STUDIES ON THE NUTRITION AND PHYSIOLOGY OF *PASTEURELLA PESTIS*

I. A CASEIN HYDROLYZATE MEDIUM FOR THE GROWTH OF *Pasteurella pestis*

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The ability of *Pasteurella pestis* to grow in a chemically defined medium is well established (Rao, 1939, 1940; Berkman, 1942; Doudoroff, 1943; Herbert, 1949; and Rockenmacher *et al.*, 1952). The culturing of the organism in casein hydrolyzate media has been described by Smith and Phillips (1943), Seal and Mukherji (1950), Sokhey *et al.*, (1950), and by Englesberg and Levy (1954). However, the amount of growth obtained has been rather low (5×10^8 to 50×10^8 cells per ml). When these values were compared to those obtained with the Brucella, where yields of 30×10^9 to 100×10^9 cells per ml have been reported (McCullough *et al.*, 1947; Sanders *et al.*, 1953), it was apparent that a better growth medium for *P. pestis* might be developed. The present report deals with the factors influencing the growth of *P. pestis* in a casein hydrolyzate medium. Results indicate that viable cell yields of 30×10^9 to 100×10^9 per ml in cultures incubated at 27 C can be obtained. The results of a similar study on a chemically defined medium will be described in a subsequent paper.

MATERIALS AND METHODS

Preparation of media. The principal component of the medium was a partial hydrolyzate of casein. The material was prepared by hydrolysis of 100 g of casein in dilute H_2SO_4 (33.3 ml conc H_2SO_4 + 500 ml H_2O) in an autoclave for 2 hr at 20 to 22 pounds steam pressure. The hydrolyzate was treated with an anion-exchange resin (Amberlite IR-4B) to remove the H_2SO_4. Approximately 200 g of resin (the amount varied with the moisture content of the resin) was rinsed several times with distilled water and then added to the acid hydrolyzate. The reaction was allowed to proceed for 30 min with continuous stirring. The addition of 20 ml of toluene to the mixture aided in preventing excessive foaming. The final pH of the solution was approximately 3.5. The suspension was then filtered and the resin washed repeatedly. The combined filtrate and washings were made up to a volume of 2 L. To the partially de-acidified hydrolyzate, 10 g of activated charcoal were added and the mixture was boiled for 5 min. The solution was cooled, filtered, and made up again to 2 L. A pH of approximately 3.5 and a "solids" content in the range of 4.5 to 5.0 per cent (determined by drying an aliquot at 100 C) were desirable. This material was designated as "5 per cent DCPH" (de-acidified casein partial hydrolyzate). DCPH was employed in the growth medium at a concentration equivalent to 2.5 per cent of casein. Certain variables in the preparation and evaluation of DCPH are described later.

The salt components of the medium (excepting $Na_2S_2O_3$) were prepared as a single tenfold concentrated stock solution. The DCPH and the salt mixture were combined, adjusted to pH 7.5 and sterilized by autoclaving for 15 min at 121 C. The carbohydrate and $Na_2S_2O_3$ were separately autoclaved and added aseptically to the medium.

Inoculum and cultures. A number of strains of *P. pestis* (both virulent and avirulent, table 1) was included in these studies, but strain A1122 (Jawetz and Meyer, 1943) was used for a major portion of the work. All strains of *P. pestis* examined have grown well in the final medium. The inoculum was grown routinely 20 to 30 hr in the DCPH liquid medium after transfer from a stock slant of blood agar base (Difco), and employed at a concentration of 1 per cent by volume.

