The Biological Synthesis of "Labile Methyl Groups"[1,2]

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IN THE FIRST REPORT OF GROWTH of the rat on a methionine-free, homocystine-containing diet supplemented with choline, in which the concept of the "biologically labile" methyl group and transmethylation was proposed, du Vigneaud, Chandler, Moyer, and Keppel (*8*) called attention to the fact that they occasionally encountered animals "capable of showing some growth on the homocystine diet without added choline." These authors stated: "It appears to us that the explanation of this behavior probably lies in the phenomenon of refection, or else certain factors are operating of which we are not as yet cognizant." It was well known that refection caused difficulties in the assay of the various members of the vitamin B complex. The tendency was, therefore, to attribute growth of some animals on a methyl-free diet to the synthesis of biologically labile methyl groups by intestinal bacteria.

Several years later, Toennies, Bennett, and Medes (*30, 3*) reported that they had repeatedly encountered growth of rats receiving methyl-free, homocystine-containing diets. When they administered Sulfasuxidine along with eight B vitamins, however, growth of the animals was interrupted (*1*). Addition of a "folic acid" concentrate and extra biotin did not cause a resumption of growth. However, when a crude liver extract was added to the methyl-free diet in place of the B vitamins, growth ensued that could not be accounted for by the methionine and choline contents of the extract. As pointed out by Bennett, Medes, and Toennies (*3*), "There may be vitamin factors, of either dietary or intestinal origin, the presence of which may enable the animal to compensate for the absence of dietary methyl donors by biosynthetic means of its own or of its intestinal bacteria."

The problem was further investigated in this laboratory in an attempt to get crucial proof of the synthesis of biologically labile methyl groups in the animal, regardless of whether it took place in the tissues or in the intestinal tract. This was accomplished in an experiment with rats, in which the deuterium concentration of the body water was kept at a level of about 3 atom percent for a period of several weeks (*11*). At the end of this time the animals were sacrificed, and the choline was isolated from the tissues and degraded to trimethylamine. It was felt that, if, under these conditions, deuterium were found in the methyl groups of choline, synthesis of the methyl group must have occurred in the animal tissues or in the intestinal tract. It was extremely unlikely that a direct exchange reaction between the hydrogens of biologically labile methyl groups and the deuterium of the D_2O in the tissue fluids could take place to bring about the appearance of the deuterium in the methyl group. Strong evidence of this unlikelihood had already been presented (*9*) and has now been further strengthened by more recent studies with methionine, in which the methyl group was doubly labeled with C^{14} and deuterium (*19*). The concentration of deuterium in the methyl groups of choline isolated from the two rats kept on heavy water rose to 7.7 and 8.5 percent of the concentration of deuterium in the body water. It was pointed out that

The present data do not distinguish between direct synthesis by the tissues and synthesis by intestinal bacteria with subsequent utilization of the methyl groups in the tissues. On the basis of the facts we now have con-

[1] The crucial experiment reported herein on germ-free animals was made possible through the collaboration of James A. Reyniers, Thomas D. Luckey, and their co-workers at LOBUND, of the University of Notre Dame. We are extremely indebted to them. At the suggestion of Professor Reyniers, this paper is submitted by us as a resumé of the present status of the synthesis of the biologically labile methyl groups, in which the results on choline from this joint experiment are incorporated. It is planned to submit jointly elsewhere the complete bacteriological and chemical details of the experiments, involving not only the choline data but also further work on the feces, urines, and carcasses.

[2] While these experiments were under way, it was announced by H. G. Wood in a Harvey Lecture, February 16, 1950, that W. Sakami and A. D. Welch have demonstrated the synthesis of biologically labile methyl groups from formate *in vivo* in the rat and by rat liver slices *in vitro*. Our results with the germ-free animals are thus in harmony with their result demonstrating tissue synthesis of biologically labile methyl groups. Since the submission of this article, their abstracts recording this work have appeared (Sakami, W., *Fed. Proc.*, 1950, **9**, 222; Welch, A. D., and Sakami, W., *Fed. Proc.*, 1950, **9**, 245).

cerning the labile methyl groups in the diet and in metabolism, we feel that the latter explanation involving intestinal bacteria is the most logical interpretation of our results.

