The Incorporation of Acetate-2-^{14}C and Mevalonate-2-^{14}C into Cholesterol During Vitamin B_{12} Deficiency (35393)

E. E. ARMSTEAD, J. M. HSU, AND B. F. CHOW

Department of Biochemistry, The Johns Hopkins University, and The Biochemistry Research Laboratory, Veterans Administration Hospital, Baltimore, Maryland 21218

Vitamin B_{12} and its coenzyme forms are essential components in various areas of intermediary metabolism but their role in cholesterol metabolism has not been clarified. Forbes and Patterson (1), Hsu and Chow (2), and Icayan and Chow (3) have presented evidence demonstrating that vitamin B_{12} is an important factor in cholesterol metabolism. The current research project was undertaken to study possible B_{12} involvement in cholesterol synthesis, the following questions being of particular interest: (a) At which steps in the cholesterol biosynthetic pathway might the presence of vitamin B_{12} be required, and (b) Is the effect of vitamin B_{12} deficiency limited to hepatic cholesterol metabolism or is it a general phenomenon?

Materials and Methods. Materials. Radiochemicals. Sodium acetate-2-^{14}C (sp act 8.56 mCi/mole) was purchased from Tracerlab-Keleket, Waltham, Massachusetts. Toluene-^{14}C standard and DL-mevalonic acid-2-^{14}C–lactone (sp act 3.35 mCi/mmole) were purchased from Nuclear-Chicago, Des Plaines, Illinois. Cholesterol purchased from Matheson Co. was recrystallized five times from absolute ethanol. PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-2-(5-phenylaxazolyl) Benzene], scintillation grade, were obtained from Packard Instrument Company. All other chemicals were ACS grade.

Experimental animals. Five-month-old McCollum strain female rats were mated and then maintained on a 62% soybean protein diet (3), unsupplemented with cyanocobalamin throughout gestation and lactation. Pregnant rats were housed separately in raised, wide-mesh, screen-bottomed cages until day 19 of gestation at which time, they were transferred to cages containing bedding. To limit coprophagy, bedding was changed once a day and fecal pellets seen at any time were removed with forceps. A 28 day lactation period was used to ensure survival of progeny. At the end of that period, progeny were separated on the basis of sex, age, and body weight. Female offsprings were discarded while male progeny were divided into two groups, one receiving the basal diet without vitamin B_{12} and the other, the same diet containing 50 μg of B_{12}/kg of diet.

At the end of the B_{12} deprivation period, rats were fasted overnight but with water *ad libitum.* On the following morning, animals were weighed, given the radioactive form of a cholesterol precursor through intraperitoneal injection, and were housed in metabolism cages under an exhaust hood for the duration of the incorporation period. Subsequently, animals were sacrificed under light ether anesthesia by cardiac puncture. Blood, along with various tissues, was either processed immediately for assay or prepared for storage in a frozen state until used.

Methods. Quantitative and radiometric measurement of cholesterol. Cholesterol was isolated according to the method of Sperry and Webb (4) with volumes of reagents being adjusted to accommodate sample size. Precipitated cholesteryl digitonide was dissolved in 1.0 ml of glacial acetic acid at 100°. For colorimetric analysis, 0.1-ml aliquots were diluted with glacial acetic acid to a volume of 2.0 ml. The Liebermann-Burchard reaction was employed for color development and its absorbance was measured in a Spectronic 20 photometer at 625 mμ. Aliquots (0.2 and 0.4 ml) were used for radioactivity tests. Samples were added to 20-ml capacity low potassium counting vials and 10 ml of chilled Diatol scintillation solvent were dispensed into each vial. The

TABLE I. Liver and Plasma Cholesterol Content in B_{12} Deficient and Treated Rats.[a]

Group	Type of diet	Total	Free	Ester	F/T
		Liver (mg/g)			
A	B_{12} supplemented	2.46 ± 0.02	1.98 ± 0.04	0.60 ± 0.02	0.76 ± 0.03
B	B_{12} deficient	2.20 ± 0.01	1.82 ± 0.02	0.40 ± 0.02[b]	0.83 ± 0.02
		Plasma (mg/100 ml)			
A	B_{12} supplemented	111 ± 4.4	32 ± 6.0	79 ± 10.0	0.29 ± 0.05
B	B_{12} deficient	77 ± 12.0[b]	38 ± 3.0	39 ± 10.9[c]	0.49 ± 0.09[b]

[a] Each value represents an average of five, 8-month-old male rats. Results are given as mean ± SEM.

