STUDIES ON THE NUTRITION AND PHYSIOLOGY OF *PASTEURELLA PESTIS*

II. A DEFINED MEDIUM FOR THE GROWTH OF *Pasteurella pestis*

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The cultivation of *Pasteurella pestis* in chemically defined media has been investigated by Rao (1939, 1940), Berkman (1942), Doudoroff (1943), Rockenmacher, *et al.* (1952), and Hills and Spurr (1952). These studies have shown that at a temperature of 27 C the minimal growth requirements of this species are satisfied by media containing relatively few amino acids and no added accessory growth factors. Although there have been some disagreements as to which amino acids were to be designated as essential, it has been established generally that phenylalanine, cysteine, and methionine are required for most strains of *P. pestis*. However, the requirement for cysteine could be satisfied by inorganic sulfur compounds such as sulfite, thiosulfate, etc., (Englesberg, 1952). The highest yields obtained in synthetic media, heretofore, have been of the order of 1 billion cells per ml. Because much higher yields (60 to 100 billion cells per ml) have been obtained in a complex medium (Higuchi and Carlin, 1957), it was believed that yields in synthetic media could be greatly improved. The present report deals with the development of a chemically defined medium which is capable of supporting growth of a number of strains of *P. pestis* to yield viable cell populations in the range of 40 to 60 billion cells per ml.

MATERIALS AND METHODS

Culture conditions. The organisms were grown in 25 ml of media in 500 ml Erlenmeyer flasks stoppered with cotton plugs, and aerated on a reciprocating shaker operating through a 3 inch stroke at a rate of 100 cycles per min. A growth temperature of 27 (±1) C was employed during most of these studies, but growth at 37 C also was investigated.

Measurement of growth. Growth was measured nephelometrically in standardized 18 mm test tubes after diluting the culture 10-fold in distilled water. The Coleman model 9 Nepho-Colorimeter was used for these measurements in conjunction with an arbitrary turbidity standard. A reading of 50 units in terms of the standard was approximately equal to an optical density value of 0.50 when measured as light transmission at 650 mμ wavelength. Correlation of nephelometric values and viable cell counts was obtained. Viable cell counts were determined by inoculating standard pour-plates of blood agar base (Difco) (fortified with 0.1 per cent glucose and 0.04 per cent Na_2SO_3) with appropriate dilutions of culture in potassium phosphate buffer (0.033 M, pH 7.3).

Preparation of medium. The amino acid components (except cysteine·HCl) were prepared as a double strength stock solution after neutralization with NaOH to pH 6.5 (table 1). The salt components were prepared as a single 5-fold concentrated stock solution and adjusted to pH 6.0. The cysteine·HCl (1.58 per cent) and D-xylose (25 per cent) solutions were sterilized separately by autoclaving at 121 C for 15 min. Labile substances, such as vitamins, were sterilized by filtration. The stock solution of amino acids and the salt mixture were combined, adjusted to pH 7.4 (±0.1) with NaOH, diluted to an appropriate volume with water and dispensed into 500 ml Erlenmeyer flasks. Addition of phenol red indicator (10 ppm) to the medium was useful in detecting changes in culture pH during the growth cycle. The medium was sterilized by autoclaving at 121 C for 15 min. Xylose and cysteine were added aseptically to each flask prior to inoculation.

Inoculum. Inocula were prepared by transferring growth from blood agar base slants to casein hydrolyzate medium (Higuchi and Carlin, 1957) and incubating for 24 to 36 hr on the shaker. The cells were then centrifuged, washed in

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TABLE 1

Composition of the synthetic medium for the growth of Pasteurella pestis at 27 C*

Amino Acids	Conc per Liter	Salts	Conc
	g		M
L-Glutamic acid	12.00	K_2HPO_4	0.025
DL-Phenylalanine	0.80	Citric acid	0.01
DL-Methionine	0.48	Sodium gluconate	0.01
DL-Valine	1.60	Ammonium acetate	0.01
DL-Leucine	0.40	$MgSO_4 \cdot 7H_2O$	0.0025
DL-Lysine·HCl	0.40	$FeSO_4 \cdot 7H_2O$	0.0001
L-Proline	0.80	$MnSO_4 \cdot H_2O$	0.00001
DL-Threonine	0.32		
Glycine	2.00	D-Xylose†	10.0 g per L
L-Cysteine·HCl†	0.158	Phenol red	10.0 ppm

* Medium adjusted to pH 7.4 (±0.1) before autoclaving.

† Sterilized separately. Additional aliquots of xylose were added as required during growth (see text).

0.033 M phosphate buffer, and inoculated into the synthetic medium at a concentration of 0.1 to 0.5 billion cells per ml of medium. A number of strains was employed in these studies but the development of the medium was based chiefly on results obtained with the avirulent strain, A1122 (Jawetz and Meyer, 1943). The nutritional characteristics of strain A1122 were typical of other *P. pestis* strains tested; however, it appeared to have a greater tendency to clump, especially during the early part of the growth cycle. The other strains of *P. pestis* employed in these studies are listed in table 2.

