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Gluconeogenesis from Propionate in Kidney and Liver of the Vitamin B_{12}-deficient Rat

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1. Kidney-cortex slices and the perfused livers of vitamin B_{12}-deficient rats removed propionate from the incubation and perfusion media at 33 and 17% respectively of the rates found with tissues from rats receiving either a normal or a vitamin B_{12}-supplemented diet. There was a corresponding fall in the rates of glucose synthesis from propionate in both tissues. 2. The addition of hydroxocobalamin or dimethylbenzimidazolylcobamide coenzyme to kidney-cortex slices from vitamin B_{12}-deficient rats *in vitro* failed to restore the normal capacity for propionate metabolism. 3. Although the vitamin B_{12}-deficient rat excretes measurable amounts of methylmalonate, no methylmalonate production could be detected (probably because of the low sensitivity of the method) when kidney-cortex slices or livers from deficient rats were incubated or perfused with propionate. 4. The addition of methylmalonate (5mM) to kidney-cortex slices from rats fed on a normal diet inhibited gluconeogenesis from propionate by 25%. 5. Methylmalonate formation is normally only a small fraction of the flux through methylmalonyl-CoA. This fraction increases in vitamin B_{12}-deficient tissues (as shown by the urinary excretion of methylmalonate) presumably because the concentration of methylmalonyl-CoA rises as a result of low activity of methylmalonyl-CoA mutase (EC 5.4.99.2). Slow removal of methylmalonyl-CoA might depress propionate uptake owing to the reversibility of the steps leading to methylmalonyl-CoA formation.

Vitamin B_{12} is required as an essential cofactor in the metabolism of propionate in the interconversion of methylmalonyl-CoA and succinyl-CoA catalysed by methylmalonyl-CoA mutase (EC 5.4.99.2) (Weissbach, Toohey & Barker, 1959; Lengyel, Mazumder & Ochoa, 1960). Experiments with sheep liver homogenates have shown that propionate utilization by the liver is impaired when there is no dietary supply of vitamin B_{12} (Marston, Allen & Smith, 1961). In man vitamin B_{12} deficiency causes an increased urinary excretion not only of propionate (White, 1965; Cox, Robertson-Smith, Small & White, 1968) but also of methylmalonate (Cox & White, 1962). This is not a normal intermediate of propionate metabolism, but is formed by a side reaction in which methylmalonyl-CoA is deacylated. Methylmalonate excretion has also been observed in vitamin B_{12}-deficient rats (Barness, Flaks, Young, Tedesco & Nocho, 1963; Williams, Spray, Newman & O'Brien, 1969).

The experiments reported in this paper demonstrate that the capacity to utilize propionate and to convert it into glucose is decreased in the isolated perfused liver and in kidney-cortex slices of vitamin B_{12}-deficient rats, but there is no detectable methylmalonate production by the vitamin B_{12}-deficient tissues. This indicates that the rate of the side reaction by which methylmalonyl-CoA is deacylated is very slow in these tissues compared with the rate of its normal conversion into succinyl-CoA.

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EXPERIMENTAL

Experimental animals. Second-generation vitamin B_{12}-deficient rats were reared as described by Williams *et al.* (1969). Deficient animals were chosen for the present experiments on the basis of a high excretion rate of methylmalonate (about 20–30mg/day). In each case litter mates receiving the same diet plus a vitamin B_{12} supplement (15μg of cyanocobalamin/kg of diet) were used as controls.

Incubation procedure. Washed kidney-cortex slices were incubated as described by Weidemann & Krebs (1969) in the bicarbonate-buffered saline of Krebs & Henseleit (1932) with phosphate omitted. For the determination of metabolites the medium was deproteinized with $HClO_4$ as described by Gevers & Krebs (1966).

Rat liver perfusions were carried out by the method of Hems, Ross, Berry & Krebs (1966).

