STUDIES ON THE NUTRITION AND PHYSIOLOGY OF PASTEURELLA PESTIS[1]

I. A Chemically Defined Culture Medium for Pasteurella pestis

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The chemically defined culture media for *Pasteurella pestis* which were formulated by Rao (1939, 1940*a*, *b*), Berkman (1942), Doudoroff (1943), and Herbert (1949) did not support the growth of any of the 27 strains available in this laboratory. This report describes the development of a chemically defined medium which was evaluated in terms of the following criteria: yields of viable cells, minimum size of inoculum, suitability for maintaining virulence on repeated subculture, and as a solid medium.

METHODS AND MATERIALS

Stock cultures were maintained at 5 C on nutrient agar-horse blood (5 per cent) slants. Virulent strains were injected into guinea pigs at approximately 6-month intervals, the organisms recovered from the infected spleens and used for preparing fresh stock slants. The virulence of the cultures for mice was assayed by subcutaneous inoculation, and LD_{50} values were calculated by the method of Reed and Muench (1938).

Only unselected, thoroughly washed organisms were used in testing the synthetic casein medium. The cells were carefully removed from stock slants and washed in 3 to 5 changes of Sorensen's phosphate buffer (0.03 M, pH 7.2). The size of the inoculum was approximated turbidimetrically and the number of viable cells was determined by surface plating. The use of small numbers of washed, unselected organisms minimized any influence on growth which might result from a carryover of nutrient materials or from the selection of variants capable of growth in the chemically defined medium.

The composition of the medium, which is designated SC (synthetic casein) because it approximates the amino acid composition of casein, is shown in table 1. The amino acid concentrations were based on data for the composition of casein from Cohn and Edsal (1943). Although added growth factors are not needed for growth in the synthetic casein medium, certain ones are listed in the table to record those which were tested and to which reference is made in the text.

The usual stringent precautions were observed in preparing glassware and

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media. Triply distilled water from a pyrex glass still, reagent grade chemicals, and amino acids (synthetic wherever possible) were used. The culture media were prepared by the following alternative methods: (1) by aseptic combination of

TABLE 1

The composition of a chemically defined medium (synthetic casein) for Pasteurella pestis

Amino Acids	mg
DL-Alanine	37
DL-Aspartic acid	120
L-Arginine·HCl	90
L-Cysteine·HCl*	13
L-Glutamic acid	642
Glycine	10
L-Histidine·HCl·H_2O	68
DL-Isoleucine	94
DL-Leucine	100
L-Lysine·HCl	155
DL-Methionine	65
DL-Phenylalanine	78
L-Proline	175
DL-Serine	100
DL-Threonine	70
DL-Tryptophan	31
L-Tyrosine	110
DL-Valine	160

	g	*Minerals**	mg
		$MgSO_4 \cdot 7H_2O$	40.0
		$FeSO_4 \cdot 7H_2O$	20.0
Glucose	3.00*	$MnSO_4 \cdot 7H_2O$	20.0
		NaCl	20.0

The amino acids were dissolved in 1 liter of Sorensen's phosphate buffer (pH 7.2 to 7.4). Concentrations of buffer from 0.03 to 0.2 M were used. The solution was autoclaved at 125 C for 15 minutes.

* Sterile solutions of glucose, cysteine, growth factors, and mineral salts were added aseptically to the cooled solution. Growth factors and mineral salts were sterilized by filtration through Selas, Seitz, Mandler, or fritted glass filters. Glucose and cysteine were sterilized separately by autoclaving at 125 C for 15 minutes or by filtering. Glucose concentrations varied from 0.1 to 1 per cent.

Growth factors were used only in the initial experiments and in later trials only where specifically mentioned: biotin, 1.0 mg; calcium pantothenate, 1.0 mg; choline chloride, 20.0 mg; folic acid, 0.5 mg; guanine, 10.0 mg; hemin, 1.0 mg; inositol, 10.0 mg; nicotinamide, 1.0 mg; *p*-aminobenzoic acid, 1.0 mg; pyridoxine, 1.0 mg; riboflavin, 1.0 mg; thiamin·HCl, 1.0 mg; uracil, 10.0 mg; glutathione, 1.0 mg. These quantities are for 1 liter of medium.

appropriate volumes of sterile, concentrated stock solutions of the various components, (2) by dissolving the amino acids (except cysteine) in the phosphate buffer and autoclaving at 121 C for 15 minutes. After cooling, sterile solutions of glucose, cysteine, and minerals (which were usually sterilized individually by filtration) were added. This latter procedure was followed because autoclaving

glucose with either phosphate or amino acids rendered the medium inhibitory to growth and produced a brownish discoloration.