RESULTS

The composition of the synthetic medium is presented in table 1. The 10 amino acids listed were selected after a number of preliminary experiments in which each amino acid was shown to be either essential or stimulatory for the growth of *P. pestis* strain A1122. Phenylalanine, methionine, and cysteine were found to be essential for growth; the others were required for optimal growth. When a mixture of 18 amino acids corresponding to concentrations present in the casein hydrolyzate medium (Higuchi and Carlin, 1957) was prepared and tested, little or no growth was obtained. Omissions of the inhibitory as well as the inert constituents in the mixture followed by determinations of the optimal concentration levels of the remaining amino acids, finally resulted in the composition presented in table 1. Yields of viable cells obtained in the synthetic medium were in the range of 40 to 60 billion cells per ml (table 2).

D-Serine. The failure to obtain growth with a mixture of 18 amino acids appeared to be due to the presence of DL-serine in the mixture. The data in table 3 show that D-serine was the inhibitory isomer. The basal medium employed was similar to that in table 1 but with the differences in the glycine content as indicated. The presence of adequate amounts of glycine (0.027 M or 2 mg/ml) appeared to overcome the D-serine inhibition.

Glycine. The importance of glycine for the

TABLE 2

Viable cell yields of several strains of Pasteurella pestis grown at 27 C in the synthetic medium

Strain	Viable Cells per ml*
	× 10^9
Avirulent	
A1122	46
Tjiwidej	56
T.S.	55
Soemedang	57
Virulent	
Charleston	52
Alexander	49
Yokohama	45
Poona	51

* Cell counts were made during 48 to 72 hr period of incubation. Inocula ranged from 0.2 to 0.8 × 10^9 cells per ml. Additional xylose was added to cultures at approximately 24, 40, and 52 hr to provide a total concentration of 4 per cent.

TABLE 3

Inhibition of growth of Pasteurella pestis at 27 C by D-*serine and the partial reversal by glycine.*

Serine	Glycine	Growth*
Molarity	*Molarity*	
D-*Serine*		
0.00	0	108
0.0004	0	13
0.0008	0	6
0.0016	0	3
0.00	0.027	217
0.0004	0.027	204
0.0008	0.027	202
0.0016	0.027	196

* Turbidity readings at approximately 50 hr.

growth of *P. pestis* was not recognized at first because of the success obtained with the casein hydrolyzate medium which contains relatively little glycine. However, it was shown that rather high concentrations of glycine (1 to 2 mg/ml) were necessary for rapid initiation of growth in the synthetic medium.

Ammonium acetate. Because the initiation of growth in the synthetic medium still was not as rapid as in complex media, various substances were tested for growth stimulatory activity. Among the chemically defined substances which were tried, ammonium acetate (0.01 M) gave a marked stimulation of early growth. Other experiments indicated that both the ammonium and acetate moieties were required.

Cysteine. The requirement for cysteine in the casein hydrolyzate medium was satisfied completely by such substitutes as sulfite, thiosulfate, etc., (Higuchi and Carlin, 1957). However, in the synthetic medium the replacement of cysteine with sodium thiosulfate resulted in a prolongation of the lag phase of growth. Therefore, 0.001 M cysteine hydrochloride was employed in the final synthetic medium.

Carbohydrates. The use of D-xylose in the medium was based on results obtained in the development of the casein hydrolyzate medium (Higuchi and Carlin, 1957). D-Glucose, even in concentrations as low as 0.5 per cent in a medium containing 0.025 M phosphate buffer, resulted in accumulation of acidic products which inhibited growth by lowering the pH of the medium. D-Xylose, on the other hand, could be added at an initial concentration of 1 per cent (table 1). Moreover, several 1 per cent increments were added during the growth cycle. These additions during the course of growth were based on observations of the culture pH and the amount of growth present. It was important to judge correctly the intervals between additions in order to avoid an excess of xylose which resulted in acid production or to avoid a deficiency which limited growth. The need for more xylose was indicated by a rising pH in the culture during rapid growth, approximately 6 to 10 hr following the previous addition of xylose. In practice, the first increment of xylose was added when the culture turbidity attained approximately ¼ to ½ that of maximum growth, followed by the second increment approximately 8 hr thereafter. The third increment was added after an additional incubation period of 8 to 12 hr. The culture attained maximum growth after a total of 36 to 40 hr incubation. The fluctuations in culture pH which occurred during growth, were maintained within the extreme pH values of 6.5 and 8.5.

Excellent growth of *P. pestis* was obtained also with D-mannitol and with D-galactose in the synthetic medium. However, there appeared to be a greater tendency for cultures containing mannitol to become acid as compared to the standard xylose medium. On the other hand, cultures containing galactose frequently became extremely alkaline, particularly during the rapid growth phase. Therefore, it appeared that D-xylose was the most appropriate carbohydrate for growth in the synthetic medium.

