THE INFLUENCE OF FOLIC ACID AND VITAMIN B_{12} ON NUCLEIC ACID METABOLISM IN MICROORGANISMS

By D. V. REGE* and A. SREENIVASAN

(From the Department of Chemical Technology, University of Bombay, Bombay, India)

(Received for publication, February 9, 1954)

A combination of purines and thymine has long been known to substitute for folic acid (FA) in the nutrition of microorganisms requiring the vitamin. The purines were found to be interchangeable, but the effect of thymine was specific and could only be duplicated by its desoxyriboside, thymidine (1–4). From these and other studies on inhibition analysis (5, 6), it was concluded that the purines and thymine are metabolic end-products of reactions mediated by FA.

Prusoff *et al.* (7) compared the nucleic acid make-up of *Lactobacillus casei* cells grown with optimal and suboptimal concentrations of FA and observed that the desoxypentose nucleic acid (DNA) content was depressed markedly in the deficient cells, whereas the level of pentose nucleic acid (PNA) was unaltered.

Growth studies with microorganisms requiring vitamin B_{12} showed that the vitamin could be replaced by purines and thymidine. The desoxyribosides and desoxyribotides of purines and of cytosine could also support partial growth (8–11). Mediation of vitamin B_{12} in nucleic acid synthesis was further shown by Roberts *et al.* (12).

These and other functional relationships between FA and vitamin B_{12} suggested an investigation of their effects on nucleic acid metabolism and are contained in part in a preliminary communication (13). The organisms chosen were *L. casei* 7469, and *Streptococcus faecalis* R 8043 requiring FA, *Lactobacillus leichmannii* 313 requiring vitamin B_{12}, as well as, reportedly (14), FA, and *Lactobacillus arabinosus* 8014 and *Escherichia coli* McLeod, neither of which requires either vitamin for normal growth.

EXPERIMENTAL

L. casei, *L. arabinosus*, and *S. faecalis* R were carried on dextrose-yeast extract-proteolyzed liver-agar stabs, *L. leichmannii* on this medium supplemented with peptone and tomato juice, and *E. coli* on peptone agar slants.

The following *basal media* were used: for *L. casei* and *S. faecalis* R, the medium of Teply and Elvehjem (15) with the omission of peptone, *p*-aminobenzoic acid, and alanine; for *L. arabinosus*, a modification of Wright and Skeggs' medium (16); for *L. leichmannii* the medium recommended by

* Research Fellow of the Raptakos Medical Research Board.

the United States Pharmacopeia (17); and for *E. coli* a synthetic salts-dextrose medium of Green and Sevag (18).

The organisms were grown in 500 ml. Erlenmeyer flasks, each containing 200 ml. of medium with additions as indicated in the text. The flasks and contents were sterilized by autoclaving at 15 pounds steam pressure for 15 minutes and cooled. Each flask was seeded with 1 ml. of an 18 to 24 hour inoculum grown on the basal medium supplemented with just the optimal amount of the required vitamin missing from the medium (for *L. casei* and *S. faecalis* 1 mμgm. and 8 mμgm. of FA, and for *L. leichmannii* 1 mμgm. of vitamin B_{12} per 10 ml. of medium). In the case of *L. arabinosus* and *E. coli* there were no additions to the basal media in preparing inocula. The incubation temperature was 37°, except for *E. coli*, for which the optimum is 30°.

After incubation for 24 hours the cells were harvested by centrifugation, washed twice with ice-cold distilled water, suspended in water, and made to a definite volume. Aliquots of these cell suspensions were taken for determinations of nucleic acids, nitrogen content, and dry weight.

The procedure for the determination of *nucleic acids* was essentially that adopted by Prusoff *et al.* (7) from Schneider (19). The hot trichloroacetic acid extract was analyzed for PNA by the orcinol method (20) and for DNA by the diphenylamine color reaction of Dische (21). In the orcinol method the concentration of ferric chloride was reduced from 0.1 to 0.02 per cent, as the higher concentration was found to mask the green color of the reaction. The color densities were measured in a Klett-Summerson photoelectric colorimeter at 660 mμ in both cases. For comparison ribose nucleic acid (Nutritional Biochemicals Corporation) and DNA (Schwarz) were used as standards.