TABLE 2

Growth of Pasteurella pestis in chemically-defined (synthetic casein) medium

PASTEURELLA PESTIS STRAINS	GROWTH*
A virulent†	
A-1122-1	+++
Java	++
K-120	+
TRU	++
Tijwidej	++
53H-1	+
A-1122-8	+++
Bombay	+++
Soemedang	+++
14	+++
Virulent	
Shasta (H-1)	+++
Shasta (H-2)	+++
Yreka	+
327	++
Webster	+++
0-25-26	++
de Rosa	+++
I-72	++
F-9650	+++
F-9581	+++
139-L	+++
Shasta 412	+++
0-9817-C	+++
0-9864	+++
B-741	++
499668	+++
499559	+++

* Growth after 72 hours was determined by visual observation of turbidity.

0 = no growth, + = barely perceptible growth, ++ to +++ = good to heavy growth.

Culture method: Ten ml of medium in 125 ml Erlenmeyer flasks were inoculated with 10^3 to 10^5 organisms from 5 times washed suspensions of the various strains and shaken at room temperature.

† Avirulent—More than 2×10^4 organisms inoculated subcutaneously into 8 to 14 weeks old mice caused no deaths.

The media were dispensed in 5 or 10 ml volumes in 50 or 125 ml Erlenmeyer flasks which contained 5 to 10 glass beads. Cultures used for studying maximum yields of viable cells were aerated by continuous shaking at room temperature (25 to 31 C). Most cultures were aerated in this manner, but even static cultures were afforded ample gaseous interchange with the atmosphere since the depth of culture medium in the flasks was less than 2 mm and the exposed surface was

large. Growth was measured visually and recorded as 0, +, ++, +++ to indicate absence of growth, scant, heavy, or very heavy turbidity. Numbers of viable cells were determined by surface plating.

The synthetic casein medium was solidified with agar to a final concentration of 2 per cent to determine if it would support growth in this form. Compounds such as hematin, cysteine, sodium thiosulfate, sodium sulfite, and ascorbic acid were tested by an auxanographic technique to determine their ability to promote growth on the synthetic casein-agar medium. Sterile solutions of these compounds were allowed to diffuse from sterile filter paper disks or penicillin assay cylinders

TABLE 3

Number of Pasteurella pestis organisms required to initiate growth in various culture media

STRAIN	MEDIUM	VIABLE ORGANISMS PER ML OF ORIGINAL SUSPENSION	GROWTH FROM 10-FOLD SERIAL DILUTION OF THE ORIGINAL SUSPENSION								APPROXIMATE NUMBER OF ORGANISMS INITIATING GROWTH
			0	−1	−2	−3	−4	−5	−6	−7	−8	
			Days after inoculation*									
A-1122	HIB†	1.5×10^8	4‡	4	4	4	4	4	4	4	4	less than 10
139-L	HIB	2.0×10^7	4	4	4	4	4	4	4	4	N.G.	less than 10
A-1122	S&P§	6.4×10^8	1	1	3	3	4	7	7	7	N.G.	64 or less
A-1122	SC	6.0×10^8	1	1	1	1	1	3	3	3	N.G.	60 or less
F-9581	SC	2.2×10^8	3	3	4	4	7	7	7	7	N.G.	22 or less
F-9581	SC	6.4×10^8	3	3	3	4	4	4	4	6	6	less than 10
139-L	SC	3.7×10^7	3	3	3	4	4	4	4	4	N.G.	less than 10
139-L	SC	1.4×10^9	3	3	3	3	4	4	4	6	6	14 or less

* Number indicates days after inoculation when cultures showed growth.

† HIB: Heart infusion broth (Difco).

‡ First examined after three days' incubation.

§ S&P: Smith and Phillips medium, modified as described in text.

N.G.—Indicates no growth after longest period of observation.

