STUDIES ON THE NUTRITION AND PHYSIOLOGY OF *PASTEURELLA PESTIS*

III. Effects of Calcium Ions on the Growth of Virulent and Avirulent Strains of *Pasteurella pestis*

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Hills and Spurr (1952) have shown that requirements for the growth of *Pasteurella pestis* were more exacting at 37 C than at 27 C; however, they did not report any characteristic, nutritional differences between virulent and avirulent strains. During studies on the nutrition of a number of virulent and avirulent strains in synthetic media, it was observed that virulent strains differed from avirulent types by exhibiting prolonged lag periods when incubated at 37 C (Higuchi and Carlin, 1958). This difference was not apparent when cultures were grown at 27 C. It appeared therefore that virulent strains when grown at 37 C required a characteristic nutritional factor(s) for growth. Subsequent work has shown that the addition of certain skim milk preparations to the basal medium permitted rapid growth of virulent strains at 37 C. The stimulatory factor in milk was identified as the calcium ion.

Attenuation of virulence in *P. pestis* upon prolonged cultivation in aerated liquid media at 37 C has been described previously (Wats and Puduval, 1940; Devignat and Schoetter, 1942; and Fukui *et al.*, 1957). This loss in virulence was attributed to the selection of avirulent variants. Preliminary findings of Kupferberg and Higuchi (1958) suggested that the presence of an adequate concentration of calcium in growth media might prevent the loss of virulence in aerated liquid cultures at 37 C. The subsequent results of this investigation, presented below, demonstrated that calcium was indeed associated with the maintenance of virulence during growth of *P. pestis* at 37 C. Ogg *et al.* (1958) have reported recently that the addition of certain spent culture filtrates to the growth medium aided in the maintenance of virulence of *P. pestis* cultures grown at 37 C. Oxygen tension and pH were shown also by these workers to markedly influence the selection of avirulent variants.

MATERIALS AND METHODS

Medium. The basal medium employed (table 1) was similar to that previously described (Higuchi and Carlin, 1958). The amino acid components except cysteine and tryptophan, each of which was sterilized separately, were combined as a double-strength stock solution. The salt components were combined as a 10-fold-strength stock solution. The Mg^{++} stock solution, however, was sterilized separately. The final medium was prepared by mixing appropriate amounts of amino acid and salt stock solutions and diluting with the proper amount of distilled water. The pH was adjusted to 7.1 to 7.3 with 5 N NaOH and aliquots (sufficient for 25 ml of final volume) dispensed into 500-ml Erlenmeyer flasks fitted with cotton plugs. After sterilization by autoclaving at 121 C for 15 min, appropriate amounts of sterile 25 per cent D-xylose, 0.1 per cent DL-tryptophan (dissolved in a minimal amount of NH_4OH), 0.025 M cysteine·HCl, Mg^{++}, and the vitamin mixture were added aseptically to give the final concentrations shown in table 1. When $CaCl_2$ was added, it was necessary to do so aseptically after separate sterilization and to maintain neutrality of the medium in order to prevent excessive precipitation. Baker's analyzed reagent grade salts of calcium and magnesium were employed.

The incorporation of phenol red indicator (10 ppm) was useful in controlling pH during preparation of the medium and in following pH changes in the culture during growth.

P. pestis strains and preparation of inocula. A list of the virulent strains employed in the present work is presented in table 3. The avirulent strains studied included EV76, T.S., A-4, and three others listed in table 5. Strain A-4 is an avirulent variant of the virulent Alexander strain

TABLE 1

Basal medium for study of nutritional requirements of Pasteurella pestis cultures incubated at 37 C

Constituent	g per L	Constituent	g per L
Amino acids		Vitamins*	
L-Glutamic acid	12.0	Thiamin·HCl	0.001
DL-Phenylalanine	0.80	Ca-pantothenate	0.001
DL-Methionine	0.48	Biotin	0.0005
DL-Valine	1.60	Other additions	
DL-Leucine	0.40	D-Xylose*	10.0†
DL-Lysine·HCl	0.40	Phenol red	0.01
L-Proline	0.80	**Salt components**	**Molarity**
DL-Threonine	0.32	K_2HPO_4	0.025
Glycine	2.0	Citric acid	0.01
DL-Alanine	0.40	Na-gluconate	0.01
L-Tyrosine	0.20	NH_4-acetate	0.01
L-Arginine·HCl	0.20	$MgSO_4 \cdot 7H_2O$*	0.02
DL-Isoleucine	1.0	$FeSO_4 \cdot 7H_2O$	0.0001
L-Cysteine·HCl*	0.157	$MnSO_4 \cdot H_2O$	0.00001
DL-Tryptophan*	0.040		

* Separately sterilized, (vitamins combined as a single solution).