Growth conditions. Adequate aeration was achieved with 25 ml of medium in a 500-ml Erlenmeyer flask placed on a reciprocating shaker operating at a rate of 100 cycles per min with a 3 inch stroke. Less aeration was reflected in poorer growth of the culture. The effect of temperature on the growth of *P. pestis* was determined by incubation of cultures at 27 ± 1 C and at 37 ± 0.5 C. Although 27 C

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TABLE 1

Viable cell yields from several strains of Pasteurella pestis grown in the DCPH medium at 27 ± 1 C

P. pestis Strain	Viable Cell Yield per ml, $\times 10^9$
Avirulent	
A1122	65
Tjiwidej	84
Soemedang	105
T.S	30
Virulent	
139L	69
Alexander	65
Poona	114
Charleston	86
Washington	61
Kuma	57
Yokohama	57

The DCPH medium (table 2) with D-xylose as carbohydrate substrate was employed for growing these cultures.

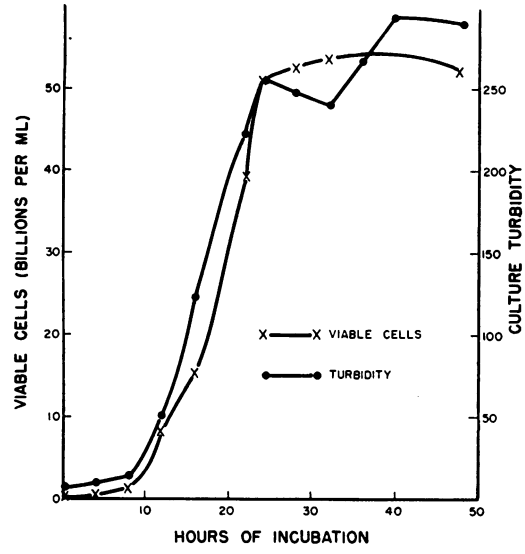

Figure 1. Growth curves of *Pasteurella pestis* in the standard DCPH medium with D-xylose as principal carbon source and incubated at 27 ± 1 C.

is believed to approximate the optimal growth temperature (Girard, 1955), considerations other than cell yields (such as production of protective antigens) have made it desirable to investigate the growth of *P. pestis* at the higher temperature.

Chemical analyses. Inorganic phosphate was determined by a modification of the procedure of Fiske and SubbaRow (1925). Reducing sugars were measured by the method of Folin and Malmros (1929). The procedures for the determination of bacterial protein and DCPH peptides were adapted from the method of Stickland (1951). The pH values were estimated by means of Hydrion pH papers or by incorporation of phenol red indicator (10 ppm) in the growth medium. The Beckman Model H-2 pH meter was employed for accurate adjustments of pH in the preparation of media.

Measurement of growth. The Coleman Model 9 Nepho-Colorimeter was employed routinely for measuring growth of cultures. Increases in growth, as measured by viable cell counts, were accompanied by parallel increases in nephelometric values (figure 1). An arbitrary turbidity standard was employed and readings were made on cultures diluted 1:10 in distilled water. (A reading of 50 turbidity units obtained with a diluted culture in a standard 18-mm pyrex test tube gave an optical density value of 0.55 in a colorimeter employing light of 650 mμ wavelength). Viable cell counts were made on the cultures by using potassium phosphate buffer (0.033 M, pH 7.3) to prepare the proper dilutions and plating in blood agar base (Difco) which had been fortified with 0.1 per cent of glucose and 0.04 per cent of Na_2SO_3. No blood was required in the agar base.

RESULTS

Effects of extent of hydrolysis and degree of ion-exchange treatment of DCPH on the growth of P. pestis. The effects of the extent of hydrolysis on the chemical and growth-promoting properties of DCPH were studied. Aliquots of a suspension of casein in dilute H_2SO_4, prepared as described under MATERIALS AND METHODS were autoclaved for varying periods of time. Samples, obtained at half-hour intervals during a period ranging from 1 to 3 hr, were tested in a standard manner after de-acidification with Amberlite IR-4B and decolorization with charcoal. The solids contents of these preparations were practically identical (4.4 ± 0.1 per cent), but as was to be expected, the amino nitrogen contents were higher in those samples hydrolyzed for longer periods. Typical examples of cell yields obtained in media prepared from these materials were 57 billion cells per ml in a medium containing a 1-hr hydroly-