[b] Significant differences between B_{12} supplemented and B_{12} deficient groups are marked: $p < 0.05$; [c] $p < 0.01$.

solvent system, Diatol, consisted of toluene, 350 ml; anhydrous methanol, 210 ml; recrystallized naphthalene, 73 g; dioxane, 350 ml; and the phosphors: PPO, 4.6 g and POPOP, 80 mg. Corrections for quench were made through the internal standard method. Samples were counted for 10-min intervals in the Tri-carb liquid scintillation counter, model 314AX. To estimate the counting efficiency, either sodium acetate-2-^{14}C or mevalonic acid-2-^{14}C–lactone standards were run as well as a commercial toluene-^{14}C standard. Total lipid determination: Soxhlet extraction was employed for the isolation of total lipid. Tissues were ground with anhydrous sodium sulfate, stored in a vacuum desiccator to dry overnight and on the next day, were extracted with chloroform for a period of 16 hr at 80°. Lipid extracts were brought to dryness through air evaporation and were stored in a desiccator until constant weight was reached. Total lipid was determined gravimetrically.

Results. Data in Table I show that in B_{12} deficient rats as compared with B_{12} treated, liver total cholesterol was lower by 15% and the ester fraction was diminished by 33% ($p<0.01$). Free cholesterol was not appreciably altered; the proportion of free to total cholesterol was comparable between the two groups. Plasma analysis revealed that total cholesterol was markedly decreased in B_{12} deficient rats, the difference between the two groups being 40%. Plasma free cholesterol was not altered but the ester fraction of the deficient rat was reduced by 50% ($p<0.05$). Further, the plasma free to total ratio was significantly higher for the B_{12} deficient group.

Data in Table II show that after 30 min, sp act of hepatic cholesterol in the deficient group was significantly higher than in the treated group ($p<0.05$). These findings were in agreement with those of Icayan and Chow (3). A significant increase in radioactivity per gram and in specific activity of cholesterol was also noted in B_{12} deficient rats 3 hr after

TABLE II. Incorporation of Acetate-2-^{14}C into Liver Cholesterol.[a]

Group	No. of rats	Incorporation period (min)	Cholesterol (mg/g of liver)	Radioactivity isolated CH (cpm/g of liver)	Sp act (cpm/mg of CH)
A	4	30	2.32 ± 0.15	1315 ± 322	562 ± 123
B	5	30	1.98 ± 0.12	1860 ± 180	971 ± 135[b]
A	6	180	2.56 ± 0.07	884 ± 199	350 ± 82
B	6	180	2.08 ± 0.06	1818 ± 296[b]	872 ± 192[b]

[a] Each rat received a single intraperitoneal injection of sodium acetate-2-^{14}C (5 μCi/0.2 mmole/100 g of body wt); and was killed by decapitation at indicated time intervals. Results are given as mean ± SEM.

[b] Significant differences between B_{12} supplemented and B_{12} deficient groups are marked: $p < 0.05$.

TABLE III. Incorporation of Mevalonic-^{14}C–Lactone into Liver Cholesterol by B_{12} Deficient and Treated Male Rats.[a]

Group	No. of rats	Type of diet	Incorporation period (min)	Cholesterol (mg/g of liver)	Radioactivity isolated CH (cpm/g of liver)	Sp act (cpm/mg of CH)
A	3	B_{12} supplemented	30	2.53 ± 0.1	$10{,}009 \pm 1424$	3918 ± 223
B	2	B_{12} supplemented	30	2.16 ± 0.08	$10{,}168 \pm 164$	4702 ± 264
C	6	B_{12} deficient	30	2.07 ± 0.17	7704 ± 1213	3724 ± 496
A	12	B_{12} supplemented	120	2.17 ± 0.04	8438 ± 379	3888 ± 106
C	14	B_{12} deficient	120	2.04 ± 0.05	7350 ± 384	3615 ± 143
A	3	B_{12} supplemented	240	2.59 ± 0.07	9619 ± 1139	3702 ± 283
B	3	B_{12} supplemented	240	2.45 ± 0.09	$10{,}758 \pm 1209$	4372 ± 294
C	6	B_{12} deficient	240	2.26 ± 0.07	6092 ± 859[b]	2679 ± 328[c]

[a] Groups A and C were progeny of mothers fed the vitamin B_{12} deficient diet during pregnancy and lactation, and Group B was progeny of mothers fed Purina Lab Chow during pregnancy and lactation. Results are given as mean ± SEM.

[b] Significant differences between B_{12} deficient rats killed at 30 and 120 min and at 240 min are marked: $p < 0.05$.