† Additional xylose (0.5 to 1.0 per cent) was added after 16 to 18 hr of growth.

TABLE 2

Effects of various commercial preparations on the growth of Pasteurella pestis, Alexander strain, at 37 C.

Materials Added to Basal Medium	Conc	Growth*
	%	
Yeast extract (Difco)	1.0	13
Yeast autolyzate (Basamin-Busch)	2.0	11
Beef extract (Difco)	2.4	8
Tryptose (Difco)	2.4	12
Heart infusion broth (Difco)	2.4	10
Peptonized skim milk (Sheffield)	2.4	134
Lactose	1.0	18
Basal medium (control)	—	14

* Turbidity of 1:10 dilution of culture at approximately 24 hr.

isolated by Fukui *et al.* (1957). Inocula were prepared by growing each strain at 27 C in a casein hydrolyzate medium (Higuchi and Carlin, 1957). In preliminary experiments, when the nature of the stimulatory factor was unknown, the cells were washed in potassium phosphate buffer prior to inoculation. Subsequent results indicated that the washing procedure was not required. The inoculated medium containing approximately 0.5×10^9 cells per ml was incubated at 37 C on a reciprocating shaker operating through a 3 in stroke at 100 cycles per minute.

Measurement of growth. Growth was determined by nephelometric measurements employing an arbitrary turbidity standard (Higuchi and Carlin, 1957) and by standard plating procedures employing 0.033 M phosphate as the diluent and

TABLE 3

Effect of calcium ions on viable cell yields of virulent strains of Pasteurella pestis at 37 C in the basal medium

Strains	Viable Cell Yield ($\times 10^9$ per ml)*	
	Without Ca^{++}	With Ca^{++} (.004 M)
Alexander	0.17	8.6
Washington	0.79	20.0
Poona	0.24	3.8
139L	0.20	13.0
Yokohama	0.48	10.0
Kuma	0.55	13.0
Saka	0.65	15.0
M-41†	6.60	8.2
Charleston	0.64	12.9

* Yields obtained at 24 hr of incubation.

† Culture contains 13 per cent avirulent types.

TABLE 4

Effects of milk factor and of divalent cations on maintenance of virulence of the Alexander strain incubated at 37 C

Medium	No. of Generations*	LD_{50} for Mice†
Basal	23	>462,000
+ 2.4% peptonized skim milk	25	17 (5.2–55)
+ 0.004 M Ca^{++}	23	5.7 (2.0–16)
+ 0.004 M Sr^{++}	23	7.5 (4.4–13)
+ 0.004 M Zn^{++}	23	11.9 (4.3–33)

* Values given were calculated on the basis of cell yields obtained during 4 serial cultures.

† Values obtained by intraperitoneal injection of Swiss Webster mice, and calculated by the method of Litchfield and Wilcoxon. Values in parentheses represent 95 per cent confidence limits.

TABLE 5

Effects of calcium on turbidity, protein production, and viability of avirulent Pasteurella pestis strains during growth at 37 C

Culture*	Turbidity†				Protein mg per ml				Viable Cell × 10^9/ml			
	hr				hr				hr			
	0	12	18	24	0	12	18	24	0	12	18	24
A1122	—	27	132	135	0.10	2.54	7.20	7.34	0.47	4.0	13.9	9.8
A1122 with Ca^{++}	—	23	112	129	0.10	2.17	5.98	5.66	0.47	2.5	11.7	13.7
Tjiwidej	—	22	86	143	0.10	2.26	5.84	9.08	0.41	3.3	8.1	6.9
Tjiwidej with Ca^{++}	—	10	62	161	0.10	1.14	3.00	7.80	0.41	1.6	8.8	20.1
Soemedang	—	20	85	137	0.11	1.88	5.58	7.36	0.49	4.8	13.8	12.5
Soemedang with Ca^{++}	—	15	78	142	0.11	1.62	5.06	8.10	0.49	2.7	15.5	24.3

* Amount of added calcium was 0.004 M $CaCl_2$.

† Of 1:10 dilution of culture.

blood agar base (Difco) as the plating medium. Protein was determined by the method of Stickland (1951) on precipitates obtained with 5 per cent trichloracetic acid.

RESULTS

Identification of calcium as the stimulatory factor in milk. A variety of natural materials was tested for factors which would reduce the prolonged lag period of growth noted for virulent strains incubated at 37 C in the basal medium. Extracts were prepared from fresh liver, raw cabbage, human plasma, sheep blood, and cow's milk. These materials and a number of commercial nutrients such as peptones, yeast extracts, and milk products were tested in the basal medium for their stimulatory effect on growth at 37 C. Only milk and certain milk products were stimulatory. The results obtained with some of the commercial preparations are presented in table 2.