TABLE 2

Composition of the casein partial hydrolyzate (DCPH) medium for the growth of Pasteurella pestis at 27 C

Component	Concentration
DCPH	2.5%*
K_2HPO_4	0.025 M
Citric acid	0.01 M
Na-gluconate	0.01 M
$MgSO_4 \cdot 7H_2O$	0.0025 M
$FeSO_4 \cdot 7H_2O$	0.0001 M
$MnSO_4 \cdot H_2O$	0.00001 M
$Na_2S_2O_3$ (sterilized separately)	0.0025 M
D-Xylose (sterilized separately)	1.0%†

* The "5% DCPH" prepared as described in the text was diluted two-fold in the preparation of the final medium.

† More xylose was added as required during growth (see text).

zate, 72 billions in a 2-hr hydrolyzate, and 69 billions in a 3-hr hydrolyzate. Highest cell yields were obtained most consistently with media prepared from two hour hydrolyzates.

The effect of the Amberlite IR-4B treatment on the properties of DCPH was studied by treating aliquots of a 2-hr acid hydrolyzate with varying amounts of ion-exchange resin. Tests were made over a range of 1 to 8 g of resin per g of casein employed. Optimal ion-exchange treatment, as reflected in cell yields in media prepared from these materials, was obtained in the region of 2 to 3 g of resin per g of casein. The final pH values of DCPH preparations with an optimal degree of de-acidification were in the neighborhood of 3.5. Solutions equivalent to 5 per cent of casein contained approximately 4.5 per cent of solids.

Effects of varying the glucose concentration in the medium. The medium finally adopted is presented in table 2. Experiments on the effect of varied glucose concentrations on the growth of *P. pestis* were made in the basal medium, except for the substitution of glucose for xylose. The growth and pH data presented in figure 2 illustrate the dual effects of glucose in the growth medium. Glucose stimulated growth at low concentrations (1 to 3 mg per ml), but the addition of 4 mg per ml or more resulted in a drastic reduction in cell yield. Very low terminal pH values were obtained in the inhibited cultures. The results were interpreted to mean that although glucose was well utilized, the oxidative processes consuming the intermediary organic acids were not keeping pace with the processes of acid production (fermentation). This appraisal of the course of glucose metabolism in the growth medium was supported by an experiment in which repeated small increments of glucose (1 to 3 mg per ml each) were fed to the growing culture. As much as 30 mg per ml, added in 17 increments, were utilized over a 90-hr incubation period. Viable cell counts of over 60 × 10^9 cells per ml were obtained. This was almost ten times the yield obtained in a medium with a single addition of glucose (figure 2). Each incremental addition of glucose was made with consideration of the fact that too much would result in excessive acidity and not enough would starve the cells (and also result in an undesirable rise in culture pH). Observations of the culture pH indicated when and how much glucose was required.

In order to avoid the laborious feeding procedures, the continuous feeding device described by Soltero and Johnson (1954) has been useful. A further elaboration of such a system would be a pH sensing unit to control the feed rate (McKee, 1955). For routine nutritional studies,

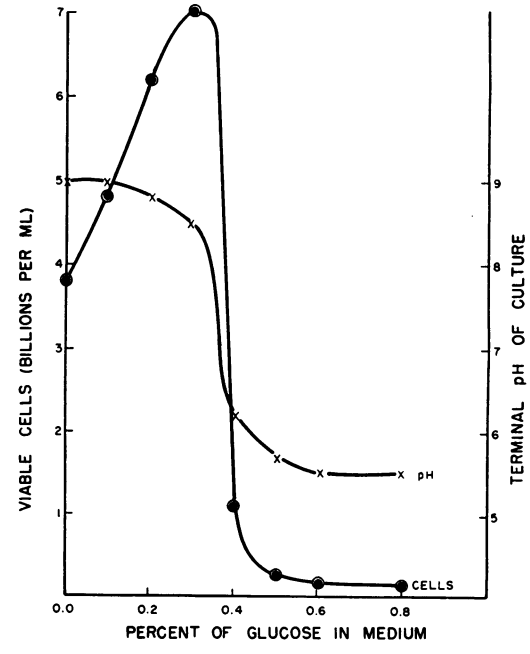

Figure 2. Effects of varied glucose concentrations on the growth of *Pasteurella pestis* in the standard DCPH medium.