Analytical methods. Glucose, lactate, pyruvate, acetoacetate and β-hydroxybutyrate were determined spectrophotometrically by enzymic methods as described by Weidemann & Krebs (1969). Attempts were made to estimate methylmalonate by the colorimetric method described for urine by Giorgio & Plaut (1965). A high blank colour contributed by acetoacetate was overcome by boiling the solutions for 1 h before the estimation of methylmalonate. In the samples obtained in liver perfusion experiments the glucose formed caused a fading of the green colour. Samples (1 ml) of the perfusate, after removal of the erythrocytes by centrifugation, were mixed with 0.3 ml of 20% (w/v) trichloroacetic acid and heated at 100°C for 60 min. After cooling 0.1 ml of glucose oxidase solution (130 units/ml) was added and the mixtures were incubated at 37°C for 10 min. The volumes were made up to 2 ml with water, the precipitates removed by centrifugation and the clear supernatant solutions (1 ml) mixed with 1 ml of 0.1 M-HCl for the estimation of methylmalonic acid. After development of the colour, the solutions were extracted with 4 ml of 'amyl alcohol' (British Drug Houses Ltd., Poole, Dorset, U.K.) and the extinctions of the 'amyl alcohol' layers were measured. Under these conditions between 104 and 124% of pure samples of methylmalonic acid (25 μg) added to 1 ml portions of six different samples of the final perfusion medium were recovered.

Propionate disappearance in experiments with kidney slices was determined by g.l.c. (Baumgardt, 1964) with valeric (pentanoic) acid as the internal standard. In the liver perfusion experiments propionate disappearance was determined by a modification of the Conway microdiffusion method as described for acetate by Serlin & Cotzias (1955).

The microbiological assay for vitamin B_{12} activity of the rat tissues was carried out as described by Booth & Spray (1960).

RESULTS

Propionate uptake and gluconeogenesis by kidney-cortex slices from vitamin B_{12}-deficient rats. Propionate removal by kidney-cortex slices of the vitamin B_{12}-deficient rats was much lower (about 33% of the normal value) than in control slices, and there was a proportional fall in the rate of glucose synthesis from 100 ± 7 to 26 ± 4 μmol/h per g dry wt. of tissue (Table 1). Under the same conditions the rates of gluconeogenesis from succinate, glutamate, α-oxoglutarate, lactate, pyruvate and fumarate (not recorded in Table 1) were not significantly different in the kidneys from rats given vitamin B_{12}-deficient and supplemented diets. Thus vitamin B_{12} deficiency affects specifically gluconeogenesis from propionate. Although addition of propionate caused an increased lactate formation in the control slices (Weidemann & Krebs, 1969) there was no corresponding increase with the vitamin B_{12}-deficient tissue. Acetoacetate (5mM) was added in these experiments to provide the major part of the respiratory fuel. Under these conditions only a small fraction of propionate was unaccounted for by the formation of glucose, lactate and pyruvate in both dietary states; this fraction must have been either completely oxidized to carbon dioxide or converted into other intermediates or products. No formation of methylmalonate could be detected with slices from either group of rats even when 1.6 ml of the incubation medium was analysed. The negative findings indicate that less than 10 μmol of methylmalonate/per g dry wt. was formed. The decreased propionate uptake (Table 1) in vitamin B_{12} deficiency indicates

Table 1. *Effect of a vitamin B_{12}-deficient diet on gluconeogenesis from propionate by rat kidney-cortex slices*

Kidney-cortex slices were incubated for 60 min in the bicarbonate-buffered saline of Krebs & Henseleit (1932) with phosphate omitted and 5 mM-acetoacetate added. For other experimental details see the text. The results are given as means ± S.E.M. (three experiments for each of the dietary treatments).

Dietary state ...	Vitamin B_{12}-supplemented diet		Vitamin B_{12}-deficient diet	
Substrates added ...	None	Propionate (2mM) + DL-carnitine (4mM) + acetoacetate (5mM)	None	Propionate (2mM) + DL-carnitine (4mM) + acetoacetate (5mM)
Metabolic changes (μmol/h per g dry wt.)				
Propionate removed		260 ±30		85 ±25
Glucose formed	9.1 ± 2.1	100 ± 7	10.0 ± 1.5	26 ± 4
Lactate formed	9.2 ± 1.3	20 ± 2	8.0 ± 1.7	10 ± 1.4
Pyruvate formed	2.1 ± 0.4	2.9 ± 0.3	1.2 ± 0.3	1.8 ± 0.4
Acetoacetate used		310 ± 9		177 ±18
β-Hydroxybutyrate formed		93 ± 5		32 ± 4
Propionate used not accounted for		37.2		21
Vitamin B_{12} content of tissue (μg/g fresh wt.)	1.19 ± 0.46		0.12 ± 0.02	

Table 2. *Activity of propionyl-CoA carboxylase in liver and kidney of normal, vitamin B_{12}-supplemented and vitamin B_{12}-deficient rats*

The capacity of kidney and liver homogenates to fix $^{14}CO_2$ in the presence and absence of added propionate was measured and the increase caused by propionate was taken to be a measure of propionyl-CoA carboxylase activity (see Baird *et al.* 1968). The activity is expressed as μmol of CO_2 fixed/min per g fresh wt. at 25°C (mean ±S.E.M. of no. of observations in parentheses). The dry wt./fresh wt. ratio was 3.7 for liver and 4.2 for kidney cortex. 'Endogenous' values represent $^{14}CO_2$ incorporation in the absence of added propionate.