In aliquots of cell suspensions, total cell *nitrogen* was determined by acid digestion, followed by direct nesslerization (22).

Separate sets were kept in tubes with the corresponding supplements, and the 72 hour *acid production* was determined by titration against 0.1 N sodium hydroxide solution with bromothymol blue as indicator.

Results

Nucleic Acid Synthesis in L. casei—The organism was grown in the basal medium supplemented with varying amounts of FA or vitamin B_{12}. The results (Table I) indicate that in FA deficiency there is a selective depression in DNA content of cells which could be made good by either FA or vitamin B_{12}, although the latter could not replace FA as a growth factor in other respects. The effect of vitamin B_{12} was apparent even in the presence of FA. Thus, the two together enhanced DNA synthesis. PNA synthesis was apparently not influenced by either vitamin.

The influence of FA in controlling DNA synthesis could have been through its mediation in thymine biosynthesis, purines being present in the basal medium. As thymine is known to replace FA in growth promotion, its effect on nucleic acid synthesis, alone and in combination with vitamin B_{12}, was studied (Table II). Inclusion of thymine increased the levels of DNA and, unlike FA, of PNA, as well, to a marked extent. While thymine did not exert any influence on nucleic acid synthesis in the pres-

TABLE I

Effects of Folic Acid and Vitamin B_{12} on Nucleic Acid Synthesis in L. casei

Supplement to 100 ml. basal medium	Cell mass	Nitrogen	PNA	DNA	0.1 N acid per 10 ml.
	gm. per l.	*per cent*	*per cent*	*per cent*	*ml.*
FA 5 mμgm.	0.24	9.23	9.54	1.68	2.5
" 200 mμgm.	0.44	9.05	9.62	2.80	12.1
Vitamin B_{12} 5 mμgm.	0.25	9.46	9.48	1.31	2.3
" " 200 mμgm.	0.27	9.19	10.06	2.88	3.7
FA + vitamin B_{12} 5 mμgm. each	0.29	9.83	9.63	2.45	2.4
" + " " 20 mμgm. each	0.40	9.57	10.37	2.55	9.1
" + " " 200 mμgm. each	0.61	9.69	9.82	3.63	12.4

TABLE II

Effects of Thymine and Vitamin B_{12} on Nucleic Acid Synthesis in L. casei

Supplement per 100 ml.	Cell mass	Nitrogen	PNA	DNA
	gm. per l.	*per cent*	*per cent*	*per cent*
FA 5 mμgm.	0.24	9.68	9.25	1.79
" 200 mμgm.	0.52	9.12	9.39	2.63
Thymine 500 γ	0.33	10.32	11.72	2.75
Vitamin B_{12} 200 mμgm.	0.22	9.28	9.90	2.51
Thymine 500 γ + FA 200 mμgm.	0.55	10.68	11.86	2.90
" 500 " + vitamin B_{12} 200 mμgm.	0.38	10.52	11.99	3.46

ence of FA, an effect in the presence of vitamin B_{12} was observable with respect to DNA. Thymine addition also caused an over-all increase in cell nitrogen.

In a medium devoid of any purine or pyrimidine additions, the effect of FA and vitamin B_{12} was seen with respect to both PNA and DNA synthesis (Table III).

In a concentration of 500 mμgm. per 100 ml., 1,2-dichloro-4,5-diaminobenzene dihydrochloride,[1] which has been reported to antagonize vitamin

[1] Obtained from Dr. D. W. Woolley of The Rockefeller Institute for Medical Research, New York, to whom we are deeply indebted.

B_{12} synthesis in microorganisms (23), did not influence nucleic acid synthesis in FA-deficient or complete media.

Results similar to those for *L. casei* were obtained with *S. faecalis* R with respect to its nutritional relationship to folic acid, vitamin B_{12}, thymine, and thymidine.

