VITAMIN B_{12} AND PROPIONATE METABOLISM *

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In the course of a comprehensive investigation of vitamin B_{12}-deficiency in sheep, Marston, by means of tolerance tests with carefully paired animals, established unequivocally that the supervening malady is the result of an impairment of the animal's capacity to deal with acetic and propionic acids; and, later with Allen and Smith, observed that massive doses of folic acid reinstated normal capacity to deal with acetic acid, but left the inability to metabolize propionic acid as an uncomplicated consequence of vitamin B_{12}-deficiency. This he considered to be the basic metabolic lesion of vitamin B_{12}-deficiency in the ruminant (Marston 1958). With Smith, the capacity of homogenates of livers from vitamin B_{12}-deficient sheep to convert propionyl-Co A to succinate was observed to be very seriously impaired. Subsequently, Smith investigated this phenomenon in rats rendered vitamin B_{12}-deficient by means devised by Marston, and observed a similar failure.

The present investigation has been concerned with an examination of the activity in the liver of vitamin B_{12}-deficient rats of the two enzymes responsible for the conversion of propionyl-Co A to succinate (Flavin and Ochoa, 1957). A series of measurements has been made with liver homogenates of the activity of propionyl-Co A carboxylase (Flavin,

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Castro-Mendoza, and Ochoa, 1957), which catalyzes the addition of CO_2 to propionyl-Co A to form methylmalonyl-Co A, and of methylmalonyl-Co A isomerase (Beck, et al., 1957, Beck and Ochoa, 1958), which catalyzes a reversible conversion of methylmalonyl-Co A to succinyl-Co A.

Vitamin B_{12}-deficient rats were bred from depleted dams and supported on a diet comprised essentially of grain and yeast (Marston, unpublished). The control animals were given the same diet supplemented with 15 μgm. of vitamin B_{12} per 100 gm. of dry diet. Growth rates on the deficient and control diets are indicated in Table 1.

TABLE 1

Growth of Vitamin B_{12}-Deficient and Control Rats
Mean body wt. in grams, and RMS variations for groups of 12 rats each.

Age	4 weeks	6 weeks	9 weeks
B_{12}-deficient	59.8 ± 4.3	105.1 ± 11.2	180.9 ± 22.1
B_{12}-supplemented	60.5 ± 3.7	145.9 ± 14.0	255.2 ± 19.7

The basis of assay of both propionyl-Co A carboxylase and methylmalonyl-Co A isomerase was the measurement of the incorporation of C^{14} into dicarboxylic acids on incubation of homogenates with $C^{14}O_2$ and propionyl-Co A. Incubation was carried out for 20 min. at 37° C in a medium containing tris buffer, 0.05 M, pH 7.4; $MgCl_2$, 0.003 M; ATP, 0.003 M; glutathione, 0.005 M; propionyl-Co A, 0.0005 M; and $Na_2C^{14}O_3$, 0.01 M (2 μc C^{14}), in a total volume of 1.0 ml.

Animals were killed by a blow on the head, the liver removed and homogenized with 9 volumes of 0.04 M tris buffer, pH 8.0, containing 0.001 M neutralized cysteine. After incubation, the reactions were terminated by the addition of 0.2 ml. of 2 N KOH. Hydrolysis of the Co A thioesters was allowed to proceed for 30 minutes at room temperature, whereupon excess $C^{14}O_2$ was discharged by the addition of 0.2 ml. of 4 N HCl. Residual $C^{14}O_2$ was removed in vacuo over sodium hydroxide, and protein precipitated by heating to 100° C for 2 minutes. Carrier dicarboxylic acids were add-

ed before diluting and centrifuging to remove the protein. Total incorporation of $C^{14}O_2$ was measured in weighed aliquots plated on stainless steel planchets and counted with a thin window Geiger counter. The distribution of radioactivity among dicarboxylic acids was determined after extraction overnight with ether. The extracted acids were resolved chromatographically on paper with the 4 N formic acid - isoamyl alcohol system of Flavin and Ochoa (1957), and radioactivity determined with a Geiger strip scanning unit.

Carboxylating activity was measured as total counts fixed by aliquots of approximately 0.5 and 1.0 mg. of homogenate protein in the presence of an excess of a partially purified methylmalonyl-Co A isomerase. Isomerase for this purpose (1.8 mg. protein per flask) was prepared from sheep kidney cortex as described by Beck, et al. (1957), the preparation being carried through the stage of ammonium sulfate fractionation. Values obtained for homogenate carboxylase activity have been corrected for the small fixation of CO_2 found with the isomerase alone, and are expressed as counts per min. fixed per mg. homogenate protein in the 20 min. incubation period. Protein was determined by the method of Lowry, et al. (1951).

Methylmalonyl-Co A isomerase in the homogenate was assayed by the rate of formation of succinic, fumaric, and malic acids by aliquots containing approximately 0.9, 1.8, and 2.7 mg. of protein. Under these conditions the major radioactive product extracted into ether is methylmalonic acid, while the accumulation of other labelled dicarboxylic acids bears an approximately linear relation to the quantity of homogenate protein present.

Comparable data indicating carboxylase and isomerase activities in liver homogenates of B_{12}-deficient and B_{12}-treated animals is given in Table 2. In the experiments listed, simultaneous measurements were made on both deficient and control animals, at ages varying between 6.5 and 9.5 weeks.

It is apparent from Table 2 that there is no significant difference

TABLE 2

Carboxylase and Isomerase Activities in Deficient and Control Rat Liver Homogenates

Cited as cpm/mg. protein

B_{12} Deficient			B_{12} Treated		
Carboxylase	Isomerase		Carboxylase	Isomerase	
	meth.mal.	malic+succ. +fum.		meth.mal.	malic+succ +fum.
10,100	5,500	300	9,600	5,000	1,300
8,500	5,100	200	10,100	4,800	1,400
10,100	6,000	200	8,300	4,400	900

in carboxylating enzyme activity between B_{12}-deficient and B_{12}-treated animals. However, in the B_{12}-deficient homogenate the accumulation of succinate, fumarate and malate is severely retarded. Apart from a reduction of methylmalonyl-Co A isomerase, two other explanations may be suggested to account for these data. Either a high rate of hydrolysis of the thioester bond of methylmalonyl-Co A in the deficient homogenate, or a high rate of oxidation of succinic and malic acids by the deficient homogenate, leading to a reconversion of C^{14} to $C^{14}O_2$, could have led to a failure to accumulate C^{14} in succinic, malic, and fumaric acids. The first is unlikely, since in the presence of excess methylmalonyl-Co A isomerase, which is inactive toward free methylmalonic acid, both deficient and control fixation products are almost completely converted to succinic, fumaric, and malic acids. The second alternative was examined by carrying out the reaction in the presence of cyanide (1.67×10^{-3} M). Under these conditions succinic acid accumulated and little malic acid was formed in both deficient and control flasks. The deficient homogenate again exhibited only a small rate of conversion of methylmalonyl-Co A to succinate.

It is concluded that in rats deficient in vitamin B_{12}, the activity of the liver enzyme catalyzing the isomerization of methylmalonyl-Co A and

succinyl-Co A is severely depressed. In some respects this reaction is analogous to the isomerization of glutamic and methylaspartic acids, catalyzed by an enzyme found in Clostridium tetanomorphum. This enzyme has been shown by Barker, et al. (1958), to exhibit a cofactor requirement for a derivative of pseudovitamin B_{12}.

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