The Amino Acid Utilization by Phase I *Bordetella pertussis* in a Chemically Defined Medium

By M. GOLDNER, CVETKA M. JAKUS, HARRIETT K. RHODES
AND R. J. WILSON

Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada

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SUMMARY

Phase I *Bordetella pertussis* was grown in a chemically defined medium containing 20 amino acids (including glutamine) plus glutathione. Growth was limited by depletion of three of the components, L-glutamic acid, L-proline and L-glutamine. Increasing the concentrations of these three components more than doubled the yield of organism. The following components were utilized, but did not limit growth: alanine, glycine, histidine, serine; cystine and glutathione were also substantially used. Ten amino acids which were not utilized to any significant extent could be omitted without diminishing growth. A preferential order of amino acid utilization is suggested. Glutamic acid appears to be important for antigenicity.

INTRODUCTION

There has been some interest in propagating phase I *Bordetella pertussis* in defined media. Rowatt (1957*a*) reviewed the growth requirements of this micro-organism and Wilson (1963) cultivated it in a chemically defined medium. The present investigation concerns the various amino acids used in the growth of *B. pertussis* phase I in Wilson's (1963) medium. Based on these utilization results, the amino acid composition of the medium was modified; the resulting changes in yield of organisms, utilization of amino acids and antigenicity are reported.

METHODS

Medium, inoculum and growth conditions. Table 1 lists the amounts of amino acids (and glutamine) and glutathione present in the medium (Wilson, 1963). In addition, this medium contains inorganic salts, nucleic acid derivatives, vitamins, adenosine triphosphate and coenzymes. The medium is defined except for the presence of a liver coenzyme concentrate which contains coenzyme A, NADP and NAD. This liver coenzyme concentrate can be replaced with equivalent amounts of these coenzymes without loss of growth of *Bordetella pertussis*. This chemically defined medium is designated medium A.

Modifications of medium A with respect to the presence and concentration of amino acids.

Medium B contained a fourfold increase in L-glutamic acid, L-glutamine and L-proline.

Medium C contained a fourfold increase in L-glutamic acid only.

Medium D. Hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine were omitted from medium A.

Medium E. The same amino acids were omitted as in medium D, and the concentration of L-glutamic acid, L-glutamine and L-proline quadrupled.

Medium F. The same amino acids were omitted as in medium D, and the concentration of L-glutamic acid only quadrupled.

The inoculum (Siebenmann strain, Connaught Medical Research Laboratories) consisted of 40×10^6 organisms/ml. medium (Wilson, 1963). The cultures were grown in 50 ml. volumes in L-shaped tubes, which were rocked gently at 35–36° for 48 hr.

Table 1. *Amino acids present in chemically defined medium for growth of phase I Bordetella pertussis (Wilson, 1963)*

	(mg./l.)		(mg./l.)
DL-alanine	100	DL-methionine	60
L-arginine	40	DL-phenylalanine	100
DL-aspartic acid	130	L-proline	60
L-cystine	10	DL-serine	130
L-glutamic acid	155	DL-threonine	80
Glycine	50	DL-tryptophan	30
DL-histidine	50	L-tyrosine	40
L-hydroxyproline	20	DL-valine	120
DL-isoleucine	120	L-glutamine	100
DL-leucine	240	Glutathione (red.)	25
L-lysine	70		

Bacterial concentrations were measured at 530 m μ (Coleman Universal Spectrophotometer, model 14) and the growth determined by relating the turbidity reading to the number of organisms/ml. based on the National Institutes of Health U.S. Standard (10 opacity units).

Amino acid analysis of culture media by paper chromatography. Fresh media and culture filtrates were concentrated in vacuum sixfold and the amino acids separated by one-dimensional paper chromatography on Whatman no. 3 filter paper (88 $\times$ 5 cm.) for 46 hr at 24° $\pm$ 1. The solvent system was *n*-propanol + ethanol + 0.05 M-pyrophosphate buffer (pH 8.9) + water (55 + 22.5 + 10 + 12.5, by vol.) containing 0.22% (w/v) NaCl (Wade, Matheson & Hanes, 1961; Hanes, Harris, Moscarello & Tigane, 1961).

One-dimensional paper chromatography was also done with solvent *n*-butanol + acetic acid + water (4 + 1 + 5, by vol.; Slotta & Primosigh, 1951) on Whatman no. 3 paper (57 $\times$ 46 cm.) for 66 hr at 24° $\pm$ 1.