[c] Significant differences between supplemented and deficient groups killed at 240 min are marked: $p < 0.05$.

isotope injection.

After 30 min and 2 hr the amount of the mevalonate radioactivity in the liver cholesterol shown in Table III was found to be the same in the B_{12} deficient and B_{12} supplemented rats. After 4 hr the concentration of radioactive cholesterol (cpm/g of liver and its sp act) decreased significantly in B_{12} deficient rats. Such changes were not observed in B_{12} supplemented animals.

To determine the effects of vitamin B_{12} on cholesterol synthesis in the organs other than the liver, the testes, adrenals, and brain were examined. Table IV shows that no observable difference in relative weights of the above 3 organs between B_{12} deficient and B_{12} supplemented rats. The total lipid and cholesterol contents in the adrenals of B_{12} deficient rats were markedly increased over those of B_{12} supplemented rats. Although differences were not noticed in radioactivity per gram of tissue, the sp act was significantly reduced in B_{12} deficient rats. Unlike adrenals, deficiency of B_{12} had no effect on brain cholesterol. In the testes, cholesterol content was unaffected by B_{12} deficiency. But the radioac-

TABLE IV. Cholesterol Content and Synthesis in Extrahepatic Tissues of Vitamin B_{12} Deficient and Treated Male Rats.

Group	Organ	Relative organ wt	Lipid (mg of CH/g)	Cholesterol (mg/g of tissue)	Radioactivity isolated CH (cpm/g of tissue)	Sp act (cpm/mg of CH)
A	Testes	0.95 ± 0.02	—	1.50 ± 0.13	207 ± 39	140 ± 8
B		1.05 ± 0.04		1.74 ± 0.08	120 ± 18	68 ± 8[b]
A	Adrenals	6.67 ± 0.41	46 ± 3	9.2 ± 1.4	9630 ± 2630	$11{,}130 \pm 890$
B		7.36 ± 0.62	140 ± 10[c]	21.8 ± 2.6[c]	$12{,}540 \pm 1600$	6050 ± 930[b]
A	Brain	0.750 ± 0.05	—	17.0 ± 0.9	628 ± 41	37 ± 3.0
B		0.738 ± 0.05		16.0 ± 0.5	667 ± 55	42 ± 4.0

[a] Data are 4-hr mevalonate incorporation. Each value represents an average of six, 8-month-old male rats. Results are given as mean ± SEM.

[b] Significant differences between B_{12} supplemented and B_{12} deficient groups are marked: $p < 0.05$; [c] $p < 0.01$.

tivity and sp act of cholesterol were substantially lowered in B_{12} deficient rats.

Discussion. The specific aim of these experiments described was to determine which reactions in the cholesterol biosynthetic pathway might require the presence of vitamin B_{12}, and if B_{12} involvement is confined to the liver or is a general phenomenon. It was observed that B_{12} deficiency lowered plasma total cholesterol as has been shown by Icayan and Chow (3), the component diminished being the ester fraction. Normally, 25 to 30% of plasma cholesterol is unbound and the remaining portion is in esterified form. Sperry (5) showed that there is enormous variation in total cholesterol content in plasma of healthy subjects but that the ratio of free to total cholesterol is within very narrow limits. It has been further shown by Friedman *et al.* (6, 7) that the primary regulator and endogenous source of blood cholesterol is the liver, and that hepatic disease results in an alteration in the plasma free to total ratio as demonstrated by Man *et al.* (8) and Gardner *et al.* (9). Data (Table I) demonstrated that B_{12} deficient rats had a significantly higher free to total ratio in comparison to that observed for treated rats. Since liver disease results in similar observations, it was thought that derangements in liver function might occur as an outcome of B_{12} insufficiency.

Data on total cholesterol content in liver, however, did not show consistent changes as a result of B_{12} deficiency. This observation made it highly improbable that the lower levels of cholesterol in the plasma were due to unavailability of liver cholesterol. It has been postulated by Brot *et al.* (10) that the liver synthesizes an enzyme that is released into the blood stream for plasma esterification of cholesterol. Therefore, the observation of lower cholesterol ester content in plasma of B_{12} deficient rats is suggestive of (i) lack of, or inactivation of, the liver esterifying enzyme, (ii) a faulty enzyme discharge mechanism, or possibly, (iii) a deficit in fatty acids essential for formation of cholesterol esters.