Nucleic Acid Synthesis in L. leichmannii—The organism did not need an external supply of FA. In a medium containing purines and uracil the effect of vitamin B_{12} could be seen only on DNA synthesis (Table IV). However, in the absence of purines and pyrimidines in the medium (Table

TABLE III

Effects of FA and Vitamin B_{12} on Nucleic Acid Synthesis in L. casei in Absence of Purines and Pyrimidines

Supplement per 100 ml.	Cell mass	PNA	DNA
	gm. per l.	*per cent*	*per cent*
FA 5 mμgm.	0.17	6.80	1.45
" 200 mμgm.	0.48	9.16	2.53
Vitamin B_{12} 5 mμgm.	0.14	6.27	1.23
" " 200 mμgm.	0.18	7.69	2.00
FA + vitamin B_{12} 200 mμgm each	0.56	9.82	3.52

TABLE IV

Effect of Vitamin B_{12} on Nucleic Acid Synthesis in L. leichmannii 313

Vitamin B_{12} supplement per 100 ml.	Cell mass	Cell nitrogen	PNA	DNA	0.1 N acid per 10 ml.
mμgm.	*gm. per l.*	*per cent*	*per cent*	*per cent*	*ml.*
5	0.25	9.87	10.32	1.79	3.7
20	0.39	9.60	9.01	2.85	9.7
100	0.53	8.74	9.89	3.05	13.4

V), the vitamin influenced synthesis of both PNA and DNA. A hydrolysate of DNA could substitute for vitamin B_{12} when used in combination with purines and uracil. Aminopterin impaired synthesis of PNA and DNA. The effect was less pronounced in the presence of vitamin B_{12} (Table VI).

Nucleic Acid Synthesis in Other Microorganisms—FA and vitamin B_{12} had no effect on nucleic acid synthesis in *L. arabinosus* and *E. coli*. The results with the latter organism are reported in Table VII.

Purine Degradation in L. casei—Although the effects of FA and vitamin B_{12} on the nucleic acid content of microorganisms could be due to their rôle in nucleotide synthesis, a check by the vitamins on the degradative mechanisms is not excluded. Inhibition of oxidation of purines by FA has

been reported in higher animals (24, 25). A study was therefore made of the oxidative decomposition of xanthine and adenine in *L. casei* by following the liberation of ammonia (26) on incubation with cell suspensions in

TABLE V

Effect of Vitamin B_{12} on Nucleic Acid Synthesis by L. leichmannii in Absence of Purines and Pyrimidines

Supplement per 100 ml.	Cell mass	Cell nitrogen	PNA	DNA
	gm. per l.	*per cent*	*per cent*	*per cent*
Vitamin B_{12} 5 mμgm.	0.19	9.21	6.51	1.43
" " 100 mμgm.	0.51	8.63	8.27	2.37
AGU* + vitamin B_{12} 100 mμgm.	0.57	9.09	10.21	2.95
" + DNA† hydrolysate	0.42	9.35	10.50	3.26
" + " " + vitamin B_{12} 100 mμgm.	0.61	9.15	10.44	3.00

* Adenine sulfate, guanine hydrochloride, and uracil 1 mg. of each.

† DNA hydrolyzed by autoclaving with NH_3 at pH 10 for 4 hours, 15 pounds; neutralized; addition per 100 ml., 2 mg. of DNA.