The original suspensions were washed 5 times. The serial dilutions were made in 0.03 M Sorensen's buffer and 1 ml used to inoculate each flask. These dilutions were also used for counting the number of viable organisms in the original suspensions.

placed on synthetic casein-agar medium previously seeded with ca 10^1, 10^2, or 10^3 washed organisms. One complete set of plates was incubated under aerobic conditions, while a duplicate set was incubated under reduced atmospheric pressure in an evacuated desiccator. The same inocula were placed on control plates of heart infusion agar containing 0.5 per cent horse blood. For definitive testing, promising compounds and certain adsorbents (starch, charcoal, or dextrin) were incorporated directly in the medium prior to pouring the plates.

Cultures grown in the synthetic casein medium were routinely checked and identified by microscopic examination of gram-stained smears, from colonial morphology on horse blood agar plates, by agglutination with plague antisera, and in some cases by the characteristic changes produced in infected animals.

RESULTS

Inocula of less than 10^3 well-washed, unselected organisms of 27 strains developed heavy growth on first transfer from blood agar into 5 to 10 ml of synthetic casein medium (table 2). The yields, which averaged 1×10^9 viable organisms per ml within 72 hours at 25 to 31 C, were equal to those routinely obtained in

TABLE 4

The effect of growth factors on the viable cell yields of Pasteurella pestis grown in the modified Smith and Phillips medium and in the synthetic casein medium*

COMPOSITION OF MEDIUM	STRAIN	VIABLE CELLS PER ML $\times 10^6$
S-P*	A-1122-1 (1)	31
S-P* + yeast extract (0.1 per cent)	A-1122-1 (1)	41
S-P* + growth factor mixture†	A-1122-1 (1)	22
SC	F-9581 (1)	0.4
	(2)	19
	139-L (1)	13
	(2)	15
SC + yeast extract (0.1 per cent)	F-9581 (1)	9.8
	(2)	22
	139-L (1)	15
	(2)	20
SC + horse serum (0.1 per cent)	F-9581 (1)	17
	(2)	23
	139-L (1)	17
	(2)	13

* Smith and Phillips medium modified by substituting 0.03 M Sorensen's phosphate buffer for the bicarbonate-phosphate buffer. The casamino acids used in this experiment were vitamin-free.

† The composition of the growth factor mixture is presented in table 1.

( ) The numbers in parentheses following the strain indicate the number of subcultures made.

Culture method: ten ml of medium in 125 ml Erlenmeyer flasks were inoculated with 10^3 to 10^5 organisms from a 3 times washed suspension and shaken at room temperature for 48 hours.

heart infusion broth cultures. No growth occurred in the media of Rao or Doudoroff which were simultaneously inoculated with more than 10^7 viable cells per ml. Two strains failed to grow in Herbert's medium. Three of the 27 strains developed faint turbidity in Berkman's medium. A synthetic medium for *Pasteurella multocida* (McKenzie *et al.*, 1948) was also unsatisfactory for all 27 strains. The addition of adsorbents (charcoal, starch, dextrin), surface active agents (bile salts, "tween 20", "tween 80", "span 20", "span 80"), blood (0.1 per cent), yeast extract (0.1 per cent), or the mixture of growth factors listed in table 1 to

the synthetic casein medium failed to increase the yield of viable cells above the average value of 1×10^9 organisms per ml. The nature of the material which is responsible for the yellow coloration which sometimes appears in the synthetic casein medium during growth was not investigated.

Fewer than 10 organisms initiated growth and produced heavy turbidity in either the synthetic casein medium or in heart infusion broth (table 3). Although Smith and Phillips (1943) reported that the minimum number which could es-

TABLE 5

Virulence and viable cell numbers of various strains of Pasteurella pestis after serial subculture in synthetic casein medium and heart infusion broth

STRAIN	CONSECUTIVE SUBCULTURES	VIABLE ORGANISMS PER ML	LD_{50}*		
			SC	HIB	Median value (HIB)†
Shasta (H-2)	2	1.1×10^9	183	—	33
Yreka	2	7.9×10^8	400	—	20
327-Lister	2	1.3×10^9	120	—	3
0-9817 C	2	5.6×10^8	143	—	32
F-9581	2)	1.9×10^9	29	—	18
	4)	5.8×10^8	96	—	
	10)	9.5×10^8	68	—	
139-L	2)	1.5×10^9	47	—	4
	4)	7.2×10^8	37	—	
	10)	1.2×10^9	24	—	
	1)	2.0×10^7	—	—	
	2)	1.4×10^9	—	25	
	4)	1.5×10^9	—	—	
	6)	1.0×10^9	—	400	
	8)	2.4×10^9	—	48	
	10)	2.1×10^9	—	42	
	15)	7.5×10^8	—	15	
	20)	6.8×10^8	—	13	

* See methods.