The chromatograms were sprayed with ninhydrin (Block, 1950) and kept in the dark for 24 hr. Those developed in the *n*-propanol system were assayed in a Photovolt automatic densitometer. Preliminary determinations with this instrument established the relation, 'area² varies as concentration of amino acid' on the basis of a statistical analysis (Block, Durrum & Zweig, 1958; D. B. W. Reid & G. Van Belle, personal communication). By this procedure, the error in the determination was of the order of 15%.

Antigenic studies. For antigenic studies, vaccines were prepared from formalin-inactivated cultures after incubation for 48 hr in the various media. The vaccines were assayed by the mouse protection test, and their potencies determined as

immunogenic units against the U.S. Standard Pertussis Vaccine according to the Wilson-Worcester statistical method (Worcester & Wilson, 1943).

RESULTS

In the results given below, the terms 'use' or 'utilization' relate to the disappearance or decrease in intensity of spots on the chromatograms.

Cultures of *Bordetella pertussis* in medium A reached a maximum population of 5×10^9 organisms/ml. by 48 hr. Where only L-isomers or the DL-mixture of the amino acids were present in the medium, the same maximum concentration of organisms was reached, but only when the concentration of the DL-mixture forms were doubled when replacing the L-forms in the original medium A. Otherwise, the growth was less.

Many of the amino acids present in medium A could be separated chromatographically in the *n*-propanol system. However, certain spots consisting of two amino acids were separable in the *n*-butanol system. Thus cystine, when separated from aspartic acid, was seen to be substantially utilized. It was also possible to separate tyrosine from tryptophan, methionine from valine and hydroxyproline from threonine; serine and glycine, however, remained together. Utilization of tyrosine and tryptophan occurred, but no utilization of methionine. The decrease in intensity of the spots of these particular components was estimated visually except for cystine whose utilization was measured with a manual densitometer (EEL densitometer).

Determinations of amino acid utilization from medium A are shown in Table 2. The % utilization results were expressed as scores from 0 to 3. (The score of 0 assigned to some DL-amino acids indicates that little or no utilization occurred even when the L-form of the amino acid was considered to be the only utilizable one.)

Since glutamic acid, proline and glutamine at the initial concentrations used were fully depleted during growth of *Bordetella pertussis*, the concentrations of these components were increased in subsequent experiments. Quadrupling the concentrations of L-glutamic acid, L-proline and L-glutamine (medium B) increased the yield of organisms in 48 hr by 100–130%. There was no indication that any significant change in the yield of organisms could be expected after further increases of these components to the medium. When L-glutamic acid alone was quadrupled (medium C), 70–90% more growth was obtained than with medium A.

Densitometric determinations of the chromatograms for these media are given in Table 2. Glutamic acid, proline, glutamine, serine-glycine and alanine were considerably or completely utilized; glutathione was substantially used. The histidine+lysine mixture seemed in some instances to be used less than in the original medium; the cystine+aspartic acid mixture was always moderately used.

Ten amino acids (hydroxyproline, isoleucine, leucine, lysine, methionine, phenyl-alanine, threonine, tryptophan, tyrosine, valine) which showed little or no evidence of utilization following growth in medium A were omitted (medium D) without a decrease in growth. Quadrupling the concentrations of L-glutamic acid, L-proline and L-glutamine (medium E), or of L-glutamic acid alone (medium F), resulted in increased 48 hr growths of 90–125% and 60–105%, respectively, as compared with that in medium A. Densitometric analysis of chromatograms of these media with

the ten amino acids omitted showed the same complete or extensive utilization of glutamic acid, proline, glutamine, serine + glycine and alanine as occurred in media A, B and C (see Table 2). Glutathione was also used extensively, while glutamine appeared to be slightly less utilized than in medium A. Histidine was utilized to a

Table 2. *Utilization of amino acids in the growth of phase I Bordetella pertussis in chemically defined media*

Incubation 48 hr at 37°. Amino acid content of concentrated coelum fluids estimated chromatographically (see text).

Components	Score*					
	Medium†			Medium††		
	A	B	C	D	E	F
Alanine	3	3	3	3	3	3
Arginine	0	0	0	0	0	0
Aspartic acid	1	—	—	—	—	—
Cystine	2	—	—	—	—	—
Cystine§ + aspartic acid	—	1	1	1	1	1
Glutamic acid	3	3	3	3	3	3
Serine + glycine	3	—	—	—	—	—
Glycine	—	3	3	3	3	3
Serine	—	3	3	3	3	3
Histidine	2	2	2	2	2	1
Proline	3	3	3	3	3	3
Glutamine	3	3	2¶	3	3	3
Glutathione	3	3¶	2	3	3	3
Hydroxyproline	0	0	0			
Isoleucine	0	—	0			
Leucine	0	—	0			
Isoleucine + leucine	—	1	—			
Lysine	0	0	0			
Methionine	0	—	—			
Valine	0	—	—			
Methionine + valine	—	1	1			
Phenylalanine	1	1	0			
Threonine	0	0	0			
Tyrosine + tryptophan	1	1	2			
Growth after 48 hr (organisms × 10 ⁻⁹ /ml.)	5	{ 10– 11·5 }	{ 8·5– 9·5 }	5	{ 9·5– 11·25 }	{ 8– 10·25 }

