THE NUTRITIONAL REQUIREMENTS OF HEMOPHILUS PARAINFLUENZAE 7901[1]

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A complex mixture of known nutrients was utilized as a basal medium in the identification of putrescine as an essential growth factor for *Hemophilus parainfluenzae* ATCC 7901 (Herbst and Snell, 1948, 1949). By the addition of an optimum concentration of putrescine to this medium it has been possible to determine the remaining nutritional requirements of this organism, and to develop a simplified synthetic medium suitable for its growth. Details of these studies are presented here.

METHODS

The basal medium is shown in table 1. The growth obtained following omission of one or more components from this medium was compared with the growth response in the intact basal medium. Growth equal or superior to that obtained with the complete medium was considered necessary to demonstrate that a given component of the medium was nonessential for growth. Cultural methods were identical to those previously described (Herbst and Snell, 1949). Growth was measured turbidimetrically in the Evelyn colorimeter after 38 hours of incubation at 37 C.

RESULTS

Amino acid requirements. The growth response of *H. parainfluenzae* following the omission of individual amino acids from the complete medium is shown in table 2. Growth was severely depressed by the omission of either arginine, isoleucine, valine, or tyrosine from the amino acid mixture; the single omission of cystine and glutamic acid caused a less pronounced depression of growth. Omission of any of the amino acids not indicated in the table failed to affect growth adversely. When a simplified mixture that contained only these essential amino acids was prepared, it failed to support growth equivalent to that given by the more complete medium (table 3). Under these conditions, additional amino acids, as shown in the table, were stimulatory (or essential) for growth. Apparently the synthesis of the latter amino acids becomes limiting when only the simplified mixture is supplied, although it is not when these amino acids are omitted individually from a complete mixture of amino acids.

The vitamin requirements. The vitamin requirements of *H. parainfluenzae* were

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TABLE 1

*Composition of the basal medium**

COMPONENT	AMOUNT PER 10 ML	COMPONENT	AMOUNT PER 10 ML	COMPONENT	AMOUNT PER 10 ML
DL-Aspartic acid	10 mg	L-Cystine	1 mg	Inositol	200 μg
L-Glutamic acid	10 mg	L-Tyrosine	1 mg	Biotin	0.01 μg
DL-Alanine	10 mg	Glycine	1 mg	*p*-Aminobenzoic acid	0.01 μg
L-Arginine·HCl	2 mg	Glucose	10 mg	Folic acid	0.1 μg
DL-Methionine	2 mg	Sodium acetate	60 mg	Coenzyme I	1 μg
L-Leucine	1 mg	Guanine hydrochloride	100 μg	$MgSO_4 \cdot 7H_2O$	1 mg
DL-Threonine	2 mg	Adenine sulfate	100 μg	$CaCl_2 \cdot 2H_2O$	400 μg
DL-Serine	2 mg	Uracil	100 μg	$FeSO_4 \cdot 7H_2O$	135 μg
		Putrescine dihydrochloride	16 μg		
L-Proline	1 mg	Thiamine chloride	1 μg	$ZnSO_4 \cdot 7H_2O$	4 μg
DL-Tryptophan	2 mg	Riboflavin	1 μg	$CuSO_4 \cdot 5H_2O$	4 μg
DL-Valine	2 mg	Nicotinic acid	5 μg	$CoCl_2 \cdot 6H_2O$	4 μg
DL-Phenylalanine	2 mg	Nicotinamide	5 μg	$MnSO_4 \cdot H_2O$	3 μg
L-Histidine	1 mg	Pyridoxine·HCl	20 μg	K_2HPO_4	15.6 mg
DL-Isoleucine	2 mg	Calcium pantothenate	10 μg	KH_2PO_4	1.4 mg
L-Lysine	2 mg	Choline chloride	50 μg		

* Ten ml of this medium were inoculated with 0.1 ml of a washed, 12-hr inoculum, diluted to a turbidity of 95 (Evelyn colorimeter). The culture receptacles were 150-ml pyrex milk dilution bottles. The medium was adjusted to pH 7.8 with NaOH and sterilized by autoclaving for 15 minutes at 120 C. Coenzyme I was sterilized by Seitz filtration and added to the cooled sterile medium (see Herbst and Snell, 1949, for further details of procedure).

