MUTANTS OF ESCHERICHIA COLI REQUIRING METHIONINE OR VITAMIN B_{12}

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The penicillin method (Davis, 1948; Lederberg and Zinder, 1948) has permitted convenient isolation of auxotrophic[1] mutants of *Escherichia coli* with specific growth requirements for most of the known water-soluble vitamins, as well as amino acids, purines, and pyrimidines. Accordingly, when crystalline vitamin B_{12} became available, a search was made for mutants requiring this nutrilite. Several strains of the desired type were promptly recovered. In all cases methionine, but not homocysteine, could be substituted for the vitamin. This paper describes certain biochemical properties of these mutants, as well as of others responding to methionine but not to B_{12}.

EXPERIMENTAL RESULTS

Methods. The mutants were isolated by the penicillin method (Davis, 1949) from the W strain of *E. coli* (ATCC 9637) following ultraviolet irradiation. The minimal medium, improved over that previously reported (Davis, 1949), had the following composition: K_2HPO_4, 7.0; KH_2PO_4, 3.0; Na_3-citrate·$3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; glucose (autoclaved separately), 2; H_2O, 1,000; pH 7.0. Solid media contained Difco agar[2] 1.5 per cent. Cultures were incubated at 37 C.

Response to methionine and homocysteine. By the use of minimal medium supplemented with 10 mμg per ml of vitamin B_{12}, three B_{12}-requiring strains were obtained in one experiment. They were all found to grow rapidly on methionine, but not on homocysteine, the known precursor of methionine in *E. coli* (Lampen *et al.*, 1947*b*; Simmonds, 1948) as well as in *Neurospora* (Horowitz, 1947). This response led to the testing of a number of previously isolated methionine auxotrophs for their response to B_{12}.

The results are presented in table 1. The mutants blocked before homocysteine did not respond to B_{12}, whereas, with one exception, those blocked between homocysteine and methionine did respond to B_{12}. The exception, 137-113, showed no perceptible growth on B_{12} alone, even after 3 days of incubation on solid medium, but its growth on a limited amount of methionine was definitely increased by B_{12}, suggesting a very limited ability to use B_{12}.

[1] The terms "auxotrophic" (Lat. *auxilium* = "aid"; Gr. *troph* = "food") and the corresponding noun "auxotroph" are suggested for convenience in denoting biochemical mutants with increased nutritional requirements.

[2] Commercial agar contains traces of a factor, presumably B_{12}, which supports slight growth of the B_{12}/methionine, but not the homocystine/methionine, auxotrophs. When necessary for the purposes of the experiment, this impurity was removed by successive washing with 50 and 95 per cent ethanol.

The available B_{12} auxotrophs (including those isolated with methionine as supplement) fall into two groups. The several "fast B_{12}" mutants, indistinguishable from one another, grow equally rapidly on B_{12} or methionine. The two "slow B_{12}" strains grow as rapidly as the others (or as the wild type) on methionine, yielding large colonies in 24 hours, but they require 48 hours to produce similar growth on B_{12}.

In all B_{12} auxotrophs the amount of DL-methionine required for full growth is 20 μg per ml; the amount of B_{12} is 0.5 mμg per ml. The slower response of a homocystineless mutant of *E. coli* to D-methionine than to L-methionine, reported by

TABLE 1

Response of mutants to B_{12}, methionine, and homocysteine

MUTANT NUMBER	SUPPLEMENT AT ISOLATION	RESPONSE TO		
		Methionine 20 μg/ml	Homocysteine or homocystine* 40 μg/ml	B_{12} 1 mμg/ml
113-3	B_{12}	++++	0	++++
113-93	B_{12}	++++	0	++++
121-176	Methionine	++++	0	++++
137-18	Acetyl-methionine	++++	0	++++
137-59	Acetyl-methionine	++++	0	++++
113-20	B_{12}	++++	0	++
26-20	Methionine	++++	0	++
137-113	Acetyl-methionine	++++	0	0
26-18	Methionine	++++	++++	0
137-28	Acetyl-methionine	++++	++++	0
122-33	Methionine	++++	++++	0
121-61	Methionine	++++	++++	0
122-79	Methionine	++++	++++	0
137-137	Acetyl-methionine	+++	+	0
66-12	Methionine	++++	++	0

++++—heavy growth in 24 hours; ++—heavy growth in 48 hours; +—moderate growth in 72 hours.