In an attempt to explain the greater frequency of growth on the methyl-free, homocystine-containing diet encountered at the Lankenau Laboratories (*30, 3*), the Cornell group furnished the Lankenau group with animals from their stock. Litters from these animals were maintained at the Lankenau Laboratories on their experimental regimen (*4*). The litters behaved similarly to the Lankenau strain, thus ruling out strain differences and indicating the importance of preexperimental nutritional conditions for the capacity to grow under these conditions. Bennett and Toennies (*4*) were also able to show that modification of intestinal flora by sulfonamide action produced a situation in which supplementation of the dietary vitamins with rice polishings extract permitted the utilization of homocystine in lieu of methionine when either choline or small amounts of certain liver fractions were fed. Further work explored the nature of the liver factor. A comparison of a variety of liver preparations led to the conclusion by Bennett and Toennies that it was not identical with the antipernicious anemia principle of liver, although its distribution paralleled that of the latter to some degree.

While these studies on homocystine and methionine were under way, other evidence that might have a bearing on the problem emerged. It began to appear that there is a relation between vitamin B_{12} and the metabolism of biologically labile methyl compounds. That the animal protein factor influenced the methionine requirement of chicks was shown by Patton and co-workers (*22*) and by Bird and co-workers (*5*). Furthermore, Shive (*28*) found an interrelationship between vitamin B_{12} and methionine in bacterial metabolism. He reported that these two compounds could function interchangeably in enabling growth of *Escherichia coli* to take place in a medium containing sulfanilamide. The sparing action of vitamin B_{12} on the dietary choline necessary for increased growth and protection against the hemorrhagic kidney syndrome in the rat was noted by Schaefer, Salmon, and Strength (*26*). Gillis and Norris (*15*) reported that the inclusion of a source of animal protein factor in their basal diet obviated the need for supplementary methylating compounds for chicks and stated that at least one metabolic function of the animal protein factor was concerned with transmethylation. Bennett (*2*) then reported that vitamin B_{12} plus folic acid gave an effect similar to that obtained earlier with the crude liver extracts with rats on the methyl-free, homocystine type of diet. Stekol and Weiss (*29*) also reported that young rats were able to grow on a labile methyl-free diet that contained vitamin B_{12} and homocystine. At the same time Jukes and Stokstad (*17*) reported that vitamin B_{12} was involved in the choline and methionine requirements of chicks.

At this stage of the investigation it was clear that labile methyl groups could be synthesized somewhere in the animal body, as evidenced by the observation of growth on a methyl-free diet under certain conditions, and by the demonstration of the formation of deuteriomethyl groups by animals in which the body fluids contained D_2O. It also appeared highly probable that vitamin B_{12} and folic acid might be involved in the synthesis of these labile methyl groups. But whether the methyl groups were synthesized in the tissues of the white rat, or whether vitamin factors mediated through the intestinal bacteria, was not clear. In every experiment reported, these two interpretations were possible. The present investigation with germ-free animals was arranged in order to obtain crucial evidence as to where the synthesis took place. The technique of detection of synthesis of methyl groups through the isolation of choline from rats whose body water contained D_2O was again utilized.

The plan of the experiment was to maintain rats of the LOBUND strain with D_2O in their drinking water under both germ-free and nonsterile conditions at LOBUND of the University of Notre Dame, and to maintain at the Cornell laboratory, for comparative purposes, animals of the Rockland Farms strain under the usual laboratory conditions on the same dietary regimen as that used at LOBUND.

