-deprived diet	B_{12}-supplemented diet	B_{12}-deprived diet
nmoles	*nmoles/min/mg protein*		*nmoles/min/mg protein*		*nmoles/min*		*nmoles/min*	
30 Acetyl-CoA	0.51	0.73	0.65	1.39	127	180	164	64
44 Malonyl-CoA	1.55	4.12	1.46	4.39	389	900	335	383
30 Propionyl-CoA	0.89	0.81	0.89	0.91	267	195	61	79
30 Acetyl-CoA + 44 malonyl-CoA	3.34	8.85	14.1	30.8	839	1934	1058	2690
30 Acetyl-CoA + 30 propionyl-CoA	0.948	0.867	0.49	0.23	341	189	364	20
44 Malonyl-CoA + 30 propionyl-CoA	3.78	11.8	16.4	43.3	950	2580	1231	3780
30 Acetyl-CoA + 44 malonyl-CoA + 30 propionyl-CoA	3.58	13.0	14.8	28.6	967	2840	2580	3960

TABLE IV

Acetyl-CoA carboxylase activity in liver and brain of B_{12}-supplemented and -deprived rats[a]

Representative acetyl-CoA carboxylase assay data from the 104,000 × *g* supernatant fraction of tissues obtained from B_{12}-deprived and B_{12}-supplemented animals. Assay conditions were as described by Lane and co-workers (19) with a reaction volume of 0.5 ml for the citrate preincubation and 1.0 ml for the assay incubation.

Assay conditions	Liver		Brain	
	B_{12}-supplemented diet	B_{12}-deprived diet	B_{12}-supplemented diet	B_{12}-deprived diet
	nmoles/min/mg protein			
Standard assay	9.1	13.8	0.48	0.47
Minus acetyl-CoA	0.13	0.18	0.03	0.03
Minus acetyl-CoA, Plus propionyl-CoA	6.0	8.7	0.83	1.14

[a] Activity in the 104,000 × *g* supernatant fraction.

total activity in the 104,000 × *g* supernatant and the partially purified fraction. As noted the B_{12}-deprived animals demonstrated an increase in both specific activity and total activity over their paired controls. Partial purification of the enzyme revealed the expected increase in specific activity, but the ratios between B_{12}-deprived and supplemented remained the same, suggesting the absence of an endogenous inhibitor in the supplemented state or activation in the B_{12}-deprived animals. The same relationships were seen in total activity for the two groups with a variety of substrate conditions.

Acetyl Coenzyme A Carboxylase Activity—Table IV shows the acetyl-CoA carboxylase activity in liver and brain of the 104,000 × *g* supernatant fraction for the B_{12}-supplemented and deprived animals as measured by $^{14}CO_2$ fixation under conditions of maximum carboxylation (19). Activity of this critical enzyme of fatty acid synthesis was also greater in the livers of the B_{12}-deprived animals than the livers of the control animals. In the absence of added acetyl-CoA, endogenous activity was very low. In both the B_{12}-supplemented and the deprived group, propionyl-CoA served as effective substrate for the reaction in liver as previously reported by Lane and co-workers (20), and at equal substrate concentrations propionyl-CoA was metabolized faster than acetyl-CoA in neural tissue.

TABLE V

Acetyl-CoA carboxylase activity in rat liver (effect of citrate on incubations)

Assay was performed on both the 104,000 × *g* supernatant fraction and an aliquot of that supernatant fraction further purified by gel filtration on Sephadex G-25 from which the void volume contained 90% of the enzyme activity.

Enzyme source and conditions	Specific activity		Total activity	
	B_{12}-supplemented diet	B_{12}-deprived diet	B_{12}-supplemented diet control	B_{12}-deprived diet
	nmoles/min/mg protein		*nmoles/min*	
104,000 × *g* supernatant fraction				
Absence of acetyl-CoA	0.18	0.06	26	9
No citrate preincubation	2.0	5.2	284	775
No citrate incubation	0.11	0.18	16	27
Complete system	5.7	12.3	809	1833
Gel-filtered supernatant fraction				
Absence of acetyl-CoA	0.02	0.05	1	3
No citrate preincubation	2.6	5.8	214	476
Complete system	6.1	13.5	503	1107

The stabilization of the polymeric active form of acetyl-CoA carboxylase has been demonstrated to require citrate or isocitrate (19, 20, 37, 38). The effect of citrate on enzyme activity, whether added during preincubation or during incubation, is shown in Table V for the 104,000 × *g* supernatant fraction as well as the partially purified gel-filtered enzyme. Fractions

TABLE VI

Acetyl-CoA carboxylase activity (liver admixture studies)

Conditions of preparation and assay are as previously described. In the admixture study aliquots of the 104,000 × *g* supernatant fraction were used. The aliquot from the B_{12}-supplemented or control group is labeled C and that from the B_{12}-deprived group is labeled B_{12}.