Fractionation of the active material on ion-exchange resins (Amberlites IR-4B and IRC-50) showed that a cationic substance was involved. Subsequent tests with ashed milk preparations indicated that the inorganic cation calcium, which is present in high concentrations in milk, was the active factor. The stimulatory effect of added $CaCl_2$ on the growth of virulent strains of *P. pestis* was shown by the data in table 3. The effects of graded amounts of $CaCl_2$ on the growth of the virulent Alexander strain is presented in figure 1. Omission of citrate from the basal medium did not appreciably reduce the amount of calcium required for optimal growth.

Effects of other cations. A number of other cations, 18 in all, were examined in order to determine the specificity of the calcium requirement. Strontium and zinc were able to replace calcium at concentrations approximately equivalent to that of calcium (0.004 M). Barium, magnesium, cadmium, and others were either ineffective or toxic at the levels tested.

Effect of calcium on maintenance of virulence. Growth of the virulent Alexander strain occurred after a long lag period in the basal medium without calcium. After a total of 4 serial passages (consisting of approximately 23 generations in the basal medium in which the culture now grew rapidly), the cells had become practically avirulent for mice (LD_{50} > 462,000; table 4). No loss in virulence occurred in parallel cultures which contained 0.004 M $CaCl_2$ (LD_{50} = 5.7). Similar results were obtained with media containing strontium, zinc, or peptonized skim milk powder.

Effect of calcium on growth of avirulent strains at 37 C. There was no requirement for calcium for the growth of 5 out of 6 avirulent strains as judged by turbidity data. Viable cell counts, however, showed invariably lower viability in calcium-deficient cultures when determined after 24 hr incubation. In a more detailed examination of this phenomenon, three avirulent strains were grown with and without added calcium (table 5). The amounts of growth were followed by turbidity measurements, total protein determinations, and viable cell counts. When examined at 12 hr of incubation, all three calcium-deficient cultures had grown more rapidly than the control cultures containing 0.004 M calcium. This was shown by all three analytical procedures (table 5). After further incubation, however; a trend toward decreased viability became evident in the calcium-deficient cultures.

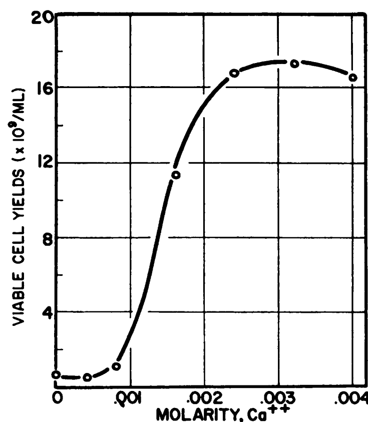

Figure 1. Effect of added Ca^{++} on the growth of the Alexander strain (virulent) in a defined medium containing 0.02 M Mg^{++}. Inoculated with 0.39×10^9 cells per ml and incubated at 37 C for 24 hr.

Microscopic examination of these cultures failed to provide an explanation for the reduced viability of the calcium-deficient cultures. No aggregation of cells or cells of abnormal appearance were noted.

Strain EV76 was the only one of 6 avirulent strains tested which required calcium for growth at 37 C in the basal medium.

Effects of calcium-magnesium ratios on the growth of P. pestis strains. When the effect of added calcium on the growth of virulent and avirulent strains was compared in media containing low (0.0025 M) and high (0.02 M) concentrations of magnesium, it was apparent that interactions occurred between calcium and magnesium. As reported above, in the absence of calcium with 0.02 M magnesium present, little or no growth of virulent strains occurred; however, with only 0.0025 M magnesium present, yields of the virulent Saka and Alexander strains were 4 to 8×10^9 cells per ml in the absence of added calcium. On the other hand, avirulent strains (with the exception of A-4) grew poorly or not at all in the absence of calcium when the magnesium concentration was 0.0025 M, but grew without calcium when the magnesium concentration was 0.02 M (except strain EV76).

DISCUSSION

The chemically defined medium previously described (Higuchi and Carlin, 1958) was based chiefly upon the requirements of avirulent strain A1122. An increased complement of amino acids was employed in the experiments reported herein (table 1) because the addition of calcium revealed that these extra amino acids promoted better growth of virulent strains at 37 C.

In calcium-deficient media, growth of virulent strains occurred only after prolonged lag periods. Selection and growth of variant types undoubtedly occurred. The results presented in this paper showed that the variants were of reduced virulence.