Two male, germ-free rats reared by the technique of Reyniers were maintained in individual metabolic compartments under germ-free conditions at LOBUND (*23*). The procedure permitted the administration of food and water, and the collection of the urine and feces under sterile conditions. Sterilized drinking water containing 10 atom percent D_2O was furnished for 4 days, followed by 4 atom percent D_2O for the rest of the experimental period. An average deuterium level in the body water of approximately 2.5 atom percent during the experiment resulted. The diet employed was essentially that already elaborated at LOBUND for the rearing of animals under germ-free conditions.[3] At the end of the periods given in Table 1 the germ-free animals were sacrificed under sterile conditions. Samples of

[3] The diet had the following composition: casein (Labco) 25 g, cellophane 2 g, corn starch 59.5 g, salt mixture 6 g, corn oil 7 g; vitamins/100 g of diet: vitamin A (esters) 800 I.U., vitamin D_3 100 I.U., vitamin C 200 mg, vitamin E 50 mg, vitamin K 10 mg, inositol 100 mg, thiamin hydrochloride 6 mg, riboflavin 3 mg, pyridoxin hydrochloride 2 mg, pyridoxamine hydrochloride 0.4 mg, calcium pantothenate 30 mg, nicotinamide 5 mg, nicotinic acid 5 mg, biotin 0.1 mg, folic acid 1 mg, *p*-aminobenzoic acid 5 mg, vitamin B_{12} 0.01 mg.

TABLE 1

DEUTERIUM CONTENT OF METHYL GROUPS OF CHOLINE

Rat No.		Experimental period	Change in body weight	Choline chloroplatinate		Trimethylamine chloroplatinate (A)		Deuterium in methyl groups of choline (B)*	Deuterium in body water (average) (C)	$\frac{B}{C} \times 100$
		days	*gm*	*mg*	*% Pt*†	*% Pt*†	*atom % excess D*‡	*atom % excess D*	*atom % excess D*§	
CUMC‖, ¶	710	21	119–237	469	32.1	37.1	0.23	0.26	3.29	7.8
CUMC¶	707	21	109–186	429	31.7	36.7	0.19	0.21	3.08	6.9
CUMC	708	21	103–186	419	32.2	36.7	0.19	0.21	2.52	8.4
CUMC	709	21	105–225	462	31.2	36.8	0.21	0.23	3.01	7.8
LOBUND	1	21	183–253	533	31.4	36.9	0.19	0.21	2.20	9.6
LOBUND	2	21	180–282	579	31.2	37.0	0.18	0.20	2.27	8.8
LOBUND (germ-free)	5	23	294–322	661	31.4	36.8	0.14	0.16	2.44	6.4
LOBUND (germ-free)	6	10	308–289	632	31.4	37.2	0.09	0.10	2.99	3.3

* Calculated value: $B = \frac{10}{9} A$.

† Theoretical Pt content for choline chloroplatinate = 31.7%; for trimethylamine chloroplatinate = 37.0%.

‡ Determined with mass spectrometer (% error = ± 0.02).

§ Determined by falling-drop procedure (% error = ± 0.06).

‖ CUMC = Cornell University Medical College, Rockland Farms strain.

¶ Received additional 1.5 μg of vitamin B_{12} daily.

the tissues and intestinal contents were examined for the presence of bacteria. No bacterial contamination was detected. The tissues were then ground under sterile conditions, ethyl alcohol was added, and the mixture was shipped along with the sterile urine and feces, by air express to New York.

To obtain the average concentration of heavy water present in the body fluids during the experimental period, the deuterium content of the urine after suitable purification was determined by the falling-drop procedure. The choline was extracted from the tissues of the sterile rats and degraded to trimethylamine with alkaline permanganate (*20*). The deuterium content of the trimethylamine salt was obtained by a micro deuterium method, which involves the combustion of several milligrams of the salt, reduction of the resulting H_2O-D_2O with zinc, and determination of the ratio of the deuterium-hydrogen mixture by means of the mass spectrometer (*24*).