	Specific activity		Total activity	
	B_{12}-supplemented diet	B_{12}-deprived diet	B_{12}-supplemented diet	B_{12}-deprived diet
	nmoles/min/mg protein		*nmoles/min*	
104,000 × *g* supernatant fraction	2.6	18.1	225	1662
Gel-filtered supernatant fraction	2.0	11.6	105	694

Admixtures of supernatant fractions from liver

104,000 × *g* supernatant fraction	Specific activity	Total activity
1C:1 B_{12}	9.9[a] (10.4)	882 (943)
1C:3 B_{12}	12.6 (14.2)	1140 (1302)
3 C:1 B_{12}	6.0 (6.3)	526 (584)

[a] These numbers indicate the values found while the numbers in parentheses indicate the values expected.

derived from B_{12}-deprived tissues behave in the expected way in response to citrate addition.

As seen in Table VI, supernatant fractions from the livers of B_{12}-supplemented animals added to supernatant fractions of livers from B_{12}-deficient animals did not cause a reduction in fatty acid-synthesizing ability. This showed that no excess quantities of a putative inhibitor existed.

Identification of Product of Propionyl Coenzyme A Carboxylation Catalyzed by Acetyl-CoA Carboxylase—When acetyl-CoA is the substrate, the product of acetyl-CoA carboxylase-catalyzed reaction is malonyl-CoA (20). Although propionyl-CoA has been shown to be a substrate for the enzyme (20) the reaction product has not been identified in the normal or B_{12}-deprived state. If the CO_2 fixation reaction is like that of propionyl-CoA carboxylase, methylmalonyl-CoA would be the expected product. The isolation and identification of the products of the reaction were performed. Since a separate propionyl-CoA carboxylase, a mitochondrial enzyme, exists, the 104,000 × *g* supernatant fraction was carefully prepared in sucrose to avoid contamination with the mitochondrial fraction and hence the propionyl-CoA carboxylase (39). As shown in Table VII the similarity of specific activities with acetyl-CoA or propionyl-CoA or an admixture of the two as substrate suggested that propionyl-CoA competes with acetyl-CoA as substrate in the acetyl-CoA carboxylase reaction, and that propionyl-CoA was nearly as effective a substrate as acetyl-CoA. In addition (Table VII) the product formed from propionyl-CoA by the acetyl-CoA carboxylase reaction was isolated and identified as methylmalonyl-CoA. These results suggest the enzyme is specific for a CO_2 fixation at the α carbon.

Effect of Methylmalonyl Coenzyme A on Fatty Acid Synthetase and Acetyl Coenzyme A Carboxylase Activity—Propionic and methylmalonic acid are known to be increased in vitamin B_{12} deficiency (40, 41). As shown above propionyl-CoA appeared to be an effective substrate for the acetyl-CoA carboxylase-

TABLE VII

Identification of propionyl-CoA product of acetyl-CoA carboxylase reaction

The acetyl-CoA carboxylase assay was performed with substrate additions as indicated. Following the assay the products were subjected to alkaline hydrolysis (21). The free acidic products were then separated by acidification to pH 2 with concentrated HCl and extraction with diethyl ether. The radioactivity was determined and an aliquot separated by thin layer chromatography.

Substrates	$^{14}CO_2$ fixed	Specific activity	Thin layer chromatography recovery		
			Malonic acid	Methylmalonic acid	Succinic acid
mM	*cpm*	*nmoles/min/mg*	%	%	%
0.2 Acetyl-CoA	7050	8.1	97	—[a]	—
0.2 Propionyl-CoA	5250	6.0	—	89	—
0.1 Acetyl-CoA + 0.1 propionyl-CoA	6650	7.6	55	42	—
0.2 Acetyl-CoA + 0.2 propionyl-CoA	7150	8.2	56	44	—

[a] This indicates that none was recovered.

TABLE VIII

Effect of methylmalonyl-CoA on fatty acid synthetase activity from rat liver

A representative study of the effect of purified methylmalonyl-CoA on fatty acid synthetase activity. The assay was performed as previously noted with substrate additions as recorded.