* The equally good response of *E. coli* mutants to homocysteine or homocystine has been previously reported by Lampen *et al.* (1947*a*). The samples of homocysteine and homocystine were kindly furnished by Dr. V. du Vigneaud.

Lampen *et al.* (1947*a*), has been verified and extended to B_{12} auxotrophs. The sample of D-methionine was kindly furnished by Dr. E. E. Howe of Merck and Company.

Response to other compounds. The block in the B_{12} auxotrophs between homocysteine and methionine suggests that vitamin B_{12} may be concerned with the formation or transfer of a labile methyl group. Accordingly, these strains were tested for growth response to other compounds containing this group. Choline, betaine, creatine, and sarcosine in concentrations of 100 and 10 μg per ml gave no response, even in the presence of homocysteine. Simultaneous tests with the wild type ensured that the absence of response was not due to the toxicity of these compounds in the concentrations tested.

Adenine thiomethyl pentoside, a component of yeast, has been suggested as a possible transmethylating agent (Lipmann, 1941). Through the kindness of Dr. F. Schlenk, this compound was made available for testing. It also failed to substitute for methionine with a fast or a slow B_{12} mutant, although it supported the growth of a purine-requiring mutant. In both these respects the behavior of *E. coli* mutants resembled that reported for naturally occurring lactobacilli (Schlenk and Gingrich, 1944).

The B_{12} auxotrophs did not respond to any other naturally occurring amino acids, water-soluble vitamins, purines, or pyrimidines, in the concentrations required by mutants with these respective requirements. Negative results were also obtained with the ribonucleosides and ribonucleotides of adenine, guanine, and cytidine, as well as with hypoxanthine, xanthine, and xanthosine. As B_{12} and desoxyribosides are interchangeable nutrilites for many thymidine-requiring lactic acid bacteria (Wright *et al.*, 1948; Shive *et al.*, 1948; Hoffman *et al.*, 1949), though not all (Kitay *et al.*, 1949; Hoff-Jorgensen, 1949; Wright, 1949; Broquist *et al.*, 1949), our mutants were tested with thymidine (kindly furnished by Dr. E. E. Snell) in concentrations of 1 to 100 μg per ml. Commercial desoxyribonucleic acid, unhydrolyzed and partially hydrolyzed by boiling with 1.0 and 0.2 N NaOH and 1.0 and 0.2 N HCl, was also tested in concentrations up to 0.4 per cent. No response was obtained, even after 3 days of incubation.

The observations reported above were made with surface inocula of various mutants on solid media. Although this method does not provide precise quantitative data, it has several advantages: the response to small inocula is more uniform; slight growth is recognized with more certainty; and the growth of the mutant strain is readily distinguished from that of occasional back mutations, permitting prolonged observation. Subsequent quantitative confirmation in liquid medium of many of the observations is presented in table 2, which also shows the regularity of the growth curves on limiting amounts of B_{12} or methionine.

Syntrophism among methionine auxotrophs. Syntrophism between mutants blocked before and after homocysteine has been reported (Lampen *et al.*, 1947*b*) on the basis of the observation that tubes with certain mixed inocula yield heavier growth than either inoculum alone on limiting amounts of methionine. Although this technique for demonstrating syntrophism is very sensitive, it provides no information on whether one, the other, or both mutants are fed. We have therefore conducted tests with the technique of adjacent streaks on agar (Davis, 1950*a*), in which syntrophism is shown by a gradient of growth of the fed strain.

Table 3 shows that the B_{12} auxotrophs under certain circumstances feed a mutant that is blocked earlier in the synthesis of methionine. This accumulation by the B_{12} mutants of some precursor of methionine, probably homocysteine, is similar to the well-known accumulation by many microbial mutants of the substrate of the deficient enzyme, but it must be emphasized that the extent of the syntrophism here is much less than that observed with several other pairs of mutants blocked at different stages in the synthesis of an amino acid (Davis, 1950*a*). It would therefore be difficult to obtain enough of the accumulated product for identification.

