ON THE NATURE OF GROWTH INHIBITION OF *ESCHERICHIA COLI* BY AN OXIDATION PRODUCT OF VITAMIN B_{12}

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AN interrelationship between *p*-amino benzoic acid (*p*ABA) and vitamin B_{12} has been suggested on the basis of observations that vitamin B_{12} (*i*) spares the *p*ABA requirement for growth of *p*ABA auxotrophs of *Escherichia coli* (Davis, 1951; Gots and Chu, 1952); (*ii*) potentiates *p*ABA action in reversing sulphonamide growth inhibition in *E. coli* (Shive, 1950); and (*iii*) is involved in the biosynthesis of certain metabolites in which *p*ABA plays a role (Alimchandani and Sreenivasan, 1955). The function of *p*ABA in the synthesis of these metabolites in *E. coli* is probably through a folic acid (FA) active compound since, it is observed that a combination of them could overcome growth inhibition of the organism by aminopterin (Shive, 1951).

A vitamin B_{12} oxidation product is known to act as an antagonist of this vitamin to micro-organisms under certain conditions (Beiler *et al.*, 1951; Rege and Sreenivasan, 1954). It was of interest to study the relationship discussed here between B_{12}, *p*ABA and FA *vis a vis* this antagonist in the metabolism of *E. coli*.

EXPERIMENTAL

E. coli (Macleod) was maintained by fortnightly transfer on peptone-yeast extract-agar slants.

The basal medium was that of Green and Sevag (1946). To 5 ml. lots of the double strength basal medium in bacteriological test-tubes the various additions as stated were made and the volume adjusted to 10 ml. with glass-distilled water. The tubes were capped and sterilised at 15 lb. steam pressure for 15 minutes.

The vitamin B_{12} oxidation product was prepared as described by Beiler *et al.* (1951). To 10 ml. of a solution containing 500 μg./ml. vitamin B_{12}, 5 ml. of concentrated hydrochloric acid and a few drops of 30 per cent. hydrogen peroxide were added. The resultant mixture was warmed, allowed to stand for 2 to 3 hours, neutralised with potassium hydroxide solution and

the volume adjusted to 50 ml. A control with water in place of the vitamin B_{12} solution treated similarly was kept alongside, and used always for comparison in all experiments with the B_{12} oxidation product.

A forty-fold dilution of a 24-hour growth of the organism in the basal medium at 30° was used to inoculate the tubes dropwise. Growth was measured turbidimetrically in a Klett Summerson photo colorimeter at 660 mμ and is expressed in terms of galvanometer deflections.

All results represent averages of three or four different sets of experiments.

RESULTS

In a preliminary experiment, the activity of the oxidation product was tested with *Lactobacillus leichmannii* and *E. coli* (Macleod). It was observed that the preparation inhibited growth of both organisms. The control preparation showed only a very slight inhibition with both the organisms indicating, contrary to reports (Hendlin and Wall, 1954; Lester Smith, 1954), that the activity of the product is independent of its sodium chloride or hydrogen peroxide concentrations. Vitamin B_{12} was effective in reversing growth inhibition of *L. leichmannii* but not of *E. coli*.

Since, as stated earlier, vitamin B_{12} may be functionally related to *p*ABA, the effect of the latter as well as of the metabolites in whose synthesis it is involved were tried with *E. coli* (Macleod) alone and in combination. These metabolites were (per 10 ml.): methionine (0·5 mg.), purine mixture (adenine, guanine and zanthine 0·1 mg. each), serine (0·2 mg.), thymine (0·25 mg.), valine (0·5 mg.), glycine (0·25 mg.) and vitamin B_{12} (10 mμg.) (Alimchandani and Sreenivasan, 1955). The concentration of B_{12} oxidation product used was equivalent to 30 μg. B_{12}/10 ml. which was just sufficient to inhibit growth completely. None of the foregoing metabolites, singly or in combination, could reverse this growth inhibition.

As B_{12} is involved in the synthesis of purine and pyrimidine desoxyribosides, the effects of deoxypentosenucleic acid and pentosenucleic acid and ammoniacal hydrolysates of these were tried (1 mg. each). The method of hydrolysis followed (Levene and Jacobs, 1910) is known to degrade the nucleic acid to the riboside stage. But no reversal could be observed.

When yeast extract (Difco), peptone (Bacto) or crushed cells extract of *E. coli* were used, it was observed that all of them possessed considerable activity in overcoming growth inhibition of the oxidation product. These supplements were also markedly stimulatory to the growth of the organism in absence of the antagonist (Table I).

TABLE I

Effect of yeast extract, peptone and E. coli *extract on growth inhibition of* E. coli (*Macleod*) *by* B_{12} *oxidation product*

Addition to 10 ml. basal medium	Growth at the end of 24 hours	
	With B_{12} oxidation product equivalent to 30 μg. B_{12}/10 ml.	Without B_{12} oxidation product
1. Nil	0	49
2. Yeast extract (Difco) (1 mg.)	106	132
3. Peptone (1 mg.)	95	120
4. *E. coli* crushed cells extract (1·2 mg. dry weight)	73	95

An attempt was made to ascertain the nature of this reversal of growth inhibition by the oxidation product.

