TOXIC EFFECT OF GLUCOSE ON VIRULENT *PASTEURELLA PESTIS* IN CHEMICALLY DEFINED MEDIA

GARNER E. WESSMAN, DONNA J. MILLER, AND MICHAEL J. SURGALLA

Fort Detrick, Frederick, Maryland

Received for publication April 25, 1958

During the course of studies on the relation of nutrition to virulence of *Pasteurella pestis*, at 37 C and above, it was noted that under certain conditions glucose appeared to have a lethal effect on virulent cultures. This report defines the conditions under which this effect has been observed, and describes concomitant population changes affecting virulence.

MATERIALS AND METHODS

The organisms used in the investigation included the following virulent strains: Alexander, Charleston, Kuma, Poona, Saka, Washington and Yokohama. Avirulent strains included A1122, A.16 (obtained from Dr. R. Devignat), AD5, AD5-S, Soemedang, SD5 and Tjiwidej. Three of the avirulent variants, AD5, SD5 and AD5-S, were isolated in our laboratory. Strains AD5 and SD5 were isolated from 37 C cultures of Alexander and Saka, respectively. AD5-S is a streptomycin-resistant variant of strain AD5.

The composition of the chemically defined medium is shown in table 1. The medium is based primarily upon the investigations of Hills and Spurr (1952), Higuchi and Carlin (1957, 1958) and our laboratory. The components of the medium do not represent minimal nutritional requirements. The amino acids and phosphate were dissolved in distilled water, the solutions of salts, vitamins, and hemin added, and the reaction of the solution adjusted to pH 7.4 with N NaOH. Distilled water was added to bring the medium to one half the final volume. This double strength solution was sterilized by passage through an ultrafine sintered glass filter. The hemin solution was made up in 1 per cent ammonia water. Sugars were sterilized separately and added at the time of inoculation at a final concentration of 0.25 per cent.

Inoculum was prepared as follows. The growth from a 24 hr blood agar base (Difco) slant, incubated at 37 C, was suspended in 8 ml of phosphate buffer, 0.066 M, pH 7.4. This suspension was adjusted turbidimetrically so that the desired initial count was obtained upon the addition of 1 ml of the inoculum to the synthetic, liquid medium.

The organisms were grown at 37 C on a shaker operating at approximately 95 strokes per minute. Twenty ml of medium were contained in 250 ml Erlenmeyer flasks. The medium was made up before inoculation by combining solutions of certain components with 10 ml of the basal medium to a total volume of 19 ml. One ml of diluted inoculum brought the medium to full volume. When glucose determinations were conducted, the amount of medium employed was 40 ml per 500 ml flask. An Aminco-Dubnoff shaker incubator was employed in the studies on the effect of temperature. Five ml of medium were contained in a 50 ml flask and sampled by withdrawing 0.1 ml.

Routine sampling for growth response was carried out by determining the viable count by the pour plate method in blood agar base medium containing 0.04 per cent Na_2SO_3 and 0.1 per cent glucose. Plates were incubated at 72 hr at 26 C before counting. The diluting fluid for viability determination was phosphate buffer, pH 7.4 0.066 M.

Virulence titrations were conducted by intraperitoneal injection of 0.2 ml of suspended organisms, appropriately diluted in phosphate buffer, into Swiss-Webster mice (18 to 22 g). The mice were held for 10 days. The LD_{50} calculations were made by the method of Litchfield and Wilcoxon (1949). For the determination of the percentage of virulent cells in a culture, a suspension of the organisms was plated out on blood agar base medium, and after incubation, 20 isolated colonies were streaked onto individual slants. The slants were incubated at 37 C for 24 hr. The cells were suspended in phosphate buffer and diluted appropriately so that an arbitrary dose of 10,000 cells was injected into each of five mice. This dosage normally resulted in 100

TABLE 1

Composition of chemically defined medium (pH 7.4)

Component	Concentration	Component	Concentration
	m moles		*μ moles*
DL-Alanine	1.5	Biotin	0.005
L-Cysteine·HCl	0.5	Ca pantothenate	1.0
DL-Isoleucine	1.0	Thiamin·HCl	1.0
DL-Leucine	1.5		
DL-Methionine	0.5	Hemin	10
DL-Phenylalanine	4.0		
DL-Serine	1.0	$CaCl_2 \cdot 2\ H_2O$	100
DL-Threonine	1.0	$FeSO_4 \cdot 7\ H_2O$	5
DL-Valine	1.0	$MgSO_4 \cdot 7\ H_2O$	2000
L-Arginine·HCl	1.0	$MnSO_4 \cdot 4\ H_2O$	20
L-Histidine·HCl	1.0		*m moles*
L-Proline	1.0	K_2HPO_4	50
L-Glutamic acid	20.0	Na citrate	1