† Data extracted from the records of the Naval Biological Laboratory. Occasional high LD_{50} values were recorded for these strains: 139-L, 120; Shasta (H-2), 150; Yreka, 230; 327-Lister, 30; 0-9817 C, 70; F-9581, 100.

—Virulence titrations not performed.

)—Consecutive subcultures.

tablish growth in their medium was 100 cells, substitution of Sorensen's phosphate mixture for the bicarbonate-phosphate buffer of the original medium reduced the required inoculum size tenfold. The yield of viable cells was not increased even though the Smith and Phillips medium (modified as described before and prepared with either crude or vitamin-free casamino acids) was supplemented with either yeast extract or the mixture of known growth factors (table 4). Accessory growth factors were not required by *P. pestis*, and growth in the synthetic casein medium in the absence of such compounds was initiated by a few, well washed, unselected cells of our strains. Likewise, the number of viable

cells in the synthetic casein medium supplemented with either 0.1 per cent yeast extract (Difco) or 0.1 per cent horse serum was not greater than in the unsupplemented synthetic casein medium (table 4).

No diminution in the yields of viable cells occurred during subculturing (table 5). The virulence of strains F-9581 and 139-L was not appreciably decreased after 10 serial subcultures; this was also observed for 4 other virulent strains after 2 serial subcultures (table 5). The fluctuations in virulence seen in the table are not significant considering the errors inherent in virulence titrations. Similar

TABLE 6

Growth of Pasteurella pestis (strain A-1122-1) on synthetic casein-agar medium supplemented with hematin and blood and incubated under aerobic conditions and under reduced atmospheric pressure

COMPOSITION OF MEDIUM	NUMBER OF CELLS INOCULATED‡	NUMBER OF COLONIES	
		Aerobic	Reduced atmospheric pressure†
SC*	4	1	3
	47	0	7
	473	1	330
SC (pour plate)	4	0	0
	47	78	121
	473	488	463
SC + horse blood‡	4	7	8
	47	59	51
	473	552	483
SC + hematin (0.02 per cent)	4	4	2
	47	43	61
	473	497	485

* Incubated aerobically for 4 hours prior to placing in evacuated desiccator.
† Incubated in evacuated desiccator at room temperature for 48 hours.
‡ Determined by plating on nutrient agar containing 0.5 per cent horse blood.

variations in the virulence of primary cultures in heart infusion broth are evident in the table. The organisms which were transferred through 10 serial subcultures remained unaltered in cellular or colonial morphology. However, occasionally these organisms did not agglutinate sharply with either plague antisera or the gamma-globulin fractions from such antisera.

About two-thirds of the cells failed to produce colonies when seeded on the surface of either synthetic casein or synthetic casein-starch agar plates unless immediately incubated under reduced atmospheric pressure (evacuated desiccator). If either hematin or blood were incorporated in the synthetic casein medium, colonies equivalent to the number of cells inoculated were obtained under aerobic conditions. Recoveries under aerobic conditions were somewhat improved (but not quantitatively) by the addition of reducing agents or charcoal (table 6).

DISCUSSION

The results presented show that the synthetic casein medium satisfied the criteria for a liquid culture medium. The failure of the 27 strains to grow in the other chemically defined media may be an expression of strain differences. A comparison revealed that the synthetic casein medium was qualitatively similar to the other culture media in amino acid composition, but contained these compounds in different relative proportions. The proportions of the amino acids in the other media were either arbitrarily chosen or were based on the composition of gelatin hydrolysates, whereas in the synthetic casein medium these proportions were based on the amino acid composition of casein. The growth of all strains in the synthetic casein medium may reflect the requirement for an amino acid balance. Amino acid antagonisms, as have been described and quantitated for strains of *Brucella* (Schuhardt *et al.*, 1949) and for *Streptococcus bovis* (Washburn and Niven, 1948), may have been responsible for the inability of the other media to support the growth of *P. pestis*. This aspect will be investigated further.