Substrate conditions	B_{12}-supplemented diet	B_{12}-deprived diet
nmoles	*nmoles/min/mg protein*	
30 Acetyl-CoA	0	0
44 Malonyl-CoA	1.2	4.2
30 Propionyl-CoA	0	0
44 Methylmalonyl-CoA	0	0
30 Acetyl-CoA + 44 methylmalonyl-CoA	0	1.2
44 Malonyl-CoA + 44 methylmalonyl-CoA	0.5	2.0
30 Propionyl-CoA + 44 methylmalonyl-CoA	0.2	1.0
30 Acetyl-CoA + 44 malonyl-CoA	5.3	18.3
44 Malonyl-CoA + 30 propionyl-CoA	5.2	15.9
30 Acetyl-CoA + 44 malonyl-CoA + 44 methylmalonyl-CoA	1.6	5.6
44 Malonyl-CoA + 30 propionyl-CoA + 44 methylmalonyl-CoA	1.5	6.1
30 Acetyl-CoA + 44 malonyl-CoA + 30 propionyl-CoA	5.2	15.2
30 Acetyl-CoA + 44 malonyl-CoA + 30 propionyl-CoA + 44 methylmalonyl-CoA	1.5	6.6

catalyzed reaction with its product being methylmalonyl-CoA. Since Forward and Gompertz (42) have demonstrated that methylmalonyl-CoA inhibited fatty acid synthetase isolated from normal rat liver, the effect of this product on the regulatory enzymes in the B_{12}-supplemented and B_{12}-deprived animals was tested. As seen in Table VIII methylmalonyl-CoA appeared to

TABLE IX

Effect of methylmalonyl coenzyme A on acetyl coenzyme A carboxylase reaction from rat liver

A representative study of the effect of methylmalonyl-CoA on acetyl-CoA carboxylase activity. Substrate addition of methylmalonyl-CoA was same as that of acetyl-CoA. Assay as by Lane and co-workers (19).

Conditions	B_{12}-supplemented diet	B_{12}-deprived diet
	nmoles/min/mg protein	
Absence of acetyl-CoA	0.04	0.10
Absence of citrate	0.02	0.05
Complete system	1.04	2.78
Complete system + methylmalonyl-CoA added in preincubation	0.54	1.90
Complete system + methylmalonyl-CoA added in incubation	0.73	2.61

be a poor substrate in the fatty acid synthetase reaction. In addition, methylmalonyl-CoA markedly inhibited the reaction in both the control and the B_{12}-deprived animals, and the degree of inhibition was the same in both groups. In Table IX the effect of methylmalonyl-CoA on the liver acetyl-CoA carboxylase reaction is delineated. Suppression to approximately 50% activity was seen in both the B_{12}-supplemented and the B_{12}-deprived 104,000 $\times$ *g* supernatant fractions of liver when the methylmalonyl-CoA was present in the preincubation mixture. In other experiments not shown similar inhibition of both enzymes was seen in the neural tissue. Thus, methylmalonyl-CoA, the product of the reaction, inhibits both enzymes of fatty acid synthesis, and the suppression is similar in degree in both the B_{12}-supplemented and the B_{12}-deprived animals.

Immunochemical Characterization of Fatty Acid Synthetase Preparations—The results of the quantitative immunoprecipitin reaction and the immunochemical titration between the antiserum and the DEAE-cellulose-purified fatty acid synthetase preparations from the livers of B_{12}-deprived and B_{12}-supplemented animals are shown in Fig. 1. The specific activity of the DEAE-cellulose-purified fatty acid synthetase preparation from the liver of B_{12}-supplemented animals was 150 nmoles per min per mg of protein and that from the B_{12}-deprived animals was 328. Total enzyme activity was 1482 nmoles per min in the B_{12}-supplemented group and 4920 in the B_{12}-deprived state. As shown in Fig. 1 the immunochemical titration demonstrated that the equivalence point, based upon enzyme activity, was the same for the B_{12}-deprived and B_{12}-supplemented preparations.

DISCUSSION

The study of the enzymes of fatty acid synthesis in vitamin B_{12} deficiency was part of a study relating to the evidence that odd chain fatty acids were synthesized in nerves obtained from patients with pernicious anemia (2, 7). Recently, similar fatty acid abnormalities were demonstrated in a case of congenital methylmalonic aciduria (3). In addition, the synthesis of odd chain fatty acids has been demonstrated in rat glial tissue grown in vitamin B_{12}-deficient tissue culture media (6). Finally, Smith *et al.* (43) have demonstrated alterations in intermediary metabolism characterized by increased methylmalonic acid and coenzyme A content in the livers of vitamin B_{12}-deficient sheep. Forward and Gompertz (42) demonstrated that methylmalonyl-CoA had a competitive inhibitory effect on fatty acid synthetase activity in normal rat liver cytosol.