The failure to discover the importance of the calcium ion in the nutrition of *P. pestis* during earlier studies was undoubtedly due to the high concentrations required for stimulation. It should be noted, however, that the concentrations of calcium required by virulent *P. pestis* are in the same range reported for its concentration in human sera (West and Todd, 1951). Moreover, the requirement of this concentration of calcium for growth at the host temperature (37 C) may have added significance.

The function of calcium ions in the nutrition of *P. pestis* remains obscure. It would appear, however, that there exists a relationship between virulence and the requirement for calcium ion under the conditions described. All of the avirulent strains tested with the exception of strain EV76 were able to grow well in the basal medium without added calcium. This exception, furthermore, may be explained. It has been reported that strain EV76 was fully virulent if the host animal was injected with inorganic iron salts at the time of inoculation with the organism (Jackson and Burrows, 1956). On the other hand, virulent strain M-41, which yielded a moderate amount of growth in the absence of calcium, was shown to contain a high proportion (13 per cent) of avirulent variants.

The culture filtrate factor of Ogg *et al.* (1958) undoubtedly was not the calcium ion. Other variables reported by these workers such as pH and oxygen tension, which were also factors in the selection of avirulent variants, did not appear to be significant in our work. The initial pH value in our medium was approximately 7.4 and a high degree of aeration was maintained by shaking during incubation.

The definite requirement for calcium shown by avirulent strains in media containing a reduced amount of magnesium (0.0025 M) suggested that these strains were able to substitute high magnesium concentrations for their calcium require-

ments. The virulent strains, on the other hand, showed evidence of magnesium antagonism of their calcium effects. These characteristic differences between virulent and avirulent strains may be useful in the development of a selective plating medium for the differentiation of virulent and avirulent cells. The application of these findings to the formulation of such a differential plating medium will be reported later.

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SUMMARY

A factor present in milk which promoted the growth of virulent strains of *Pasteurella pestis* at 37 C was identified as the calcium ion.

Virulent strains of *P. pestis* required 0.002 to 0.004 M calcium ions for growth at 37 C in the basal synthetic medium containing 0.02 M magnesium salt. Avirulent strains grew without added calcium. The loss in virulence of *P. pestis* upon serial transfer in aerated liquid medium at 37 C was prevented by the presence of calcium salts.

Strontium and zinc salts were able to replace calcium in the growth medium. In contrast to the apparent lack of a requirement for calcium by avirulent strains in the basal medium, a reduction in magnesium content to 0.0025 M resulted in a requirement for calcium by these strains. An antagonism by magnesium for calcium was indicated in the growth of virulent strains at 37 C.

REFERENCES

DEVIGNAT, R. AND SCHOETTER, M. 1942 Le bacille de yersin en milieu aere. Rec. trav. Sci. Med. Congo Belge, **1**, 161–181.

FUKUI, G. M., OGG, J. E., WESSMAN, G. E., AND SURGALLA, M. J. 1957 Studies on the relation of cultural conditions and virulence of *Pasteurella pestis*. J. Bacteriol., **74**, 714–717.

HIGUCHI, K. AND CARLIN, C. E. 1957 Studies on the nutrition and physiology of *Pasteurella pestis*. I. A casein hydrolyzate medium for the growth of *Pasteurella pestis*. J. Bacteriol., **73**, 122–129.

HIGUCHI, K. AND CARLIN, C. E. 1958 Studies on the nutrition and physiology of *Pasteurella pestis*. II. A defined medium for the growth of *Pasteurella pestis*. J. Bacteriol., **75**, 409–413.

HILLS, G. M. AND SPURR, E. D. 1952 The effect of temperature on the nutritional requirements of *Pasteurella pestis*. J. Gen. Microbiol., **6**, 64–73.

JACKSON, S. AND BURROWS, T. W. 1956 The virulence-enhancing effect of iron on non-pigmented mutants of virulent strains of *Pasteurella pestis*. Brit. J. Exptl. Pathol., **37**, 577–583.

KUPFERBERG, L. L. AND HIGUCHI, K. 1958 Role of calcium ions in the stimulation of growth of virulent strains of *Pasteurella pestis*. J. Bacteriol., **76**, 120–121.

OGG, J. E., FRIEDMAN, S. B., ANDREWS, A. W., AND SURGALLA, M. J. 1958 Factors influencing the loss of virulence in *Pasteurella pestis*. J. Bacteriol., **76**, 185–191.

STICKLAND, L. H. 1951 The determination of small quantities of bacteria by means of the biuret reaction. J. Gen. Microbiol., **5**, 698–703.

WATS, R. C. AND PUDUVAL, T. K. 1940 A study of some virulent and avirulent strains of *Pasteurella pestis*. Ind. J. Med. Research, **27**, 823–831.

WEST, E. S. AND TODD, W. R. 1951 *Textbook of biochemistry*. The Macmillan Co., New York.