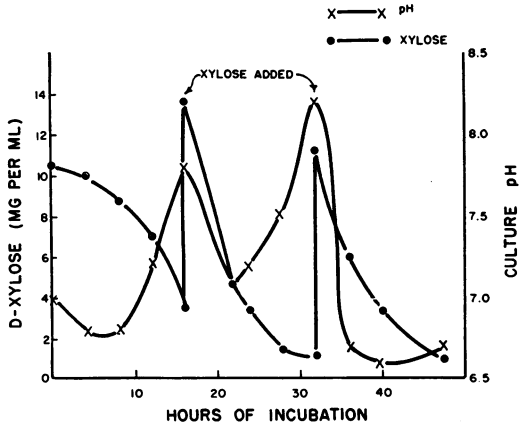

Figure 3. Relation between xylose utilization and pH of culture in the standard DCPH medium.

however, continuous feeding setups are not convenient; therefore, a search was made for carbohydrates with lower potentials for acid accumulation in the growth medium.

Alternate carbohydrates in the DCPH medium. Substances which appeared promising as a constituent of the growth medium were dextrin, D-xylose, and D-mannitol. Excessive acid did not accumulate when these materials were tested in the DCPH medium at a concentration of 10 mg per ml. Upon further study, however, consideration of dextrin for the medium was abandoned because of the lack of uniformity in the commercially available dextrins. The inertness of a large fraction of the dextrin which necessitated concentrations as high as 60 mg per ml (6 per cent) was another disadvantage of this material.

The most suitable carbohydrate was D-xylose. An initial concentration of 10 mg per ml was employed, and as this was utilized, further additions were made in 10 mg per ml increments until a total of 20 to 40 mg per ml was consumed. Because larger additions of xylose were permissible there was less danger of inadvertently failing to maintain a supply of carbohydrate reserve in the growth medium. A typical growth curve together with some of the accompanying physicochemical changes in a growth medium containing xylose are shown by data presented in figures 1 and 3 and table 3. The pH changes which occurred upon the additions as well as upon depletions of xylose are illustrated by the data. Xylose was supplied to the culture at 0, 16, and 32 hours. Data on the uptake of inorganic-P, utilization of biuret-positive peptides, and synthesis of bacterial protein are presented in table 3. Peptide utilization was determined by measurements of biuret-positive materials in the supernatant fluids after precipitation of the bacterial proteins with trichloracetic acid.

D-Mannitol was also found to be a suitable substrate in the growth medium. As much as 10 mg per ml (initial concentration) was readily tolerated. It was therefore superior to glucose with respect to ease of control of pH, but it was somewhat less suitable than xylose. Yields of cells obtained with mannitol were 10 to 20 per cent lower than those obtained with xylose. However, mannitol possessed an important advantage because of its stability. It could be autoclaved in the medium whereas it was necessary to autoclave xylose separately.

Glycerol was a suitable substrate for only certain strains of *P. pestis* (Girard, 1955). Two so-called glycerol-fermenting strains (Kuma and Yokohama) were grown in a glycerol DCPH medium and yields of approximately 50 × 10^9 cells per ml were obtained. The initial concentra-

TABLE 3

The utilization of peptides, uptake of inorganic phosphate, and production of bacterial protein in the DCPH growth medium by Pasteurella pestis at 27 C

Culture Age	Peptide*	Phosphate-P†	Protein‡	Viable Cells
hr	*mg/ml*	*mg/ml*	*mg/ml*	*×10⁹/ml*
0	15.3	0.80	0.2	0.3
4	15.2	0.78	0.7	0.4
8	13.4	0.74	1.6	1.2
12	12.4	0.67	4.2	8.3
16	10.0	0.66	5.9	15.4
22	8.2	0.56	8.2	39.3
24	8.5	0.58	9.1	50.8
28	8.0	0.59	10.0	52.6
32	7.5	0.58	9.8	53.8
36	8.0	0.55	10.0	—
40	7.5	0.55	10.1	—
48	7.3	0.56	10.1	52.3

* Measured as biuret-positive material in the supernatant fluid after trichloroacetic acid precipitation of cells.