Another area considered for exploration was cholesterol formation from its precursor, acetate, the simplest metabolite participating in the biosynthesis of cholesterol and also a reactant in other metabolic processes. B_{12} deficiency resulted in increased incorporation of acetate into liver cholesterol and this enhancement was of at least 3-hr duration. Additional experimentation for which the data are not shown demonstrated that acetate pool size was unaltered in deficient animals. Other studies indicate that incorporation of mevalonate into cholesterol after 4 hr was reduced in the B_{12} deprived rats. Since evidence of Icayan and Chow (3) and our acetate data demonstrated that B_{12} resulted in an increased cholesterol synthesis from acetate, it seemed that the observed reduction of mevalonate incorporation into cholesterol was linked to the availability of this intermediate.

The testes were further selected for study because it has been shown by Lepkosky *et al.* (11) and Jones *et al.* (12) that B_{12} deficiency results in impairment of reproductive processes. Although cholesterol content in the testes of deficient rats was equal to levels observed in treated rats, radioactivity of cholesterol and per gram of tissue were significantly lowered. These observations suggest that B_{12} is involved in cholesterol biosynthesis in organs other than the liver.

Cholesterol is a precursor of adrenal steroid hormones. According to Dryden and Hartman (13), only the kidneys and pituitary deposit more B_{12} than the adrenals. The high B_{12} concentration suggests its role in adrenal metabolism. Our findings indicating a significant increase in adrenal cholesterol content and a marked drop in incorporation of mevalonate into the cholesterol of B_{12} deficient rats support this possibility. Moreover, the increased ratio of cholesterol to total lipid in B_{12} untreated animals further suggests that B_{12} deficiency may cause an alteration in the amount of some other lipid components in the adrenals.

Since cholesterol is a major constitutent of the central nervous system and B_{12} deficiency causes marked neurological changes [Holton *et al.* (14) and Holmes (15)], it therefore seemed likely that alterations in brain cholesterol content might occur. On analysis, there was no demonstrable difference in brain cholesterol content, and mevalonate incorpo-

ration into the brain cholesterol was negligible. Additional work is needed to clarify this relationship.

Summary. Cholesterol biosynthesis was studied in male rats from mothers fed a vitamin B_{12} deficient diet during pregnancy and lactation. Deficient males had significantly lower plasma cholesterol levels, the ester fraction being diminished. Adrenal cholesterol was appreciably higher, relative to levels observed in the B_{12} fed rats, while cholesterol content of testes and brain was not significantly different between the two groups. Vitamin B_{12} deficiency enhanced the rate of acetate incorporation into liver cholesterol while mevalonate incorporation remained unchanged after 30 min and 2 hr but was markedly lower in the deficient group after 4 hr. Mevalonate incorporation into adrenal and testicular cholesterol was also reduced at 4 hr but incorporation into brain cholesterol was unchanged.

1. Forbes, J. C., and Patterson, O., Va. J. Sci. **11**, (1953).

2. Hsu, J. M., and Chow. B. F., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **16**, 63 (1957).

3. Icayan, E. E., and Chow, B. F., J. Nutr. **78**, 109 (1962).

4. Sperry, W. M., and Webb, M. A., J. Biol. Chem. **187**, 97 (1950).

5. Sperry, W. M., J. Biol. Chem. **114**, 125 (1936).

6. Friedman, M., Byers, S. O., and Michaelis, F., Amer. J. Physiol. **164**, 789 (1951).

7. Friedman, M., and Byers, S. O., J. Clin. Invest. **34**, 1369 (1955).

8. Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P., J. Clin. Invest. **24**, 623 (1945).

9. Gardner, H. T., Swift, W. E., Modica, M., Lavintow, L., and Knowlton, M., Amer. J Med. **8**, 584 (1950).

10. Brot, N., Lossow, W. J., and Chaikoff, I. L., J. Lipid Res. **3**, 413 (1962).

11. Lepkosky, S., Borson, H. J., Bouthilat, R., Sengman, D., Dimick, M. K., Pencharz, R., and Robbins, R., Amer. J. Physiol. **165**, 79 (1951).

12. Jones, C. C., Brown, S. O., Richardson, L. R., and Sinclair, J. G., Proc. Soc. Exp. Biol. Med. **90**, 135 (1955).

13. Dryden, L. P., and Hartman, A. M., P. Nutr. **90**, 382 (1966).

14. Holton, E. K., Norum, K. R., Schrumpf, A., and Skaugg, O. E., Acta. Med. Scand. **177**, 689 (1965).

15. Holmes, J. M., Brit. Med. J. **2**, 1394 (1956).

Received Oct. 29, 1970. P.S.E.B.M., 1971, Vol. 136.