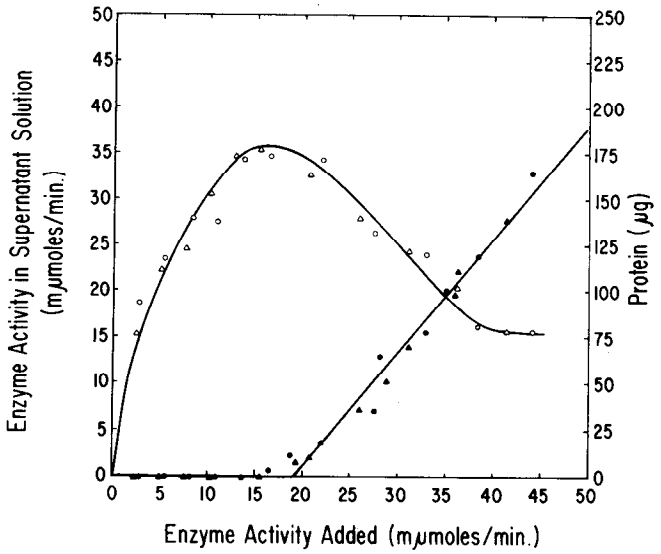

FIG. 1. Quantitative immunoprecipitation reaction and immunochemical titration between the antiserum and the DEAE-cellulose purified fatty acid synthetase preparations. ○, protein content of the antigen-antibody precipitate (27) from the preparation from the liver of B_{12}-supplemented animals; △, protein content of the antigen-antibody precipitate from the B_{12}-deprived group. The immunochemical titration employed increasing amounts of the purified enzyme preparation from the livers of B_{12}-supplemented (●) or the B_{12}-deprived animals (▲).

Our studies demonstrated that the activity of both fatty acid synthetase and acetyl-CoA carboxylase activity was approximately 3-fold greater in the cytosol of the B_{12}-deprived animals as compared to the B_{12}-supplemented controls. Several mechanisms for these differences in enzyme activity were excluded. First, since the dietary intake critically affects the levels of enzymes of synthesis of fatty acids (18, 19, 35–37), the present studies were carried out in pairs of animals under the same conditions of dietary intake and glucose supplementation (37) in order to eliminate the variables of diet as a cause of differing enzyme activity (44). Further support for the adequacy of the dietary control and the specificity of the enzyme changes for B_{12} deficiency *per se* is the fact that serial enzyme assays during the early period of deprivation failed to demonstrate any significant differences. The enzyme changes were noted only after evidence of tissue depletion of B_{12} and altered propionate catabolism was obtained. That these changes were not the result of altered protein content of the cell (45) in the B_{12}-deprived state was supported by the evidence of increased total activity as well as specific activity. The increased fatty acid synthetase activity in the cytosol of the B_{12}-deprived animals was not due to an activator since partial purification of the enzyme by ammonium sulfate precipitation and subsequent "admixture" studies failed to demonstrate increased activity of the enzyme in the B_{12}-supplemented animals. Similarly no inhibitor could be demonstrated in the cytosol of the B_{12}-supplemented animals. The same was true for acetyl-CoA carboxylase (Table VI). The findings thus suggest that B_{12} deprivation results in an increase in the total quantity of the enzymes, fatty acid synthetase, and acetyl-CoA carboxylase. This conclusion is strongly supported by the immunochemical evidence (Fig. 1) that the difference in fatty acid synthetase activity in the B_{12}-supplemented and B_{12}-deprived animals is associated with proportionate changes in the quantity of immunologically identifiable enzyme.

The reasons for the increased enzyme activity in broken cell

preparations are not known. It should be noted, however, that fatty acid synthesis as measured with the tritiated water method (46) was also higher in the B_{12}-deprived animals[2] suggesting that the unusual activity of the acetyl-CoA carboxylase and fatty acid synthetase was physiologically significant.

The direct demonstration that propionyl-CoA can serve as an effective substrate for both the fatty acid synthetase and the acetyl-CoA carboxylase enzymes in B_{12} deprivation is of interest. The presence of propionic aciduria in B_{12} deficiency suggests that the parent CoA derivative accumulates intracellularly to levels capable of providing significant substrate for fatty acid synthesis. If so, it follows that the synthesis of odd chain fatty acids in this condition occurs over the normal fatty acid-synthesizing mechanisms and does not require a unique pathway. Presumably this is also true in nerve, where abnormal fatty acids have been shown (2, 3, 7) to accumulate in B_{12} deficiency in man.

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[2] E. P. Frenkel, R. L. Kitchens, and J. M. Johnston, unpublished observations.