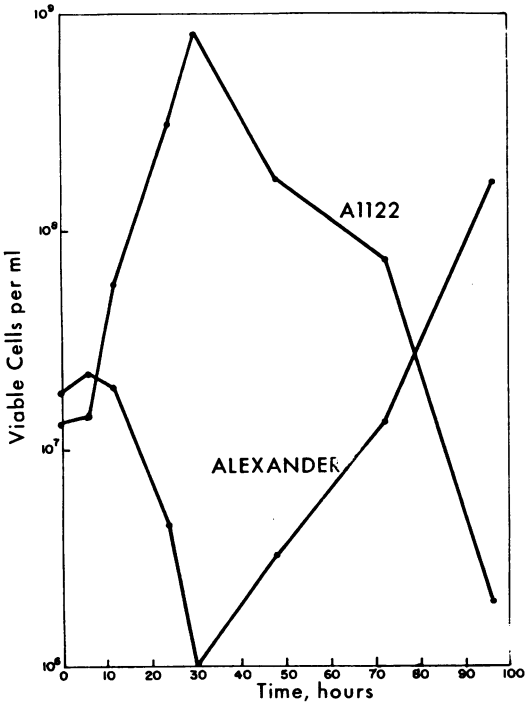

Figure 1. Growth curves for *Pasteurella pestis*, strains Alexander and A1122, with glucose as source of energy.

per cent deaths in 3 or 4 days if the organisms were virulent and no deaths if avirulent.

RESULTS

P. pestis strain Alexander, cultured in the synthetic medium containing 0.25 per cent glucose as the energy source, exhibited a small rise in number during the first few hours of incubation, then underwent a reduction in viable count beginning approximately 5 to 12 hr after inoculation. The magnitude of this decrease was usually about one log although in some cases a two log loss of viability was noted. The organism then recovered, and logarithmic growth followed (figure 1). The reduction in count did not occur with the avirulent strain A1122. Furthermore, a normal growth curve was obtained for both strains in the presence of xylose. The virulent strain grew more slowly with xylose than strain A1122, but no drop in count was detectable. All avirulent strains exhibited the same type of curve as strain A1122 when seven avirulent strains were compared (table 2). With the exception of two glycerol fermenting strains, Kuma and Yokohama, the virulent strains responded in the same manner as strain Alexander.

Direct microscopic examination of the cell suspension (Petroff-Hauser counter) proved that the rise in count following inoculation was due to multiplication of the organism during this period, rather than to an apparent increase resulting from the disintegration of cellular clumps present in the inoculum. During the period when the loss in viability was taking place, considerable lysis of cells apparently occurred because microscopic examination revealed a definite reduction in total count correlating with the decline in the number of viable cells (figure 2).

The range in initial count within which the reduction in number could be observed most

TABLE 2

Growth response of virulent and avirulent Pasteurella pestis with glucose as the energy source

Strain	Viable cells per ml × 10^6						
	0 hr	6 hr	12 hr	24 hr	36 hr	48 hr	72 hr
Virulent:							
Alexander	4.3	3.2	0.12	0.10	0.40	8.0	560
Charleston	3.6	14	3.7	3.7	13	120	720
Kuma	1.3	7.9	13	26	200	1100	2600
Poona	4.0	6.4	3.3	10	93	770	200
Saka	1.9	4.7	0.23	0.10	0.86	16	660
Washington	2.1	3.0	0.55	0.45	9.9	200	81
Yokohama	2.4	4.4	9.0	16	27	44	1600
Avirulent:							
A1122	1.9	2.1	5.0	9.0	72	290	
A.16	4.8	4.5	6.2	12	96	190	
AD5	3.2	3.3	4.4	61	170	2.9	
AD5-S	3.1	8.0	15	180	130	210	
Soemedang	2.9	6.0	8.2	53	430	820	
SD5	2.5	13	68	230	70	42	
Tjiwidej	1.6	12	26	86	160	12	