It is of particular interest to note the relationship of syntrophism to concentration of the nutrilite of the feeder strain. At very low concentrations the growth of the feeder organism is too slight to yield detectable amounts of precursor.

TABLE 2

Growth on B_{12}, methionine, and homocystine

Eighteen-hour turbid cultures, grown in minimal medium supplemented with 0.2 per cent casein hydrolyzate, were inoculated (2×10^{-4}) into 10 ml of minimal medium. Readings are percentage of transmission in $\frac{7}{8}$-inch-diameter tubes in Evelyn photoelectric colorimeter, filter 515 mμ.

SUPPLEMENT		MUTANT INOCULATED					
		113-3 (Fast B_{12})		113-20 (Slow B_{12})		26-18 (Homocystine)	
		18 hr	48 hr	18 hr	48 hr	18 hr	48 hr
$CoCl_2$	1 μg/ml	101	100	100	100	100	R
DL-methionine	40 μg/ml	48.5	44	48	44	46	34
"	20	48.5	44	48	42	47	35
"	10	54	47.5	56	47.5	52	36
"	5	63	64	62.5	63	62	R
"	2	77	R	77.5	R	75	R
"	1	85	R	85	R	84	R
"	0.5	92.5	90	93	90	90	R
B_{12}	2.5 mμg/ml	50	45	100	51	100	98
"	0.8	49	46	99	52	—	—
"	0.4	49	47	—	—	—	—
"	0.2	70	75	100	66	—	—
"	0.1	83.5	85	—	—	—	—
"	0.05	90	89	—	—	—	—
"	0.025	97	94	—	—	—	—
Thymidine	10 μg ml	100	99.5	101	99	—	—
Choline	40	100	99	100	99	—	—
Creatine	40	100	100	99.5	98.5	—	—
Betaine	40	99.5	99	100	100	—	—
Homocystine	50 μg/ml	99	98.5	101	101	99.5	63
Adenine thiomethyl pentoside	100 μg/ml	100	100	100	99	—	—

R represents tubes with a delayed increase in density due to the outgrowth of reversions, determined by streaking on minimal medium at 48 hours. Growth at 18 hours is not due to reversions.

At 0.2 and 1 mμg per ml of B_{12}, both the fast and the slow B_{12} mutants feed the homocystine-responding mutant. But at excessive concentrations of B_{12}, feeding by the fast B_{12} mutant is abolished. Presumably under such circumstances the homocysteine is methylated as rapidly as it is formed, and the resulting methionine, subject to the same regulatory mechanisms as in the wild type, is not ex-

creted. With the slow B_{12} mutant, however, an excess of B_{12} neither provides normal growth rate nor abolishes excretion of the methionine precursor. These facts, together with the fast growth of this strain on methionine, suggest that the genetic block in this mutant results in a growth-limiting rate of utilization of B_{12}, presumably by conversion into a coenzyme for methylation of homocysteine. In consequence, the precursor of methionine is excreted since its rate of formation exceeds its rate of conversion.

In the presence of limiting amounts of methionine the background growth of the homocystine-responding indicator strain is also stimulated. The resulting decrease in sensitivity of the test may possibly account for the failure to demonstrate syntrophism under these circumstances by this technique (table 3).

TABLE 3

Syntrophism

Streaks of the wild type and the fast and slow type of B_{12} mutant were made parallel to streaks of the homocystine-responding mutant. The results recorded were visual estimates of heaviness of growth and heaviness of the syntrophic gradient. The results were noted at 48 hours; no syntrophism was observed at 24 hours, and at 72 hours there was intensification only of the previously positive responses.

SUPPLEMENT TO MINIMAL MEDIUM	GROWTH ALONE				FEEDING OF 26-18		
	Wild type	113-3 (fast B_{12})	113-20 (slow B_{12})	26-18 (homocystine)	By wild type	By 113-3	By 113-20
B_{12} 100 mμg/ml	++++	++++	++++	0	0	0	++
" 10 mμg/ml	++++	++++	++++	0	0	0	++
" 1 mμg/ml	++++	++++	+++½	0	0	++	++
" 0.2 mμg/ml	++++	++½	++	0	0	+	+
" 0.05 mμg/ml	++++	+	+	0	0	0	0
Methionine 5 μg/ml	++++	++	++	++½	0	0	0
" 1 μg/ml	++++	+	+	+	0	0	0

Adjacent streaks of all possible pairs of five fast and slow B_{12}-responding mutants exhibited no syntrophism. The evidence therefore does not show whether or not they are blocked at different stages in the synthesis of vitamin B_{12}, as accumulation of the substrate of the deficient enzyme is not observed with all nutritionally deficient mutants.

