SHORT COMMUNICATIONS

Vitamin, Purine and Pyrimidine Requirements of Oral Fusiforms

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Fusiforms isolated from saliva and gingival debris were allocated on morphological, cultural and biochemical grounds to *Fusobacterium nucleatum*, *F. polymorphum*, *Leptotrichia buccalis* A and *L. buccalis* B (Hadi & Russell, 1968*a*). The viable count of such fusiforms increases significantly in both saliva (Hadi & Russell, 1968*b*) and gingival material (Hadi & Russell, 1969) in cases of acute ulcerative gingivitis (A.U.G.).

Few investigations have been made of the nutritional requirements of fusobacteria (Omata, 1953, 1959; Coles, 1968) and no work done on leptotrichiae. The present study was undertaken to determine the vitamin, purine and pyrimidine requirements of oral fusobacteria and leptotrichiae.

METHODS

Test organisms and their maintenance. Three strains of each of the following were employed: *Fusobacterium nucleatum*, *F. polymorphum*, *Leptotrichia buccalis* A and *L. buccalis* B. These organisms were originally isolated from saliva and gingival material from healthy individuals, and from cases of A.U.G.

The test strains were maintained in Reinforced Clostridial Medium (R.C.M.) (Oxoid) containing 8% CaCO₃. The cultures were stored at 4° and transferred to a freshly prepared medium every two weeks.

Basal nutritional test medium. The composition of the basal nutritional test medium (pH 7.2) was as follows (g./l.): vitamin-free caseitone (Difco), 10.0; glucose, 5.0; KH₂PO₄, 1.2; K₂HPO₄, 1.2; DL-tryptophan, 0.2; sodium thioglycollate, 0.5; FeSO₄.7H₂O, 2.5×10^{-3} ; MnSO₄.4H₂O, 2.5×10^{-3} ; MgSO₄.7H₂O, 0.025; thiamine HCl, riboflavin, nicotinic acid, pyridoxine HCl, *p*-aminobenzoic acid, each 0.001; folic acid, 10^{-5} ; biotin, 5×10^{-6} ; calcium pantothenate, 0.002; choline chloride, 0.1; inositol, 0.025; adenine sulphate.H₂O, guanine HCl.H₂O, xanthine, uracil, each 0.012.

All glassware was immersed in chromic-sulphuric acid for 8 h., washed with tap-water and then with distilled water. Culture tubes were further filled with distilled water and autoclaved at 121° for 30 min. Stock solutions of vitamins (apart from biotin) were prepared in 50% ethanol. Biotin solution was made in 0.001 N-HCl, such a solution being stable for 18 months (Gallant & Toennies, 1949). Adenine, xanthine, guanine and uracil were dissolved in 0.5 N-NaOH. Stock solutions were

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stored at -20° . Test media made from the stock solutions were distributed in 10 ml. amounts into optically matched tubes (15×125 mm.) plugged with cotton-wool and autoclaved at 116° for 10 min.

Inoculum and incubation conditions. The organisms were grown in 20 ml. aliquots of R.C.M. (adjusted to pH 7.2 to 7.4) in McCartney bottles. The inoculated medium was incubated at 37° for 48 h. After incubation, the cultures were centrifuged at 1040 g. for 15 min. and washed three times in 0.067 M-phosphate buffer (pH 7.2) containing 0.05 % sodium thioglycollate. Nitrogen gas (passed through glycerol and sterile cotton-wool) was bubbled into the cell suspension during the washing procedure in order to remove any traces of oxygen.

Appropriate volumes of cell suspensions were transferred to optically matched tubes and brought to suitable volumes, in 0.067 M-phosphate buffer (pH 7.2) containing 0.05 % sodium thioglycollate, to give a turbidity reading of 80 on an E.E.L. (Evans Electroselenium Ltd, Halstead, Essex) nephelometer. An amount of 0.1 ml. of this suspension was then inoculated into tubes of test media. All cultures were incubated at 37° for 4 to 5 days (as found to be optimal for maximum growth) in B.T.L. (Baird & Tatlock Ltd, Chadwell Heath, Essex) anaerobic jars filled with a mixture of 95 % H_2 and 5 % CO_2 . The growth was homogenized by means of a 'Rotamixer' (Hook and Tucker Ltd, London S.W.9) and estimated nephelometrically. In all experiments the organisms were inoculated in duplicate tubes and each nephelometer reading stated was an average of two. 'Essentiality' of a factor was indicated by a reduction of growth in its absence to one-quarter of the control; a fall to about one-half showed stimulation.