TABLE 2

Amino acid requirements of H. parainfluenzae

OMISSION FROM COMPLETE MEDIUM	TURBIDITY*
None	80
L-Arginine hydrochloride	94
DL-Isoleucine	99
DL-Valine	99
L-Tyrosine	95
L-Glutamic acid	87
L-Cystine	89
L-Leucine	80
L-Lysine	79
DL-Phenylalanine	80
DL-Aspartic acid	80

* Per cent of incident light transmitted; uninoculated medium = 100.

found to be relatively simple and not at all unusual (Peterson and Peterson, 1945). Omission of either biotin or calcium pantothenate from the vitamin mixture resulted in complete growth failure. The effects of omitting thiamine and

pyridoxine were less pronounced, but normal growth was not obtained in their absence, and vitamin B_6 appears essential when a restricted mixture of vitamins

TABLE 3

Stimulatory effect of "nonessential" amino acids when added to a minimal mixture

AMINO ACIDS IN MEDIUM	TURBIDITY*
(1) "Complete" mixture of basal medium (table 1)	82
(2) Simplified mixture of essential amino acids (table 2)†	95
(3) Same as (2) + L-leucine	88
(4) Same as (3) + DL-phenylalanine	85
(5) Same as (4) + L-lysine	82
(6) Same as (5) + DL-aspartic acid	80

* Per cent of incident light transmitted; uninoculated medium = 100.

† L-Arginine, DL-isoleucine, DL-valine, L-tyrosine, L-glutamic acid, and L-cystine at the concentrations indicated in table 1.

TABLE 4

The vitamin requirements of H. parainfluenzae

VITAMIN MIXTURE IN MEDIUM	TURBIDITY*
(1) "Complete" mixture of basal medium (table 1)	76
(2) Same as (1) minus thiamine chloride	79
(3) Same as (1) minus calcium pantothenate	100
(4) Same as (1) minus biotin	100
(5) Same as (1) minus pyridoxine hydrochloride	77
(6) Thiamine chloride, calcium pantothenate, and biotin	97
(7) Same as (6) + pyridoxine hydrochloride	76

* Per cent of incident light transmitted; uninoculated medium = 100.

TABLE 5

The effect of uracil on the growth of H. parainfluenzae

ADDITIONS PER 10 ML OF MODIFIED MEDIUM*	TURBIDITY†
None	97
100 μg adenine sulfate, guanine hydrochloride, and uracil	76
1 μg uracil	96
5 μg "	89
10 μg "	81
25 μg "	78
50 μg "	76
100 μg "	77

* Basal medium (table 1) minus purine bases and uracil.

† Per cent of incident light transmitted; uninoculated medium = 100.

is used (table 4). The remaining vitamins of the basal medium were neither essential nor stimulatory for growth.

Purine and pyrimidine bases. Almost complete growth failure resulted when

adenine, guanine, and uracil were omitted from the basal medium (table 5). However, maximum growth could be obtained in the absence of the purine bases if an optimum concentration of uracil (25 to 100 μg per 10 ml of medium) was added to the basal mixture. The specificity of this requirement is shown in table 6. Cytosine and thymine could not be utilized as substitutes for uracil, either

TABLE 6

A comparison of compounds effective in supplying the pyrimidine requirement of H. parainfluenzae

COMPOUND*	REQUIREMENT FOR HALF-MAXIMUM GROWTH	
	μg/10 ml	μM/10 ml
Uracil	7	0.069
Uridine	14	0.060
Uridylic acid	19	0.055
Cytidine	10	0.041
Cytidylic acid	16	0.050
Thymidine	>250	>1.0
Desoxycytidine	20	0.090

* For the basal medium used, see first footnote of table 5.

TABLE 7

*The effect of purine bases on the growth of H. parainfluenzae in media containing uracil**

ADDITIONS TO MODIFIED BASAL MEDIUM†	TURBIDITY‡
None	70
Adenine sulfate and guanine hydrochloride	70
Adenine sulfate	85
Guanine hydrochloride	94
Hypoxanthine	73
Xanthine	74
Adenine sulfate and hypoxanthine	73
Adenine sulfate and xanthine	84
Guanine hydrochloride and hypoxanthine	74
Guanine hydrochloride and xanthine	96

* Basal medium (table 1) minus adenine and guanine (contains 100 μg of uracil per 10 ml).

† One hundred μg of each compound added to 10 ml of medium.