† 0.80 mg per ml of P is equivalent to 0.0257 M K_2HPO_4 in the growth medium.

‡ Measured as the trichloracetic acid precipitable material in the growth medium. Casein was employed as a standard in both peptide and protein determinations.

tion of 10 mg per ml of glycerol was well tolerated in these tests, and a second 10 mg per ml was added and utilized before the cultures reached the stationary phase of growth. The glycerol fermenting strains, Kuma and Yokohama, also grew well in the xylose medium (table 1).

Concentrations of the constituents in the medium. The concentrations of the various components of the medium were determined by experiments in which the effects of graded amounts of each component were tested in media which were believed to be otherwise adequate. The composition of the final medium was a result of these studies and is presented in table 2.

Varying the DCPH concentration of the medium had a marked effect on viable cell yields. A concentration of approximately 2.5 per cent of DCPH appeared to be optimal, yielding approximately 70 billion cells per ml. On the other hand, 2 per cent and 4 per cent of DCPH yielded only about 40 billion cells per ml. Evidence that DCPH was a particularly favorable material for the growth of *P. pestis* was seen when several samples of commercial peptones and digests were substituted for the DCPH in the standard medium at an equivalent concentration (2.5 per cent). The growth obtained in the DCPH medium was better than with the commercial preparation tested.

The concentration of phosphate buffer employed in the standard medium (0.025 M) was based on results of experiments which showed higher concentrations to be inhibitory.

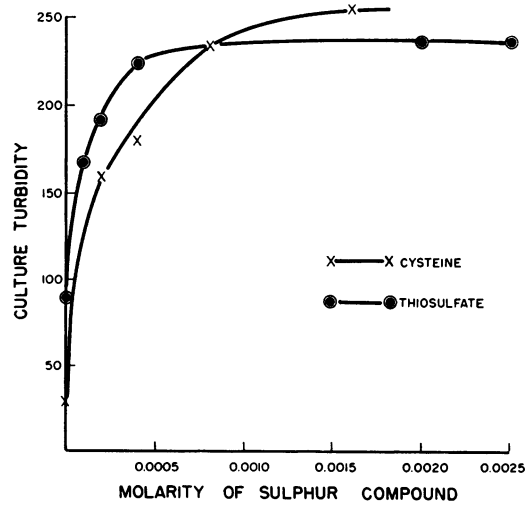

Figure 4. The requirement for either thiosulfate or cysteine for the growth of *Pasteurella pestis* in the DCPH medium.

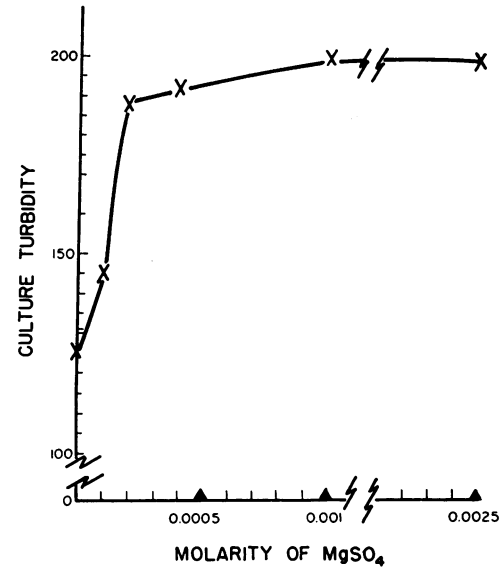

Figure 5. The requirement for additional Mg ions for the optimal growth of *Pasteurella pestis* in the DCPH medium.