TABLE VI

Effect of Aminopterin on Nucleic Acid Synthesis in L. leichmannii

Supplement per 100 ml.	Cell mass	Cell nitrogen	PNA	DNA
	gm. per l.	*per cent*	*per cent*	*per cent*
Vitamin B_{12} 5 mμgm.	0.28	9.64	10.05	1.62
" " 5 " + aminopterin 25 γ	0.19	9.26	7.74	0.79
" " 100 mμgm.	0.60	8.86	9.88	2.86
" " 100 " + aminopterin 25 γ	0.47	8.93	8.68	2.08

TABLE VII

Effects of FA and Vitamin B_{12} on Nucleic Acid Synthesis in E. coli

Supplement per 100 ml.	Cell mass	PNA	DNA
	gm. per l.	*per cent*	*per cent*
None	0.44	8.92	3.78
FA 200 mμgm.	0.39	8.68	3.90
Vitamin B_{12} 200 mμgm.	0.48	8.99	4.26
FA + vitamin B_{12} 200 mμgm. each	0.47	9.22	3.80

Conway microdiffusion units (27). The results showed that FA and vitamin B_{12} depressed purine degradation by 10 to 30 per cent; the inhibition was observed best with xanthine. This effect did not, however, parallel the stimulation of nucleic acid synthesis by the two vitamins.

DISCUSSION

The foregoing observations demonstrate that FA and vitamin B_{12} are concerned with the control of nucleic acid synthesis in microorganisms. The failure of Prusoff *et al.* (7) to observe an effect of FA on PNA synthesis may be explained as due to the presence of preformed purines in the basal medium. The present study would also emphasize that the vitamins are concerned primarily in the biosynthesis of the purine and thymine components and that vitamin B_{12} further mediates in DNA synthesis.

The effect of vitamin B_{12} on *L. casei* and *S. faecalis* R is somewhat unexpected. These organisms do not require vitamin B_{12} for growth or other general activity, as shown by acid production. Yet the vitamin exerts an influence on the formation of nucleic acids with or without FA. A similar case of stimulation, specifically of nucleic acid elaboration with cobalt, was recently reported by Levy and coworkers (28, 29) with yeast and *Proteus vulgaris*.

A rôle of vitamin B_{12} in the biosynthesis of purines from 4-amino-5-imidazolecarboxamide has been suggested from studies with sulfonamide-inhibited *E. coli* and *E. coli* mutants requiring purines (30). However, its FA-replacing action reported here would indicate that it is also concerned in thymine synthesis, although growth replacement and related studies implicate it in thymidine formation. It is tempting to speculate that an alternative pathway not involving thymine might be operative in *L. casei* for thymidine synthesis when vitamin B_{12} replaces PGA.

In *L. arabinosus* and *E. coli*, FA and vitamin B_{12} singly or in combination do not influence nucleic acid synthesis. These organisms are known to elaborate FA-active compounds in considerable quantities (31, 32). *L. arabinosus* also produces appreciable amounts of vitamin B_{12} (33). Hence it may be expected that added vitamins may have no effect. On the other hand, *E. coli*, *L. casei*, and *S. faecalis* R are known to produce only a feeble vitamin B_{12} activity (33). Although this amount may be sufficient to stimulate normal growth, requirements for growth and for optimal metabolic activity with respect to isolated systems need not necessarily be parallel. However, this may mean that enhanced synthesis of nucleic acids may not be essential for normal life processes.

In this connection the recent observations of Bendich and coworkers (34) are of interest. Tissue DNA is reported to be heterogeneous, the types being differentiated by chemical characteristics. Physiological differences are postulated. Such heterogeneity has also been demonstrated with DNA of microbiological origin; thus, part of the DNA in *S. faecalis* cells was found to be firmly bound to polysaccharide material and could not be dissolved in alkali (35).

The lack of any influence of vitamin B_{12} on *E. coli* could be due to a

difference in permeability or metabolic pattern which may exist, especially in view of its gram-negative character.

SUMMARY

The influence of folic acid and vitamin B_{12} on nucleic acid synthesis was studied in the microorganisms *Lactobacillus casei* and *Streptococcus faecalis* R, requiring folic acid, *Lactobacillus leichmannii*, requiring vitamin B_{12}, and *Lactobacillus arabinosus* and *Escherichia coli*, not requiring either vitamin.

In *L. casei* and *S. faecalis* R, the desoxypentose nucleic acid content varied with availability of folic acid. In *L. leichmannii*, a similar relationship was observed with respect to vitamin B_{12}.