RESULTS

In preliminary work none of the organisms grew in semi-synthetic medium containing vitamin-free Casamino acids (Difco) as source of amino acids, and only two strains

Table 1. *Growth of fusiforms in deficient media*

Substance deleted	Nephelometer reading							
	<i>Fusobacterium nucleatum</i>		<i>Leptotrichia buccalis</i> A			<i>L. buccalis</i> B		
	A2	A3	C1	C2	C3	D1	D2	D3
None	66.0	29.0	27.5	31.5	31.0	29.0	29.0	29.0
Thiamine HCl	61.0	30.5	25.0	29.5	30.5	30.0	29.5	27.5
Riboflavin	62.0	28.0	28.5	30.5	28.5	26.5	26.0	27.0
Ca-pantothenate	<u>7.5</u>	<u>0</u>	(12.0)	(14.0)	(14.0)	(11.0)	(11.0)	(10.0)
Inositol	62.5	28.0	21.0	28.0	25.0	28.0	23.5	24.0
Choline chloride	70.0	27.0	20.5	27.5	25.5	27.0	27.0	26.5
Biotin	66.0	20.5	<u>4.5</u>	<u>4.5</u>	<u>1.0</u>	<u>2.0</u>	<u>3.5</u>	<u>2.5</u>
Nicotinic acid	(24.0)	(11.0)	24.5	32.0	32.0	24.5	25.5	27.0
PABA	53.5	28.5	<u>2.5</u>	<u>3.0</u>	<u>2.5</u>	<u>3.5</u>	<u>3.0</u>	<u>1.0</u>
Pyridoxine .HCl	51.0	22.0	<u>0</u>	<u>1.5</u>	<u>0</u>	<u>2.0</u>	<u>0</u>	<u>0</u>
Folic acid	61.5	21.0	24.5	30.5	30.5	28.0	29.5	25.5
Adenine (A)	<u>12.0</u>	<u>7.5</u>	29.5	23.5	23.5	24.0	23.5	22.0
Guanine (G)	59.5	28.5	29.0	24.0	27.5	26.5	24.5	24.0
Xanthine (X)	55.0	27.0	25.0	30.0	31.0	25.0	25.0	24.5
Uracil (U)	60.5	27.5	24.5	24.5	28.5	26.0	29.0	29.5
A, G, U, X	<u>14.5</u>	<u>7.0</u>	27.5	25.0	30.0	29.0	27.5	27.0

Essentiality indicated by underlined figures and stimulation by figures in parentheses.

of *Fusobacterium nucleatum* and three strains each of *Leptotrichia buccalis* A and *L. buccalis* B grew in the basal medium with casitone. In experiments (Table 1) on the single deletion of vitamins from the basal medium, *F. nucleatum* A2 and A3 required calcium pantothenate and were stimulated by nicotinic acid. *Leptotrichia buccalis* (A and B) differed considerably from *F. nucleatum* and required biotin, pyridoxine hydrochloride and *p*-aminobenzoic acid. Furthermore, calcium pantothenate – essential for *F. nucleatum* – was found stimulatory for *L. buccalis* and nicotinic acid was not required.

Since the omission of *p*-aminobenzoic acid or folic acid did not affect the growth of *Fusobacterium nucleatum* (see Table 1), a medium deficient in both factors was tested. The two strains of *F. nucleatum* (A2 and A3) grew as well in the doubly deficient as in the complete medium, indicating that the lack of a requirement for either vitamin was not due to a mutual replacement effect.

The lack of requirement for biotin by isolates of *Fusobacterium nucleatum* could have been due to the presence of CO₂ in the atmosphere and the presence of asparagine in the vitamin-free casitone. A medium deficient in biotin was inoculated with the test organisms and incubated in an atmosphere of pure hydrogen. The control medium was that without biotin but incubated in a mixture of 95 % H₂ and 5 % CO₂. The complete medium, incubated in pure hydrogen, was also included as an additional control. It was observed that *F. nucleatum* A2 and A3 grew equally well in the above three media. This suggests that there was no requirement for biotin either in the presence or absence of CO₂.