Growth at 37 C. The nutritional requirements of *P. pestis* were shown to be more exacting at 37 C than at temperatures below 30 C (Hills and

TABLE 4

Recommended modifications of the standard synthetic medium for growth of Pasteurella pestis strain A1122 at 37 C

Supplement	Concentration
	mg/ml
DL-Isoleucine	1.00
$MgSO_4 \cdot 7H_2O$	4.31*
Thiamin·HCl	0.001
Ca-Pantothenate	0.001
Biotin	0.0005

* In addition to amount present in the standard medium (table 1).

Spurr, 1952). The modifications in the standard medium (table 1) which permitted good growth of strain A1122 at 37 C are listed in table 4. Isoleucine, which had no effect on growth at 27 C in the standard synthetic medium, was essential for growth at 37 C. The increased amount of magnesium sulfate (0.02 M) produced turbidity in the medium but was stimulatory specifically for the growth of the A1122 strain at 37 C and appeared to have no deleterious effects on several other strains tested. No definite requirements for any of the vitamins could be demonstrated, but the addition of vitamin supplements appeared to promote better growth occasionally. Yields of viable cells in the modified synthetic medium at 37 C were considerably lower than the yields obtained in the standard medium at 27 C. The values ranged from 10 to 30 billion cells per ml. A part of the irregularity in viable cell yields may be ascribed to clumping since the turbidity values were more uniform. A characteristic difference in the length of the lag phase between the virulent and avirulent strains of *P. pestis* was observed when cultures were grown at 37 C. The growth curves plotted in figure 1 show that the 4 avirulent strains were able to initiate growth rapidly, whereas the 7 virulent strains attained heavy growth only after a prolonged lag period. These data indicate the possibility of a relationship between virulence and some nutritional characteristic in *P. pestis*. Inoculum size was not a factor in these experiments.

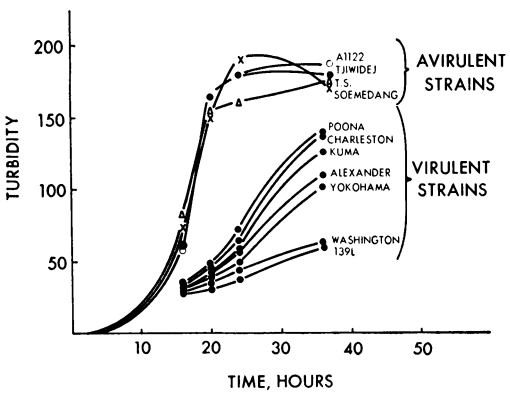

Figure 1. Characteristic differences in the length of the lag phase between virulent and avirulent strains of *Pasteurella pestis* in the synthetic medium at 37 C. (The medium in table 1, modified as in table 4, was further supplemented for this particular experiment with DL-tryptophan, (10 μg/ml); L-arginine·HCl, L-histidine·HCl, L-tyrosine, (50 μg/ml); DL-alanine and DL-aspartic acid, (100 μg/ml), in order to exclude consideration of strain differences in amino acid requirements as an explanation for the results).

DISCUSSION

The development of a synthetic medium capable of supporting high yields of *P. pestis* was based on the earlier studies with a casein hydrolyzate medium (Higuchi and Carlin, 1957). The selection of an appropriate carbohydrate (D-xylose) which had been a major factor in obtaining good growth in the casein medium was likewise of great importance in the synthetic medium. Although the employment of racemic amino acid mixtures as substitutes for the so-called natural L-forms has been satisfactory in the preparation of chemically defined media for many bacteria, examples are known, (Maas and Davis, 1950) where the D-isomers were more or less inhibitory. The strong inhibition of the growth of *P. pestis* by D-serine in media deficient in glycine together with the reversal of the inhibition by adequate amounts of glycine revealed an interesting aspect of glycine metabolism which deserves further study.

The characteristic difference in the length of the lag phase between virulent and avirulent strains of *P. pestis* when grown in the modified synthetic medium at 37 C may indicate the existence of a specific nutritional requirement in virulent strains. Such a theory may provide an explanation for the rapid loss in virulence observed in *P. pestis* strains when cultured under certain conditions *in vitro* at 37 C (Fukui *et al.*, 1957).

An earlier modification of the synthetic medium adapted for cultivation at 37 C has been employed in the production of protective antigens by *P. pestis* (Silverman *et al.*, 1954). The modified medium described in the present report may contribute further to similar efforts in antigen production.

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SUMMARY

A defined medium was developed which is capable of supporting 40 to 60 billion cells per

ml of a number of strains of *Pasteurella pestis* at a growth temperature at 27 C.

The standard medium contains D-xylose, a combination of organic and inorganic salts, and a mixture of 10 amino acids.

The growth of *P. pestis* at 37 C also was investigated. The standard medium was modified to contain 11 amino acids and a combination of thiamin, pantothenate and biotin. Yields ranged from 10 to 30 billion cells per ml at 37 C.

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