With media devoid of purines an effect of the vitamins on pentose nucleic acid synthesis was observable in *L. casei* and in *L. leichmannii*.

In *L. casei* and *S. faecalis* R, vitamin B_{12} could apparently substitute for folic acid in nucleic acid synthesis but not in other respects. There was an additive effect when both the vitamins were supplied.

Neither folic acid nor vitamin B_{12} influenced nucleic acid synthesis in *L. arabinosus* and *E. coli*.

Liberation of ammonia from purines by resting cells of *L. casei* was inhibited by folic acid and vitamin B_{12} when present in the growth media. The effect, however, was not pronounced.

BIBLIOGRAPHY

1. Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **27**, 1 (1941).
2. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475 (1941).
3. Stokes, J. L., *J. Bact.*, **48**, 201 (1944).
4. Krueger, K., and Peterson, W. H., *J. Biol. Chem.*, **158**, 145 (1945).
5. Rogers, L. L., and Shive, W., *J. Biol. Chem.*, **172**, 751 (1948).
6. Stokstad, E. L. R., Regan, M., Franklin, A. L., and Jukes, T. H., *Federation Proc.*, **7**, 193 (1948).
7. Prusoff, W. H., Teply, L. J., and King, C. G., *J. Biol. Chem.*, **176**, 1309 (1948).
8. Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, **175**, 475 (1948).
9. Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).
10. Kitay, E., McNutt, W. S., and Snell, E. E., *J. Bact.*, **59**, 727 (1950).
11. Shive, W., Sibley, M. E., and Rogers, L. L., *J. Am. Chem. Soc.*, **73**, 867 (1951).
12. Roberts, I. Z., Roberts, R. B., and Abelson, P. H., *J. Bact.*, **58**, 709 (1949).
13. Rege, D. V., and Sreenivasan, A., *Nature*, **166**, 1117 (1950).
14. Franklin, A. L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., and Jukes, T. H., *J. Am. Chem. Soc.*, **71**, 3549 (1949).
15. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, **157**, 303 (1945).
16. Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944).
17. The pharmacopeia of the United States of America, fourteenth revision, Easton, 3rd suppl., 15 (1951).
18. Green, M. N., and Sevag, M. G., *Arch. Biochem.*, **9**, 129 (1946).
19. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
20. Albaum, H. G., and Umbreit, W. W., *J. Biol. Chem.*, **167**, 369 (1947).

21. Dische, Z., *Mikrochemie*, **8,** 4 (1930).
22. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1946).
23. Woolley, D. W., *Proc. Soc. Exp. Biol. and Med.*, **75,** 745 (1950).
24. Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, **176,** 1095 (1948).
25. Williams, J. N., Jr., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **71,** 303 (1949).
26. Barker, H. A., and Beck, J. V., *J. Biol. Chem.*, **141,** 3 (1941).
27. Conway, E. J., and Byrne, A., *Biochem. J.*, **27,** 419 (1933).
28. Levy, H. B., Skutch, E. T., and Schade, A. L., *Arch. Biochem.*, **24,** 199, 206 (1949).
29. Levy, H. B., Hale, M. G., Wallerstein, J. S., and Schade, A. L., *Wallerstein Lab. Communicat.*, **14,** 43 (1951).
30. Bergmann, E. D., Volcani, B. E., and Ben-Ishai, R., *J. Biol. Chem.*, **194,** 521 (1952). Bergmann, E. D., Ben-Ishai, R., and Volcani, B. E., *J. Biol. Chem.*, **194,** 531 (1952).
31. Mitbander, V. B., and Sreenivasan, A., *Current Sc.*, India, **20,** 129 (1951).
32. Miller, A. K., *Proc. Soc. Exp. Biol. and Med.*, **57,** 151 (1944).
33. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **108,** 634 (1948).
34. Bendich, A., Russell, P. J., Jr., and Brown, G. B., *J. Biol. Chem.*, **203,** 305 (1953).
35. Sherratt, H. S. A., and Thomas, A. J., *J. Gen. Microbiol.*, **8,** 217 (1953).