Unselected strains of *P. pestis* require amino acids as sources of nitrogen for growth. Media which provide ammonium salts as the sole source of nitrogen have been reported as unsatisfactory for the cultivation of *P. pestis* (Rowland, 1914; Herbert, 1949). Growth of *P. pestis* in an ammonium salt medium on first transfer from stock culture required phenylalanine and cysteine and in subsequent subcultures in this medium on the selection of variant organisms (Doudoroff, 1943). The essential amino acids for particular strains have been determined by Rao (1939) and Herbert (1949). Our findings will be reported later.

Lack of added growth factors had no influence on the establishment of growth in the liquid synthetic casein medium, on yields of viable cells, on the minimum size of the inoculum, or on virulence. The other chemically defined media failed to support growth even on the addition of these compounds. Hematin was unnecessary for growth in the liquid synthetic casein medium although Herbert reported hematin as essential for growth in his medium. Blood serum was not required for the maintenance of virulence as was reported by Rowland (1914).

The contrast between the beneficial effects of aerobic conditions on growth in liquid medium and the reduced atmospheric pressure requirements for growth on the lightly inoculated surface of solidified media in the absence of blood, tissue extracts, reducing agents, or hematin still needs elucidation. In liquid cultures, *P. pestis* can tolerate normal atmospheric oxygen tensions (Girard, Neal, and Chevalier, 1946). Aeration by continuous shaking in thin layers in flasks or by sparging with sterile air produced increased yields of *P. pestis* (Smith and Phillips, 1943; Devignat, 1942; Devignat and Schoette, 1942; unpublished data of the authors). The higher yields were the result of oxygenation since shaking the cultures in a vacuum or bubbling nitrogen through them did not improve growth (Devignat and Schoette, 1942). These findings are difficult to reconcile with those of Sokhey (1948) who indicated that increased CO_2 tension and a certain optimum range of O_2 tension are essential to the growth of plague organisms in liquid medium. Unless blood, tissue extracts, or reducing agents are present, *P. pestis* organisms die rapidly on the surface of solid medium if exposed to a partial pres-

sure of oxygen exceeding 1 per cent (Wright, 1934). The beneficial effects of reduced atmospheric pressure (lower oxygen tension) on the aerobic growth of *P. pestis* on the surface of solid medium which was observed in our experiments can perhaps be correlated with the favorable results reported when reducing agents were incorporated in the medium (Drennan and Teague, 1917; Schüetze and Hassanein, 1929; Meyer and Batchelder, 1928). Reducing agents were not definitively tested in the synthetic casein medium. Under aerobic conditions, small numbers of *P. pestis* did not yield an equivalent number of colonies on plates prepared by the addition of agar to complex media which were satisfactory in liquid form (Drennan and Teague, 1917; Taylor, 1933; Samsonow, 1935; Schüetze and Hassanein, 1929; Wright, 1934; Sokhey, 1939; Herbert, 1949). Similar results were reported with chemically defined media for *P. pestis* (Rao, 1940*b*) and for *Hemophilus pertussis* (Verwey *et al.*, 1949). Hematin was identified as the constituent of blood which produced the "protective" effect permitting the growth of small numbers of plague organisms on solid medium under aerobic conditions (Herbert, 1949). This worker established that variable quantities of hematin were lost from complex media by slight changes in procedure during preparation, thus accounting for the erratic appearance of colonies on agar-solidified complex media. The results with solid synthetic casein medium confirm the observation of Wright and support the findings of Herbert. Some evidence of possible growth inhibition by certain batches of agar was noted. In any case, a chemically defined solid medium is available for possible application in such studies as bacterial genetics, mechanism of drug resistance, intermediary metabolism and immunology. The use of silica gel or "methocel" (Dow) as solidifying agents for the synthetic casein medium would permit very rigid criteria for a chemically defined solid medium.

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SUMMARY

A chemically defined culture medium which duplicates the amino acid composition of casein, but lacks added growth factors, has been developed and tested with 27 virulent and avirulent strains of *Pasteurella pestis*. The medium yielded high concentrations of viable cells, required only a small inoculum, supported growth in serial subcultures, and maintained the virulence of the cultivated organisms. Hematin, blood, or incubation under reduced atmospheric pressure was required when the medium was used as a solid medium.

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