	Enzyme activity	
	Liver	Kidney
Endogenous	0.25, 0.39	0.59
Normal diet	1.87±0.14 (4)	1.92 (1)
Vitamin B_{12}-supplemented diet	1.36	1.65
Vitamin B_{12}-deficient diet	1.43±0.26 (3)	1.80±0.20 (3)

Table 3. *Effect of methylmalonate on gluconeogenesis from propionate by rat kidney-cortex slices*

Kidney-cortex slices from 48h-starved rats were incubated for 60min in the phosphate-buffered saline of Krebs & de Gasquet (1964). In addition to the substrates given, all cups contained acetoacetate (5mM) and DL-carnitine (4mM). For other experimental details see the text.

	Metabolic changes (μmol/h per g dry wt.)	
Additions	Oxygen uptake	Glucose formation
None	1023	27.4
Methylmalonate (1mM)	1145	32.3
Methylmalonate (5mM)	1073	38.5
Propionate (2.5mM)	1200	89.3
Propionate (2.5mM)+ methylmalonate (1mM)	1240	89.0
Propionate (2.5mM)+ methylmalonate (5mM)	1170	66.1

that it is the first step of propionate metabolism that is inhibited, i.e. the formation of propionyl-CoA. The cause of the inhibition is not yet understood. The assay of propionyl-CoA carboxylase (see Table 2) gave virtually equal values in normal and deficient rat liver and kidney. This assay determines enzyme activity under standardized optimum conditions. The low activity of the enzyme *in vivo* may be due to product inhibition (by methylmalonyl-CoA) or to unfavourable substrate concentrations (low propionyl-CoA concentration).

Two effects of vitamin B_{12} deficiency can be attributed to the decreased propionate uptake: the removal of acetoacetate was decreased by 43% and the formation of β-hydroxybutyrate was decreased by 67%. Because acetoacetate provides the main fuel, a decreased rate of its removal would be expected in view of the decreased energy required for a lower rate of gluconeogenesis. The lower rate of acetoacetate reduction to β-hydroxybutyrate may be connected with the fact that in tissue from the vitamin B_{12}-supplemented animals the dehydrogenation of succinate derived from propionate normally spares NADH oxidation (Kulka, Krebs & Eggleston, 1961).

Effect of adding methylmalonate to the incubation medium. Methylmalonate at 1mM or 5mM caused a small increase in gluconeogenesis by normal kidney-cortex slices (Table 3). Gluconeogenesis from propionate was not affected by the addition of methylmalonate at the lower concentration, but at the higher concentration it was inhibited by 25%. Thus a rate of methylmalonate formation below the limit of detection by the method employed (<25nM) could not explain the decreased uptake of propionate by the vitamin B_{12}-deficient tissue.

Effect of adding vitamin B_{12} to the incubation medium in vitro. Hydroxocobalamin (10μg/ml) had no effect on either propionate uptake or gluconeogenesis. The addition of dimethylbenzimidazolylcobamide coenzyme (10μg/ml) increased propionate uptake by the vitamin B_{12}-deficient slices slightly (Table 4). The failure of the added cofactor to restore the normal function in the deficient tissue is in contrast with the striking effect *in vitro* of vitamin B_1 on pyruvate metabolism (Peters & Thompson, 1934).

Glucose formation from propionate by the isolated perfused rat liver. Optimum conditions were established in preliminary tests with livers from normal rats. A concentration of 4mM-propionate was used throughout; this avoided the inhibitory effects on gluconeogenesis of high propionate concentration and permitted an accurate measurement of propionate uptake to be made. Carnitine was not included in the perfusion medium as, in contrast with experiments with kidney-cortex slices (Weidemann & Krebs, 1969), its addition had no effect on gluconeogenesis by the perfused liver. Livers from normal rats and from rats on the experimental diet supplemented with vitamin B_{12} formed glucose from propionate at a rate of 0.3–0.4μmol/min per g in excess of the endogenous rate (0.14μmol/min per g fresh wt.). In contrast, propionate failed to stimulate gluconeogenesis above the endogenous rate in livers from vitamin B_{12}-deficient rats (Table 5). Propionate was

Table 4. *Effect of dimethylbenzimidazolylcobamide coenzyme and hydroxocobalamin on propionate uptake and gluconeogenesis by rat kidney-cortex slices*

Kidney-cortex slices were incubated under the conditions given in Table 1. Each cup contained propionate (2.0mM), DL-carnitine (4.0mM) and acetoacetate (5.0mM). In Expt. 2 the incubation was carried out under red light (Kodak no. 1 Safelight). For other experimental details see the text.