Among the mutants responding to homocysteine, 26-18 and 137-28 slightly feed adjacent streaks of 121-161, 122-33, and 122-79, indicating blocks in these strains at different stages in the synthesis of homocysteine, presumably before and after cystathionine. Although Lampen *et al.* (1947) observed syntrophism between strains blocked before and after homocysteine, as well as between the former group and cystineless strains, none of their four homocysteineless strains fed each other, implying that they were blocked in the same reaction. This interpretation is compatible with the fact that none of their strains responded to cystathionine. Simmonds (1948), however, has described another methionineless

mutant of *E. coli* that does respond to either homocysteine or cystathionine. Apparently our series includes mutants of both groups.[3]

As shown by syntrophism, excretion of an amino acid can be forced to occur by the addition of an excess of its precursor (Davis, 1950*a*). With methionine the extent of this phenomenon is more limited than with some other amino acids. In the presence of an excess of homocysteine (100 μg per ml) the mutants blocked immediately behind this compound (26-18, 137-28) moderately fed an adjacent streak of a methionine/B_{12} mutant (113-3) after 3 days, whereas a mutant blocked earlier in this synthesis (122-33) failed to do so. The reason for this failure is obscure. The amounts involved were too small for chemical identification, but it seems probable that the compound excreted is methionine.

Response to components of B_{12}. Since several degradation products of B_{12} have become available, it was of interest to determine whether some of the mutants were blocked so early in B_{12} synthesis that one or another of these compounds might satisfy its growth requirements. 1,2-Aminopropanol was kindly furnished by Dr. E. Lester Smith of the Glaxo Laboratories, and 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene by Dr. K. Folkers of Merck and Company. All the B_{12}-responding mutants were tested on each of these three compounds in concentrations of 10, 0.1, and 0.01 μg per ml. No response was obtained. Similarly, no effect was produced by high concentrations of vitamin B_{12} that had been autoclaved in 6 N HCl for 20 hours in a sealed capillary (kindly furnished by Dr. T. H. Jukes) or a sample that had been autoclaved in N/5 NaOH for 20 minutes. Simultaneous tests with the wild type organism showed no toxicity from any of these supplements.

Acceleration of the slow B_{12} mutants. The slow B_{12} mutants (113-20 and 26-20) yield only microscopic growth on B_{12} at 24 hours; at 48 hours their growth on plates or in tubes is heavy and requires only slightly more B_{12} than that of the fast B_{12} mutants. The presence of even a great excess of B_{12} (100 mμg per ml) does not accelerate the slow mutants. However, the addition of methionine in a very small amount (0.3 μg per ml), which by itself yields barely visible growth of any methionineless mutant, permits full growth of the slow B_{12} mutants in 24 hours on plates in the presence of B_{12}. The mechanism underlying this effect is under further investigation.

Antagonism of sulfonamide inhibition. Shive (1949) has shown that B_{12} and methionine exert a similar degree of antisulfonamide activity with wild type *E. coli*. We have confirmed this effect with auxotrophs for B_{12} and homocysteine as well as with the wild type; all of these show, in the presence of B_{12} or methionine, an approximately 20-fold increase in the minimal inhibitory concentration of sulfathiazole. The maximal antisulfonamide effect is exerted by the lowest concentration of either compound that is adequate for full growth of the appropriate mutant.

Since the relation between sulfonamides and B_{12} is noncompetitive, the known

[3] *Note added in proof.* With a sample of DL-cystathionine kindly furnished by N. H. Horowitz it was possible to confirm the prediction that 121-161, 122-33, and 122-79 respond to this compound, whereas 26-18 and 137-28 do not.

function of PABA in the synthesis of methionine may actually be participation in the synthesis of B_{12}. It is particularly interesting that the B_{12} mutants do not respond to PABA, and PABA auxotrophs do not respond to B_{12}. Folic acid (pteroyl glutamic acid) supports the growth of neither group.