‡ Per cent of incident light transmitted; uninoculated medium = 100.

in the presence or absence of the purine bases. However, cytidine, cytidylic acid, uridine, and uridylic acid were utilized as efficiently as was uracil. Thymidine showed very low growth-promoting activity; the requirement for half-maximum growth was 20 to 30 times that of the active compounds. This slight activity may possibly result from impurities in the preparation used. By contrast, the desoxyriboside of cytosine was highly active. Uracil was most active on a weight basis; however, on a molar basis cytidine, cytidylic acid, uridylic acid, and uridine were all slightly more active than uracil.

The single addition of either guanine or adenine to media containing uracil inhibited growth (table 7). When both were added together, however, no inhibition was apparent. Hypoxanthine and xanthine were not inhibitory under the same conditions, and hypoxanthine, but not xanthine, effectively overcame the inhibitory effects of adenine and guanine. These interesting relationships are highly reproducible and are somewhat similar to those observed in other organisms (e.g., Pennington, 1942; Loring and Pierce, 1944); their explanation, however, is not apparent at the present time.

Composition of a simplified medium. The information obtained in the foregoing experiments permitted the development of the simplified medium of table 8. The inorganic nutrition of the organism has not been critically examined in media rigorously freed of individual ions. However, minor changes in the salts

TABLE 8

*Composition of a simplified synthetic medium for H. parainfluenzae 7901**

COMPONENT	AMOUNT PER 10 ML	COMPONENT	AMOUNT PER 10 ML
L-Glutamic acid	10 mg	Uracil	100 μg
DL-Aspartic acid	10 mg	Putrescine dihydrochloride	16 μg
L-Arginine hydrochloride	2 mg	Thiamine chloride	10 μg
DL-Isoleucine	2 mg	Calcium pantothenate	10 μg
DL-Valine	2 mg	Biotin	0.01 μg
L-Cystine	1 mg	Pyridoxine hydrochloride	20 μg
L-Tyrosine	1 mg	Coenzyme I	1 μg
L-Leucine	1 mg	$CaCl_2 \cdot 2H_2O$	40 μg
L-Lysine	2 mg	$MgSO_4 \cdot 7H_2O$	1 mg
DL-Phenylalanine	2 mg	$FeSO_4 \cdot 7H_2O$	135 μg
Glucose	10 mg	$NaNO_3$	10 mg
Sodium acetate	60 mg	K_2HPO_4	31.2 mg
		KH_2PO_4	2.8 mg

* The cultural conditions are described briefly in table 1, and in detail by Herbst and Snell (1949).

mixture of the original basal medium (table 1) have been made as a result of experiments that are not given in detail. The Zn^{++}, Cu^{++}, Co^{++}, and Mn^{++} of the basal medium were neither essential nor stimulatory and were omitted from the simplified mixture. The Mg^{++}, Fe^{++}, and Ca^{++} salts were retained since the omission of any one of them resulted in slightly reduced growth. The Ca^{++} level was reduced to avoid formation of the undesirable inorganic precipitate in the original medium during autoclaving. The concentration of K_2HPO_4 and KH_2PO_4 was doubled since the higher level gave uniformly better results with the simplified medium. $NaNO_3$ was added since it stimulated growth very slightly in several experiments. Glucose and sodium acetate were essential for maximum growth in either medium.

The growth of H. parainfluenzae in a simplified medium. The response of *H. parainfluenzae* to putrescine in the simplified medium and in the original basal

medium (both minus putrescine) is compared in figure 1. Low concentrations of putrescine are considerably less effective in promoting growth in the simplified medium than in the complex medium. However, the maximum growth obtained with excess putrescine is similar in the two media.

The cause of this decreased response to low concentrations of putrescine is not fully known. Presumably, the increased demands placed on the bacteria grown in the less complex medium for the synthesis of a number of essential metabolites have increased the requirement for putrescine. However, the addition (singly) of those compounds absent from the simplified medium did not improve growth of the organism. Similarly, the single addition of either the complete vitamin mixture, the amino acid mixture, or the purine bases did not enhance the maximum growth obtained with an excess of putrescine.