* Score Utilization after 48 hr
 0 Between 0 and 15 %
 1 Between 16 and 45 %
 2 Between 46 and 75 %
 3 Between 76 and 100 %

† Components are listed as present in each medium (see text under 'Methods' for details).

‡ Ten amino acids present in medium A were omitted.

§ Not assayed separately.

|| Most utilized components in medium A.

¶ Could score either 2 or 3.

substantial extent, as in medium A. Aspartic acid was moderately used, even in respect to the L-form only.

Vaccines were prepared from cultures grown in all the media described. The results of mouse protection tests showed that media B and C produced more immunogenic vaccines than did medium A (Table 3). Antigenicity tests also indicated

that the vaccines prepared from cultures grown in the other media (D, E, F) were in some cases more antigenic than those prepared from cultures in medium A.

Table 3. Vaccine potency of phase I *Bordetella pertussis* cultures from regular and modified chemically defined media

Vaccine prepared in medium	Potency* (immunogenic units/ml.)
A	1.5
	2.0
	4.6
B	4.1
	8.0
	7.9
C	7.5
	6.7

A, Regular medium; B, concentration of L-glutamic acid, L-proline and L-glutamine quadrupled; C, concentration of L-glutamic acid, only, quadrupled.

* Based on 20 opacity units of bacterial concentration/ml. in the mouse protection tests; U.S. Standard Pertussis Vaccine contained 8 immunogenic units/ml.

DISCUSSION

With respect to the amino acid requirements of phase I *Bordetella pertussis*, a position of primary importance has been assigned to glutamic acid (Rowatt, 1957*b*; Jebb & Tomlinson, 1951). Ungar *et al.* (1950), who used a paper chromatographic technique, observed that alanine, aspartic and glutamic acids, glycine, proline, serine and threonine were used when *B. pertussis* was cultivated in the partially defined liquid medium of Cohen & Wheeler (1946). In the present work, proline, glutamine and glutamic acid were found to be limiting components for growth of phase I *B. pertussis* in a chemically defined medium, in the presence of a full complement of amino acids. Fukumi, Sayama, Tomizawa & Uchida (1953) suggested that proline was converted to glutamic acid by *B. pertussis*. Such a conversion may also apply to glutamine. Alanine, glycine, serine and, to a more limited extent, histidine, are utilized by the organism. The use of these components would probably be related, to a great extent, to the availability of glutamic acid, proline and glutamine. The utilization of aspartic acid could not be considered as comparable in importance to that of glutamic acid. The requirement for cystine (cysteine) and glutathione in preference to methionine as sulphur and nitrogen sources may be in part a function of their effect on the redox potential during bacterial growth. The advantage of these particular sulphur-containing components for the nutrition of *B. pertussis* has already been noted (Jebb & Tomlinson, 1955; Rowatt, 1957*b*).

Jebb & Tomlinson (1955), who used small inocula, found that the growth of *Bordetella pertussis* was consistently better in media prepared with L-amino acids than with the DL-amino acids. The diminished growth observed in our medium when L-acids were replaced by equal weights of DL-amino acid, must have been due to the decrease, by half, of the amount of one or more of the L-amino acids (cystine, glutamic acid, glutamine, proline) present in medium A. It should be noted that *B. pertussis* used the D-isomers, D-alanine, D-serine and probably D-histidine. D-amino acids (e.g. D-alanine) are known to occur in bacterial cell walls.

The grouping of a preferential order of amino acids used as a result of metabolism and growth by phase I *Bordetella pertussis* is shown on Table 1 of the accompanying paper (Vajdic, Goldner & Wilson, 1966). Omission from the chemically defined medium A of the ten amino acids shown in the lower level of the order did not adversely affect growth. The addition of increased amounts of glutamic acid, proline and glutamine provided similar maximal yields, whether with or without the full complement of amino acids.

The antigenicity results (Table 3) were based only on a small number of protection tests. However, increased amounts of certain amino acids (e.g. glutamic acid) in the original medium (medium A) appeared to increase antigenic potency as well as growth.

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