Absorption of B_{12} from the medium by E. coli. Syntrophic tests between the wild type and various mutants have shown that several strains of wild type *E. coli* excrete relatively large amounts of certain vitamins, but not of others (Davis, 1950*a*). B_{12} belongs to the latter class. Furthermore, wild type *E. coli* not only fails to excrete B_{12} but rapidly removes this vitamin from the medium. These studies will be reported in detail elsewhere; certain facts relevant to problems discussed in this paper are briefly mentioned here.

Essentially B_{12} is equally effectively removed from the medium by the wild type or by the fast or slow B_{12} mutants. The rate of absorption therefore cannot be responsible for the slow growth of the latter mutant on B_{12}. The absorption capacity is large, a given inoculum being capable of removing within 2 hours approximately 50 times the amount of B_{12} required to yield the same number of cells of a B_{12} auxotroph. The absorption does not require the presence of either a carbon or a nitrogen source in the medium, although it takes place faster with growing cells and requires a temperature high enough to permit active metabolism. Recovery of B_{12} from the saturated cells by boiling indicates that its disappearance from the medium is due to absorption rather than to destruction.

In connection with this uptake of B_{12} from the medium, it may be of interest to note that the growth of wild type *E. coli* in minimal medium is accelerated by the addition of either methionine or B_{12}. In contrast, very few other amino acids, and no other vitamins, singly produce such acceleration (Davis, 1950*b*).

Microbiological assay for B_{12}. As was shown in table 2, a fast B_{12} mutant gave a regular turbidimetric response to graded amounts of B_{12} or methionine at 18 hours; many other experiments have given similar results at 18, 24, or 48 hours. Only an occasional tube shows a burst of secondary growth by 48 hours due to reversions to nutritional independence (table 1, table 4). The organism is therefore stable enough for use in assay and has proved satisfactory in the studies mentioned above on the removal of B_{12} from the medium. With mixtures of B_{12} and methionine, or B_{12} plus a mixture of other amino acids, the growth response is less regular; occasional tubes produce more growth than can be accounted for by the amounts of B_{12} and methionine present, yet show no reversions. It is therefore not an ideal assay organism for mixtures. Nevertheless, as it does not respond to the desoxyribosides that stimulate most other B_{12} assay organisms, and as the available assays of B_{12} are reported not to be too satisfactory, these mutants may be useful for this purpose.

For assay with materials containing both B_{12} and methionine, the correction for methionine can be made from simultaneous assays with another organism that does not respond to B_{12}, such as a homocysteine-requiring mutant of *E. coli*. Table 2 shows the regular response of such a mutant to graded concentrations of methionine. (Interference in this assay by other substances has been described by Lampen *et al.*, 1947*a*.) An alternative possibility is elimination of B_{12} activity

followed by methionine assay. Although this effect is probably most conveniently attained by chemical means (e.g., alkaline hydrolysis), it may be worth bearing in mind the specific adsorptive capacity of *E. coli*. Quantitative elimination of B_{12} can be accomplished by brief exposure (e.g., 2 hours at 37 C) to a suspension of *E. coli* in the following proportions: washed cells from 4 ml of turbid culture for each 10 ml of solution to be tested. Under conditions that do not permit growth (absence of glucose), the methionine concentration in the supernatant remains unaltered. With growing cells, however, part of the methionine is also removed, and after prolonged exposure methionine may be contributed.

TABLE 4

Effect of shaking on response to B_{12} or methionine

Inoculum: fast B_{12} mutant 113-93. Experimental conditions as in table 2. Cotton-plugged tubes. Shaking: 120 cycles per minute, amplitude 3 inches. All tubes tested at 48 hours for presence of prototrophic reversions; positive tubes noted as "R."