The requirement for either cysteine or thiosulfate in the DCPH medium was demonstrated by data in figure 4. The amount of sodium thiosulfate (0.0025 M) employed in the final medium was in excess of the requirement. The need for added magnesium in the growth medium is shown by the results in figure 5. The amount employed in the final medium (0.0025 M) was, therefore, in excess of the requirement.

In order to demonstrate a requirement for iron in the DCPH medium, extraction of the DCPH component of the medium with a 0.1 per cent solution of 8-OH-quinoline in $CHCl_3$ was necessary (table 4). However, even in media which had not been extracted, iron deficiency occasionally has been observed; therefore the standard DCPH medium was supplemented with 10^{-4} M added $FeSO_4$ to insure sufficiency. Growth media prepared with DCPH which had been extracted with 8-OH-quinoline did not appear deficient with respect to manganese, but 10^{-5} M $MnSO_4$ was added routinely as a precaution.

A requirement for potassium ions in the medium was observed when Na_2HPO_4 was substituted for K_2HPO_4. On the other hand, a requirement for sodium ions was not demonstrable in a

TABLE 4

The requirement for iron for the growth of Pasteurella pestis in an iron-deficient medium prepared by extraction with a chloroform solution of 8-OH-quinoline

$FeSO_4 \cdot 7H_2O$ Added	Cell Yields per ml
$\times 10^{-4}$ M	$\times 10^9$
none	17.2
0.25	34.4
1.00	48.0
5.00	60.1

Only the DCPH component of the standard medium was extracted with the 8-OH-quinoline solution in chloroform. Possible iron contamination was further reduced by the omission of citrate and gluconate from the test medium.

Concentrations of $FeSO_4$ above 5×10^{-4} M were not tested because this was presumed to be in great excess.

medium when sodium salts were replaced with potassium and ammonium salts. The employment of citrate and gluconate in the medium was not essential, but citrate aided in preventing precipitation of the cations and gluconate appeared to promote an earlier initiation of growth. (Gluconate was readily oxidized by resting cell suspensions of *P. pestis*; citrate was not).

Yields of viable cells of several P. pestis strains. Typical yields obtained with several strains of *P. pestis* grown in the standard DCPH medium upon incubation at 27 ± 1 C are presented in table 1. Xylose was the carbohydrate employed in these cultures. The growth characteristics of these cultures were similar to those illustrated by data in figures 1 and 3 and table 3. No differences were noted in the growth of the virulent and avirulent cultures.

Effects of inoculum size. The size of inoculum employed in most of these studies was rather large. Approximately 1×10^8 to 5×10^8 cells were usually inoculated per ml of medium. It was found that initiation of growth from extremely small inocula (1 to 10 cells per ml) was difficult in the standard DCPH medium. There appeared to be inhibition by the concentration of the casein hydrolyzate employed. When stepwise reductions in only the DCPH concentration of the medium were made, there was a progressive decrease in the time required for appearance of visible growth from small inocula. It was evident that in order to obtain initiation of growth from small inocula (1 to 10 cells per ml), it was necessary to employ only 0.25 per cent of DCPH in the growth medium even though the final yield of cells was thereby limited.

Growth of P. pestis at 37 C. The optimal temperature for the growth of *P. pestis* is generally believed to be in the range of 26 to 30 C (Girard, 1955). Increased nutritional requirements have been observed in cultures grown at 37 C (Hills and Spurr, 1952). Nevertheless, the culturing of *P. pestis* at 37 C has deserved much attention for several reasons. A soluble capsular antigen is elaborated in cultures grown at 37 C but is produced only in insignificant amounts at 27 C. (Chen *et al.*, 1952). Furthermore, the *in vivo* state of physiology of the organism in a host animal probably is more closely approached by culturing at 37 C rather than at 27 C. It was desirable, therefore, to investigate the growth of *P. pestis* at 37 C in the DCPH medium. The standard DCPH medium (table 2) frequently was unsatisfactory for the growth of strain A1122 at 37 C. The modifications made in the standard medium for more consistent and improved growth at 37 ± 0.5 C are listed in table 5. The unusually high magnesium concentration (0.02 M), though resulting in a rather turbid medium, promoted the growth of A1122 at 37 C. Glycine, supplemented at a concentration of 0.027 M, also was distinctly beneficial. Although the employment of cysteine in place of thiosulfate was not advantageous, there were occasional indications that the change was beneficial. The addition of biotin and pantothenate is in accord