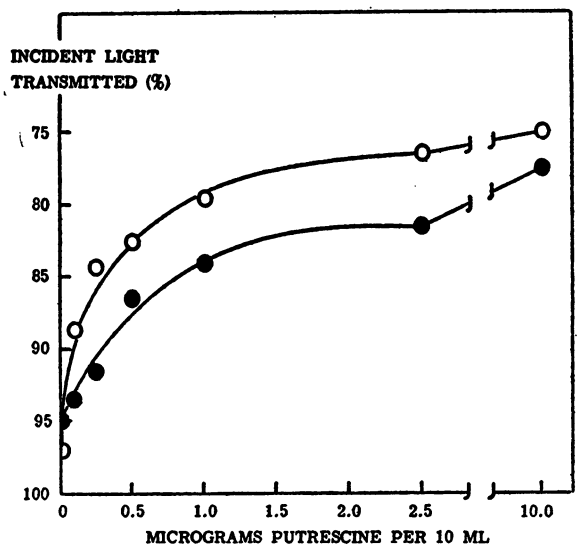

Figure 1. The comparative response of *H. parainfluenzae* to putrescine in the original (○) and simplified (●) medium.

DISCUSSION

The nutritional requirement of *H. parainfluenzae* for (a) coenzyme I (Lwoff and Lwoff, 1937) and (b) putrescine (or its congeners, spermine or spermidine —Herbst and Snell, 1948, 1949) are quite unusual. Several *Hemophilus* species require coenzyme I (Knight, 1945), but no other organisms having this requirement are known. In all cases examined, however, bacteria which do not require the pyridine nucleotides as essential nutrients can synthesize them. Perhaps putrescine represents an additional requirement common to many organisms of the *Hemophilus* group. This possibility does exist since only *H. pertussis* (Hornibrook, 1940) and *H. parainfluenzae* have been cultured in purified media.[2]

[2] A requirement of putrescine for *H. pertussis* is not eliminated, since growth of this organism in Hornibrook's medium was stimulated by the addition of hydrolyzed casein or of yeast extract. Both supplements contain putrescine (or spermine or spermidine), as indicated by assay with *H. parainfluenzae* 7901.

The specificity of the requirement of *H. parainfluenzae* for uracil is very similar to that of *Tetrahymena geleii* (Kidder and Dewey, 1948) and to that of uracilless *Neurospora* mutants (Loring and Pierce, 1944). For *Tetrahymena geleii*, as for *H. parainfluenzae*, cytidine, cytidylic acid, uridine, and uridylic acid were, on the molar basis, roughly equivalent to uracil in activity, but cytosine was inactive. For certain strains of uracilless *Neurospora*, however, uridine and cytidine were many times more active than uracil in promoting growth. Here again, cytosine was inactive. These similarities in widely diverse organisms point to a common mechanism for the synthesis and interconversion of pyrimidines and pyrimidine nucleosides.

An antagonism between purine bases has previously been reported in nutritional investigations with bacteria (Pennington, 1942) and molds (Fairley and Loring, 1949). Pennington observed that if the ratios of adenine to hypoxanthine or of guanine to hypoxanthine were high, the growth-promoting effect of hypoxanthine for *Spirillum serpens* was completely masked. Neither adenine nor guanine alone was active in promoting growth, but a mixture of approximately equal parts of the two would replace hypoxanthine. Fairley and Loring showed a definite inhibition of the growth of a purine-deficient strain of *Neurospora* when the ratio of guanine to hypoxanthine was greater than one. These results, though similar, are not entirely comparable to the purine antagonisms observed in *H. parainfluenzae* since the latter organism, in contrast to *S. serpens* and the mutant *Neurospora* discussed above, grows in the absence of added purine bases, i.e., is able to synthesize them. However, the cases cited do emphasize the extent to which imbalances in the medium employed may affect the apparent nutritional requirements of a given test organism.

SUMMARY

A simplified synthetic medium has been developed which supports growth of *Hemophilus parainfluenzae* 7901.

Arginine, isoleucine, valine, tyrosine, glutamic acid, cystine, leucine, phenylalanine, lysine, and aspartic acid are essential for maximum growth of this organism. Biotin, calcium pantothenate, thiamine, and vitamin B_6 are essential vitamins. Coenzyme I (or II) and putrescine (or spermine or spermidine) are also essential. A suitable pyrimidine is essential for growth; uracil, uridine, uridylic acid, cytidine, cytidylic acid, or desoxycytidine can serve in this role. Cytosine is inactive. Glucose and sodium acetate are also required for maximum growth.

Growth of *H. parainfluenzae* in the synthetic medium is severely inhibited by the single addition of either adenine or guanine. Such inhibition of growth is alleviated by the simultaneous addition of hypoxanthine, or of appropriate combinations of other purine bases.

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