B_{12} mμg/ml	DL-METHIONINE μg/ml	UNSHAKEN			SHAKEN		
		18 hr	24 hr	48 hr	18 hr	24 hr	48 hr
—	—	100	100	100	100	100	100
1.0	—	49	50	47.5	25	23	23
0.4	—	51	50	49	25	22	22
0.2	—	61.5	63.5	65	35.5	32	27.5
0.1	—	75.5	75.5	79	59	52	R
0.05	—	88	87	87.5	77	70	64
0.02	—	93.5	93	92.5	91	86.5	81
0.01	—	95	94	94	96	93	88
0.005	—	98	98	96.5	99	99	97.5
—	40	49	48	45	25.5	23.5	23
—	20	50.5	48	45	28	26	25
—	8	60	58	50	33	32	33
—	4	65.5	65.5	63	50	48	50
—	2	76	70.5	75	68	66	R
—	1	86	82	82	80	78	78.5
—	0.5	93	91.5	91.5	89	88	88
—	0.2	96	95	95.5	95	94.5	95

Effect of aeration. In the medium used here the final turbidity of the wild type attained in unshaken tubes is limited by the amount of glucose present, rather than by pH. In shaken tubes the more extensive aerobic utilization of glucose yields much heavier growth, as expected, and greater response of the mutants to the larger amounts of either B_{12} or methionine. In addition, it is of particular interest that shaking produces a regular, slow further response to B_{12} during the second 24 hours of cultivation, which is not seen with methionine or with unshaken tubes (table 4). This observation is consistent with the interpretation of B_{12} as a catalyst. It would be expected that the amount per cell of a building block, such as methionine, would strictly limit growth, whereas slow

further growth on suboptimal amounts of a catalyst would not be surprising. The absence of this phenomenon in unshaken tubes may well depend on the rapidity with which the available glucose is fermented once the culture has become turbid (Ryan and Schneider, 1949).

DISCUSSION

The results presented show that vitamin B_{12} and methionine serve as alternative nutrilites for certain mutants. In view of the known function of several vitamins as components of coenzymes, it appears reasonable to assume that here, too, the relationship is of this type. Specifically, B_{12} would be postulated to function in a coenzyme participating in the manufacture or transfer of labile methyl groups, since this last stage in methionine synthesis is the one blocked in the B_{12} auxotrophs.

Further support of this interpretation would be desirable, as an alternative growth response to either of two compounds does not tell which one is concerned with the synthesis of the other. Such support is furnished by the existence of mutants with an excellent response to methionine but a slow response to B_{12} (113-20, 26-20). Studies on absorption of B_{12} from the medium appear to have excluded any permeability defect in these strains; the difference therefore concerns their metabolic activity. It would be difficult to understand the superiority of methionine over B_{12} in these mutants if the sole relation between the two compounds were the function of methionine as a precursor or cofactor in the formation of B_{12}. On the other hand, this phenomenon can most readily be explained by the assumption in these instances of a metabolic alteration that interferes with efficient utilization of B_{12} in the manufacture of methionine, the methionine being used normally. This interpretation is further strengthened by the evidence from syntrophism that an excess of B_{12} abolishes the accumulation of a methionine precursor by a fast B_{12} mutant, but fails to do so with a slow B_{12} mutant. Presumably the slow mutant cannot provide enough coenzyme from B_{12} to permit homocysteine to be methylated as fast as it is made.

One mutant blocked in the methylation of homocysteine (137-113) does not grow at all on B_{12} alone, leading to the suspicion that it might be genetically blocked in the synthesis of the apoenzyme of this reaction, or in an enzyme for converting B_{12} to the postulated coenzyme. Yet the response of this mutant to limiting amounts of methionine is slowly increased by the addition of B_{12}, indicating the retention of some ability to utilize B_{12}.

It must be emphasized, however, that although all our observations point toward the methylation of homocysteine as the sole indispensable function of B_{12} in *E. coli*, one cannot exclude the possibility of a more obscure mechanism underlying these phenomena, particularly the slow growth of certain strains on B_{12}. Instances of internal inhibition are known in which an accumulated metabolite interferes with a synthetic reaction (Bonner, 1946). Some atypical mutants we have encountered suggest that occasionally a compound that provokes a growth response may not be serving as the product of a genetically blocked reaction, but may rather be restoring synthesis by upsetting the equilibrium that

produces the internal inhibition. A suggestive case of this sort, related to the present discussion, is a mutant (66-12) that, in addition to the fast growth on methionine and slow growth on homocystine listed in table 1, also grows very slowly on thiamine or its pyrimidine moiety. Yet no other methionine auxotrophs respond to thiamine, and no other thiamine auxotrophs to methionine.