TABLE 5

Modifications of the standard medium for improved growth of Pasteurella pestis A1122 at a temperature of 37 C

Modifications*		
Compound	Concentration	Nature of change
Glycine	0.027 M	Supplement
$MgSO_4 \cdot 7H_2O$	0.020 M	Instead of 0.0025 M
Cysteine·HCl	0.004 M	To replace $Na_2S_2O_3$
Ca-Pantothenate	1.0 μg/ml	Supplement
Thiamin·HCl	1.0 μg/ml	Supplement
Biotin	0.5 μg/ml	Supplement

* The modifications are applicable to the standard medium (table 2).

TABLE 6

Viable cell yields of several strains of Pasteurella pestis in the modified DCPH medium incubated at a temperature of 37 C

Strain	Cell Yield per ml
	$\times 10^9$
A1122*	21, 30
Soemedang*	50
139L	29
Alexander	32
Yokohama	20

* Avirulent strains.

The growth medium is described in tables 2 and 5.

with the report of Hills and Spurr (1952), although the rather complete complement of amino acids supplied in the medium may have made the growth factors dispensable. Thiamin was added because distinct differences were obtained in the terminal pH values of unsupplemented cultures which indicated that thiamin may be limiting. The organism appeared to grow well in the absence of added thiamin but there was a tendency for these cultures to become more acid.

The various modifications described above were made in order to improve the growth of strain A1122 at 37 C. Possibly, all of the changes were not required for the other strains. In any case, the data presented in table 6 show that the modified medium was suitable for the growth of several *P. pestis* strains at 37 C. Yields of 20×10^9 to 30×10^9 viable cells per ml were usually obtained.

DISCUSSION

The results presented indicate that the primary cause of low cell yields of *P. pestis* obtained by other workers was the incapacity of the organism to maintain a balance between the production and utilization of the intermediary acids arising from glucose. The concentration of glucose permissible in the medium was limited to only a few mg per ml even though the culture was capable of ultimately utilizing larger amounts and thereby attain correspondingly greater growth. This situation is, of course, not uncommon in other aerobic microbial cultures.

The data showing the beneficial effect of reduced DCPH concentration on the initiation of growth from small inocula provide another example in support of the observation that a concentration of nutrient which provides for maximal cell yield is not always consistent with optimal growth conditions.

The improvements in the medium described in this work may contribute to studies on the physiology and immunology of the plague organism.

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SUMMARY

An improved casein hydrolyzate medium capable of supporting yields as high as 1×10^{11} viable cells per ml has been developed for the growth of *Pasteurella pestis*.

A study of the metabolism of carbohydrates by *P. pestis* aided in selecting appropriate carbon sources and growth conditions for increased cell yields. D-Xylose, D-mannitol, D-glucose, and glycerol were useful under certain conditions.

Determinations of optimal concentrations of the principal components of the growth medium were made. Casein hydrolyzate corresponding to 2.5 per cent of casein, potassium phosphate at 0.025 M, $MgSO_4$ at 0.0025 M, $Na_2S_2O_3$ at 0.0025 M, and $FeSO_4$ at 0.0001 M; each was at optimal (or noninhibitory excess) concentration.

A partial acid hydrolyzate of casein, particularly suitable for the requirements of *P. pestis* in the growth medium, has been developed.

The differences in the growth of *P. pestis* at two temperatures (27 C and 37 C) were investigated and a modified medium suitable for growth at 37 C has been developed.

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