The two animals maintained at LOBUND under nonsterile conditions were sacrificed at the times indicated in Table 1, and processed in the same manner as the sterile animals. In order to compare the colony of animals under nonsterile conditions at LOBUND with the colony and experimental conditions at the Cornell Laboratories, two animals were maintained at Cornell on the same diet as that used at LOBUND. The experimental diet was made up at the Notre Dame Laboratories and shipped by air express to New York to eliminate any possible difference in source of dietary material. Another pair of animals was maintained at Cornell on the LOBUND diet, which already contained generous amounts of vitamin B_{12} and folic acid, with an additional supplement of B_{12}.

From the data presented in Table 1 it is obvious that methyl groups have been synthesized by the germ-free animals. The enrichment of deuterium in the methyl groups of the choline isolated from the LOBUND germ-free rat after 23 days was 6.4 percent of that in the body water. In the 10-day experiment involving the rat under germ-free conditions, a value of 3.3 percent was obtained, an amount that agrees quite well with that of the longer experiment. A somewhat higher value was obtained in the LOBUND animals under nonsterile conditions, but this may not be a significant difference. On the other hand, it may well be that additional synthesis by intestinal bacteria of labile methyl groups took place in the case of the nonsterile animals. However, the germ-free animals were older. Of ultimate importance, of course, is the fact that deuterium was present in significant amounts in the methyl groups of choline in the germ-free animals. It is also notable that the strain of animals maintained at Cornell on the LOBUND diet synthesized methyl groups to about the same degree as the LOBUND animals at Notre Dame. The additional amount of vitamin B_{12} administered to two of the Cornell animals did not increase the degree of methyl synthesis.

We believe that the data obtained with the germ-free animals justify the conclusion that biologically labile methyl groups can be synthesized by the tissues of the rat. It might be of interest to review briefly the significance of the establishment of the tissue syn-

thesis of biologically labile methyl groups in relation to the earlier work. In the original paper, in which it was shown that homocystine plus choline could serve in lieu of methionine for growth purposes in the rat, it was noticed that an occasional animal could grow without choline, although the vast majority required a source of labile methyl groups (*8*). It was inferred that the latter were synthesized by intestinal bacteria, but it is now obvious that they were being synthesized, at least in most part, in the tissues rather than in the intestinal tract by the bacteria. The suggestion first put forth by the Lankenau group that vitamin factors may be involved in the synthesis of labile methyl groups in the tissues of the rat would appear to offer an explanation of these results. It is now realized that the diet employed in this early work was extremely low in vitamin B_{12} and folic acid. If vitamin B_{12} and folic acid bear a relationship to labile methyl metabolism in the tissues, the possibility exists that the occasional animal that did grow may have been enabled to do so because of bacterial synthesis of these vitamin factors resulting from a difference in the intestinal flora. On the other hand, a difference in degree of storage of these vitamin factors from the preexperimental period could be involved. The more frequently encountered growth in the Lankenau Laboratories may be attributable, as suggested by the Lankenau workers, to the fact that their preexperimental diet carried relatively large amounts of vitamin factors which may have been stored in the liver and later used in the experimental period. This is consistent with the now well-known storage of vitamin B_{12}. Of course, the preexperimental diet, as these workers well realized, may also have affected the flora of the intestinal tract in such a way as to favor the synthesis of these vitamin factors. Earlier cases of growth on diets low in methyl groups with homocystine in lieu of methionine may be recalled. White and Beach (*31*), on adding homocystine to an arachin diet low in methionine (0.5 percent) and supplemented with Ryzamin B and powdered liver extract, LEL No. 343, obtained growth almost equivalent to that produced by an equal amount of methionine. Brand (*6*) also demonstrated growth with homocystine in lieu of methionine on a purified amino acid diet with a vitamin supplement of dried yeast and milk concentrate. It is also interesting that, although Rose and Rice (*25*), like du Vigneaud, Dyer, and Kies (*10*), could not obtain growth on a purified diet containing homocystine as the sole source of dietary sulfur with the purified B vitamins and the small amounts of Ryzamin B used, they did report suboptimal growth on a diet with homocystine and a vitamin supplement of tikitiki and milk concentrate. What part of this growth response was due to the labile methyl compounds, and what part to vitamin factors present in the diets employed, is difficult to evaluate fully.