Using mixtures of amino acids, purines and pyrimidines and vitamins of the B group, it was observed that the amino acid mixture alone was active in overcoming growth inhibition though only partially. The amino acid mixture also stimulated growth in the absence of the oxidation product (Table II).

TABLE II

Effect of amino acids, purines, pyrimidines and B vitamins on growth inhibition of E. coli (*Macleod*) *by* B_{12} *oxidation product*

Addition to 10 ml. basal medium	Growth at the end of 24 hours	
	With B_{12} oxidation product equivalent to 30 μg. B_{12}/10 ml.	Without B_{12} oxidation product
1. Nil	0	51
2. Amino acid mixture* (0·2 mg. each) ..	71	118
3. Purine and Pyrimidine† mixture (0·1 mg. each)	0	50
4. B Vitamins mixture‡	0	53

* Alanine, asparagine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, norleucine, methionine, norvaline, phenylalanine, proline, serine, threonine, tryptophane and tyrosine. In the case of the optically active amino acids the recemic forms were used.

† Adenine, guanine, xanthine, uracil and thymine.

‡ Thiamine HCl, riboflavin, nicotinic acid, pantothenic acid, pyridoxine HCl and *p*ABA (10 μg. each), pteroyl glutamic acid (PGA) (3 μg.), Leucovorin (LV), Lederle (6 μg.) and Vitamin B_{12} and Biotin (10 mμg. each).

With increasing concentrations of amino acid mixture increased reversal was obtained (Table III).

TABLE III

Effect of increasing concentrations of amino acid mixture on growth inhibition of E. coli *(Macleod) by* B_{12} *oxidation product*

Addition to 10 ml. basal medium	Growth at the end of 24 hours	
	With B_{12} oxidation product equivalent to 30 μg. B_{12}/10 ml.	Without B_{12} oxidation product
1. Nil	0	49
2. Amino acid mixture*—		
0·2 mg. each	69	117
0·4 mg. each	87	126
0·6 mg. each	99	127
1·0 mg. each	106	124

* Component amino acids same as in Table II.

Using the amino acids at 0·4 mg. each/10 ml., it was observed that, in spite of a ten-fold increase in the concentration of the oxidation product, no complete growth inhibition could be obtained (Table IV).

TABLE IV

Increasing concentrations of B_{12} *oxidation product and growth of* E. coli *(Macleod) in basal medium supplemented with amino acid mixture*

B_{12} oxidation product in terms of equivalent μg. B_{12}/10 ml. ..	Nil	30	60	100	200	300
Growth at end of 24 hours, in 10 ml. basal medium*+ amino acid mixture (0·4 mg. each)	128	89	77	64	54	47

* Component amino acids same as used in Table II.

When the amino acids were tried individually (0·4 mg. each) they were all found to be ineffective in overcoming growth inhibition by the B_{12} oxidation product at a concentration equivalent to 30 μg. vitamin B_{12}/10 ml.

Discussion

The considerably greater inhibition obtained with the oxidation product over that of the control preparation without vitamin B_{12} discounted the possibilities (Hendlin and Wall, 1954; Lester Smith, 1954) that inhibition was non-specifically caused by sodium chloride or by hydrogen peroxide.

The inability of B_{12} to overcome growth inhibition by the B_{12} oxidation product suggested that the B_{12} oxidation product was probably not just an antagonist of the vitamin. However, this failure to achieve reversal of the action of an antimetabolite in organisms which do not show a nutritional need for the metabolite has been known for glucoascorbic acid (related to ascorbic acid) (Woolley and Krampitz, 1943; Woolley, 1944), phenyl-pantothenone (analogue to pantothenic acid) (Woolley and Collyer, 1945) and hexachlorocyclohexane (related to inositol) (Fuller *et al.*, 1950). This may be explained as due to the fact that the analogue acts as an antagonist of a derivative of the vitamin, which is an intermediate more closely related to the functionally active form. It is also possible that the vitamin cannot be absorbed by the cell.

The inactivity of PGA, LV, nucleic acids, nucleic acid hydrolysates, *p*ABA and the metabolites in whose synthesis the latter is involved indicates that growth inhibition caused by the oxidation product is not due to an interference in the biosynthesis of these metabolites. The inactivity of PGA and LV, however, could be due to their not being available as FA source for *E. coli* (authors: unpublished data).

The activity of the amino acid mixture in partially reversing growth inhibition suggests that the oxidation product functions by inhibiting the synthesis of certain amino acids. The potency of the natural materials tried seems to be due to their contribution of these amino acids.

The inactivity of individual amino acids suggested that the biosynthesis of more than one amino acid is blocked by the antagonist. Presumably the oxidation product blocks the biogenesis of a common precursor of two or more amino acids.

Summary

1. An oxidation product of vitamin B_{12} inhibits growth of *E. coli* (Macleod).

2. Of various substances tried, it is observed that yeast extract, peptone, *E. coli* crushed cells extract or a mixture of amino acids, overcome growth inhibition of the B_{12} oxidation product to an appreciable extent.

3. The amino acids when tried individually are found to be inactive.

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