The purine and pyrimidine requirements were first tested in media deficient in a single factor. The results are given in Table 1. It is clear that *Fusobacterium nucleatum* showed an absolute requirement for adenine. A subsidiary experiment showed that complete growth was obtained in the presence of adenine alone and that guanine, uracil and xanthine (singly or in combination) did not substitute for adenine: in contrast, deletion of adenine, guanine, uracil and xanthine as a group from the basal medium still did not affect the growth of *Leptotrichia buccalis*.

Media containing the compounds found essential and stimulatory for the organisms were prepared. *Leptotrichia buccalis* (A and B) grew nearly as well in the medium with only biotin, *p*-aminobenzoic acid, pyridoxine and calcium pantothenate as in the complete medium (with ten vitamins). On the contrary, *Fusobacterium nucleatum* A2 and A3 failed to grow when only calcium pantothenate, nicotinic acid and adenine were added. An experiment was then conducted in which single vitamins were added to the minimal medium used for *F. nucleatum*; in the case of folic acid there was a very slight response. The further addition of pyridoxine improved the growth of this organism to nearly one-half that in the complete medium.

DISCUSSION

The lack of growth of *Fusobacterium nucleatum* A1 and the three strains of *F. polymorphum* in medium containing vitamin-free casitone or Casamino acids made it impossible to study the nutrition of these organisms in such a medium. However, Omata (1959) reported that *F. nucleatum* did grow in a medium essentially similar to the basal medium used in this study (with Casamino acids). Similarly, Coles (1968) found that *F. polymorphum* responded to Omata's (1959) medium when glucose was

heat-sterilized with the medium and the adenine concentration reduced to $0.5 \mu\text{mole/ml.}$, i.e. 185 mg./l. from the $5 \mu\text{moles/ml.}$ employed by Omata (1959); he also stated that adenine was inhibitory at concentrations higher than $0.5 \mu\text{mole/ml.}$ In fact, Omata sterilized all the components of his medium together by autoclaving at 116° , and reported that *F. nucleatum* gave maximum response to adenine at a concentration of $5 \mu\text{moles/ml.}$ (1.85 g./l.) and no growth at $0.3 \mu\text{mole/ml.}$ (111 mg./l.). It should be noted that in the present work, even when the concentration of adenine was matched to those used by Omata (1959) and Coles (1968) respectively, one strain of *F. nucleatum* and three strains of *F. polymorphum* still failed to grow.

The requirement of *Fusobacterium nucleatum* for pantothenate is in agreement with the findings of Omata (1959), who found that this vitamin is a nutrilit for six strains. In the present study, however, the growth of the two strains of *F. nucleatum* studied was stimulated by nicotinic acid, although Omata did not indicate any stimulation by this vitamin. In addition, this organism requires adenine for active growth – a requirement also shown by Omata (1959).

It is strange to find that the highly fastidious organism *Fusobacterium nucleatum* apparently requires only one vitamin (pantothenic acid) and a purine (adenine). The use of casitone which had been treated with activated charcoal led to a reduction in growth, even though all the vitamins listed in Table 1 were present. It is clear there are unknown growth factors in the 'vitamin-free' casitone. However, the present work showed that pantothenic acid and adenine (plus nicotinic acid, being stimulatory) were not in fact the minimal vitamin requirements in the medium used. The further addition of both folic acid and pyridoxine resulted in moderate growth (approximately one-half the control).

It has been shown that the growth of *Fusobacterium nucleatum* in the experimental medium deficient in both folic acid and *p*-aminobenzoic acid is as good as that in the complete medium. It was not possible to eliminate components such as methionine and adenine from the medium; the former is presumably present in casitone, whereas the latter is essential for growth. This non-requirement for folic acid or *p*-aminobenzoic acid must imply the synthesis of these compounds by *F. nucleatum*. The failure to demonstrate a biotin requirement either in the presence or in the absence of CO_2 and, it may be added, in the absence of unsaturated fatty acids may be related to the fact that asparagine was not removed from the medium.

In the case of *Leptotrichia buccalis*, biotin, pyridoxine, *p*-aminobenzoic acid and calcium pantothenate could meet the minimal vitamin requirements. Similar observations were made on *Bacteroides ruminicola* by Bryant, Small, Bouma & Chu (1958). These workers found that this organism also grew well in the absence of purines and pyrimidines, and that medium containing vitamin-free enzymic digest of casein with biotin and *p*-aminobenzoic acid supported as good growth as medium with nine vitamins.

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