Since the present work was undertaken, other reports have strengthened the possibility of a relationship between vitamin B_{12} and folic acid and the metabolism of the biologically labile methyl group. The additive action of vitamin B_{12} and folic acid on sparing choline was measured by Schaefer, Salmon, Strength, and Copeland (*27*) by determining the protection these factors afforded against renal kidney damage in rats and against perosis in chicks on a diet low in labile methyl-containing compounds. In a very recent report Jukes and co-workers (*18*) have shown that vitamin B_{12}-deficient chicks were unable to utilize homocystine or homocystine plus betaine in lieu of methionine for growth, but could do so with vitamin B_{12} as a supplement. It has recently been observed by Davis and Mingioli (*7*) that vitamin B_{12} is capable of replacing the methionine requirements of an *E. coli* mutant "blocked" in the methylation of homocysteine.

In one of the earlier studies on transmethylation in 1941, methionine labeled with deuterium in the methyl group was administered to rats on an amino acid diet, otherwise free of labile methyl groups, for approximately 3 months (*9*). This diet, in the light of present knowledge, must have been low in vitamin B_{12} and folic acid. At the end of the experimental period, the concentration of deuterium in the methyl groups of the choline and the creatine from the tissues rose to 89% of that of the deuterium in the methyl groups of the administered methionine. Obviously considerable transmethylation had occurred during this period. In addition to ruling out formaldehyde as an intermediate in transmethylation, this high concentration of deuteriomethyl groups in choline and creatine would lead one to suspect that not much synthesis of methyl groups had occurred under these conditions.

A consideration of the tissue synthesis of biologically labile methyl groups naturally raises the question as to the nature of the precursors or intermediates in the synthesis. It has been reported by du Vigneaud and Verly (*12*) that methyl alcohol may serve as a precursor of the labile methyl groups. In an extension of the study of the one-carbon compounds from this standpoint, it has been found that the carbon labeled with C^{14} of formaldehyde or formic acid[4] makes its appearance in the methyl groups of choline (*14*). However, labeled bicarbonate gave a negative result. The effect of the level of methionine and of other labile methyl compounds on the degree of synthesis is yet to be explored. The level of methionine in the

[4] In the presentation of their paper on formate, Welch and Sakami also reported formaldehyde as a precursor of labile methyl groups.

diet affects tremendously the rate of oxidation of the labile methyl group to CO_2 (*21*), and it may well be that a large supply of these methyl groups in the diet brings about some inhibition of synthesis and favors oxidation of potential precursors.

In recent experiments in which the methyl group of methanol was doubly labeled with C^{14} and deuterium (*13*), the ratio of deuterium to radiocarbon in the methyl group of choline was considerably less than that in the methanol administered. This indicates that oxidation of methanol, followed by reductive conversion to the biologically labile methyl group, occurred probably through formaldehyde and/or formic acid.

The experience at Cornell Laboratories has been that even when fairly high levels of B_{12} and folic acid are present, a source of labile methyl groups must be present in the diet immediately after weaning for the majority of animals to survive. From the work of Griffith (*16*) it is known that the demand for choline is higher in the young rat shortly after weaning than later, to prevent hemorrhagic kidney. Undoubtedly, some synthesis of the methyl group at this critical stage does take place but, in the majority of animals, apparently at a rate not fast enough to support normal development of the young animal. These results tend to place the biologically labile methyl groups in the same position that arginine holds with respect to the concept of the essential amino acids, in that it can be synthesized but not at a rate fast enough for the demands of the young growing rat. Thus, in this sense, it can be regarded as an essential dietary component. A supply of preformed labile methyl groups in the diet may also become of considerable significance under special dietary or pathological conditions. Finally, it might be added that the behavior of the biologically labile methyl group that is synthesized within the body and used in the synthesis of a labile methyl compound is not distinguishable, so far as we know, from that of the biologically labile methyl group that is presented to the body in the diet from the standpoint of the metabolic process of transmethylation.

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