Despite the possibility of alternative explanations, the growth responses of the more typical microbial mutants have permitted definite establishment of many biosynthetic reactions. We therefore feel justified in assigning to B_{12} a function in a coenzyme of methylation and, somewhat more tentatively, in interpreting the slow response of some mutants to B_{12} in terms of inefficient manufacture of this coenzyme.

Other evidence of a relation between B_{12} and methionine in *E. coli* has been noted in their similar antagonism of sulfonamide inhibition (Shive, 1949). It may be of interest to note that methionine does not replace the B_{12} requirement of the chick (Jukes and Stokstad, 1949), but B_{12} does permit homocystine to replace the methionine requirement (Jukes, 1950). Similarly, a growth response of rats to B_{12} on a "labile-methyl"-free diet containing homocystine has been reported (Bennett, 1949), as well as a possible substitution of B_{12} for methionine in the pig (Cunha *et al.*, 1949).

The failure of choline and other possible methyl donors to permit certain methionine auxotrophs of *E. coli* to utilize homocystine has been reported previously (Green and Sevag, 1946; Lampen *et al.*, 1947*b*). To this group of compounds we have added adenine thiomethyl pentoside, with negative results. In this connection it may be pointed out that we have failed to obtain mutants of *E. coli* requiring choline, betaine, or creatine, despite repeated attempts with the efficient penicillin method and despite the previous successful isolation of cholineless *Neurospora* mutants (Horowitz and Beadle, 1943). Furthermore, sarcosine fails to serve as a growth factor for a glycine-requiring mutant of *E. coli* (unpublished data). These results therefore do not necessarily militate against the postulated function of B_{12} in a coenzyme of methylation, but rather suggest that the metabolism of *E. coli* may not involve many of the labile-methyl compounds known to participate in animal metabolism (Du Vigneaud, 1948).

The replacement of B_{12} by methionine in *E. coli* and by desoxyribosides in various lactobacilli (grown on media containing methionine) provides an extraordinary contrast, since both *E. coli* and the lactobacilli must similarly require desoxyribosides in their protoplasm and would scarcely be expected to synthesize them by fundamentally differing mechanisms. Inhibition analysis (Shive, 1949) has suggested that certain cofactors (e.g., PABA) are required at different effective concentrations for the synthesis of the various products with which they are concerned. This concept suggests that B_{12} might be required at one concentration for desoxyriboside synthesis and at a higher concentration for methionine synthesis. The mutants here reported might have only a relative block in B_{12} synthesis, making enough for the desoxyriboside reaction only. Such a picture would be satisfactory for the mutants isolated with the aid of methionine. But it would be surprising (though by no means impossible) that the several mutants

isolated with the aid of B_{12} supplementation should all be partly blocked to the same degree, for any completely B_{12}-deficient mutants should also survive in this medium. The matter remains obscure. In this connection it may be noted that desoxyribosides are reported to be practically inactive as substitutes for the B_{12} requirement of *Euglena gracilis* (Hendlin and Woodruff, 1949; Hutner, 1950).

SUMMARY

A number of mutants of *Escherichia coli* have been isolated that require vitamin B_{12} or methionine. These do not respond to homocysteine, the immediate precursor of methionine. Furthermore, mutants responding to homocysteine do not respond to B_{12}. Most of the B_{12} auxotrophs grow equally rapidly on the vitamin or methionine, but two grow rapidly on methionine and slowly on B_{12}.

Tests for syntrophism show that the B_{12}-requiring mutants accumulate a precursor of methionine that feeds the homocysteineless mutants. An excess of B_{12} abolishes this accumulation with the fast B_{12}, but not the slow B_{12}, mutants.

In contrast to the behavior of many B_{12}-requiring lactic acid bacteria, B_{12}-requiring *E. coli* do not respond to thymidine.

It is concluded that B_{12} is concerned with the methylation of homocysteine. The nature of the genetic block resulting in slow growth on B_{12} is less clear but may involve slow conversion of B_{12} to a coenzyme of methylation.

The mutants are stable enough to be useful for B_{12} assay. The quantitative response to B_{12} is greater and more prolonged in shaken than in unshaken tubes. With such assays it could be shown that suspensions of wild type *E. coli* rapidly absorb large amounts of B_{12} from the medium.

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