			Metabolic changes (μmol/h per g dry wt.)					
		Additions	None		Dimethylbenzimidazolyl-cobamide coenzyme (10μg/ml)		Hydroxocobalamin (10μg/ml)	
Expt. no.	Dietary state	Changes measured	Control animal	B_{12}-deficient animal	Control animal	B_{12}-deficient animal	Control animal	B_{12}-deficient animal
1	Well-fed	Propionate used	311	134	—	—	304	131
		Glucose formed	100	34	—	—	96	35
2	Starved (48h)	Propionate used	208	53	218	64	—	—
		Glucose formed	87	22	99	22	—	—

Table 5. *Gluconeogenesis from propionate by the perfused livers of normal, vitamin B_{12}-supplemented and vitamin B_{12}-deficient rats*

All animals were starved for 48h before perfusion. Propionate (4mM) was added after 40min and perfusion was continued for a further 90min. The results are given as means ± S.E.M. (numbers of experiments in parentheses). The endogenous rate of gluconeogenesis without substrates in normal rats is 0.14±0.03μmol/min per g wet wt. of tissue (5).

	Metabolic changes (μmol/min per g fresh wt.)		
Dietary state	Normal	Vitamin B_{12}-supplemented	Vitamin B_{12}-deficient
Propionate removed		0.75±0.08 (3)	0.13±0.06 (3) ($P<0.0025$)
Glucose formed	0.55±0.09 (4)	0.44±0.06 (4)	0.13±0.02 (4) ($P<0.0025$)
Vitamin B_{12} content of tissue (μg/g fresh wt.)		0.12±0.04 (3)	0.02±0.01 (3)

removed by the livers of vitamin B_{12}-supplemented animals at a rate of 0.75μmol/min per g or slightly in excess of the total amount required to form 0.3μmol of glucose/min per g. The rate of propionate removal by the livers of vitamin B_{12}-deficient rats was only 17% of that in liver of vitamin B_{12}-supplemented litter mates. As with the kidney-cortex experiments no methylmalonate was found in the perfusion medium. Rates of formation less than 0.02μmol/min per g could not be detected by the available method.

DISCUSSION

The failure of liver and kidney preparations from vitamin B_{12}-deficient rats to metabolize propionate at the normal rate is expected in view of the involvement of vitamin B_{12} as a cofactor in the reaction catalysing the conversion of methylmalonyl-CoA into succinyl-CoA. The inability to detect significant methylmalonate formation by the vitamin B_{12}-deficient tissues implies that its rate of formation is less than the rate of propionate removal, as methylmalonate formed at this rate would have been detected by the method used. When 1mmol of propionate is given to intact vitamin B_{12}-deficient rats by intraperitoneal injection about 30% can be accounted for as methylmalonate. Had this proportion been formed in kidney-cortex slices it would not have been measurable.

As already mentioned methylmalonate is not a normal intermediate whereas its CoA derivative is. The side reaction by which methylmalonate is formed must normally be very slow, probably because of the low concentration of methylmalonyl-CoA. When the concentration of methylmalonyl-CoA rises, because of the low activity of the mutase in vitamin B_{12} deficiency, the proportion of methylmalonyl-CoA undergoing deacylation would be expected to rise.

The decreased rate of removal of propionate in vitamin B_{12} deficiency can be explained in terms of a slow removal of methylmalonyl-CoA by the combined activities of the mutase and deacylase. The initial stages of propionate metabolism leading to the formation of intramitochondrial methylmalonyl-CoA are not necessarily directly affected by vitamin B_{12} deficiency. Indirectly they may be affected by unfavourable equilibrium conditions or by feedback inhibitions on an early stage of propionate metabolism. However, an increased steady-state concentration of methylmalonyl-CoA might depress propionate uptake owing to the ready reversibility of the steps leading to its formation (Weidemann & Krebs, 1969), or by directly inhibiting one of the enzymes involved in propionyl-CoA formation and carboxylation. A further possibility is that the steady-state concentration of free CoA falls as the methylmalonyl-CoA concentration rises and that this decreases the rate of the formation of intramitochondrial propionyl-CoA.

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