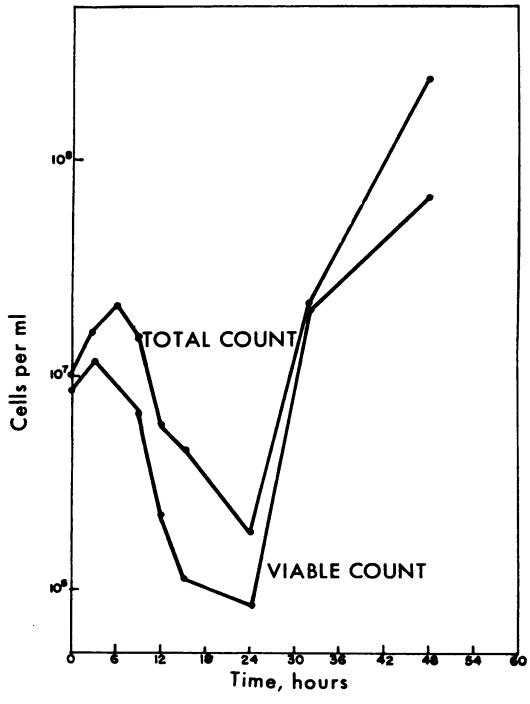

Figure 2. Comparison of total and viable counts during growth of *Pasteurella pestis* strain Alexander with glucose as source of energy.

clearly was found to be between 10^6 and 10^7 cells per ml. Recovery following the early decline in count normally did not occur when the inoculum was 10^8 cells per ml or higher. The early development of an acid reaction interfered if 10^9 cells per ml were present initially. Growth curve experiments with strains Alexander, Saka, and Washington showed that virulent organisms normally did not grow if the initial count was much less than 10^6 cells per ml. Avirulent cultures, as shown with strains A1122 and Tjiwidej, generally grew from an inoculum approximately one tenth that required for virulent cells.

It was necessary to include glucose in the medium if the typical decline in viability were to occur. The result of incubation of the organism in the synthetic medium with no added energy source, compared with cultures to which varying amounts of glucose were added, is shown in figure 3. The curve representing the culture containing no glucose did not fall abruptly, but dropped at a much slower rate within the first 30 hr. No correlation between the amount of glucose added and the degree of reduction in viable count was noted. Other experiments showed that when glucose was added with an equal amount of xylose, the number of viable cells decreased to an

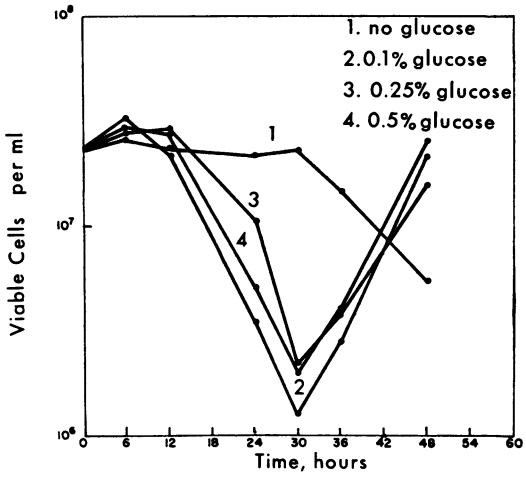

Figure 3. Effect of added glucose on the growth response of *Pasteurella pestis* strain Alexander.

extent equal to that in cultures with glucose alone. As pointed out above, xylose alone supported growth with no reduction. Addition of glucose produced a lethal effect even when introduced during the logarithmic growth phase of a culture growing in the presence of xylose.

The reduction in viable count occurring with most virulent strains when they were incubated with glucose was dependent upon the temperature of incubation. A series of experiments was conducted under carefully controlled temperature conditions by incubating the cultures in an Aminco-Dubnoff shaker-incubator apparatus, using virulent strains Alexander and Saka, and avirulent strains A1122 and AD5. All cultures exhibited normal curves at 36 C, but at 37 C and 38 C the virulent organisms underwent the usual reduction in viable count.

It could be expected that a rapid metabolism of glucose occurred, resulting in a rapid shift in pH, which might account for the drop in count. However, the shift in pH was no greater than that with xylose. During the period of incubation in which the count was declining, the pH changed only 0.1 to 0.2 units. The amount of glucose metabolized during this period was very small. Samples were taken and analyzed for glucose at regular intervals during growth, using the method of Folin and Malmros (1929). Utilization of glucose was detected irregularly until the organism had begun its phase of rapid growth, following the lowest point in the growth curve.

The reduction in count could also be demonstrated in static cultures, governed by the same temperature relationships. The decline in cell number was usually smaller, and the cultures exhibited greater shifts in pH.

Preliminary attemps to prevent the reduction in count under the described conditions were made. There were indications that a number of inorganic cations could prevent this reduction or limit its extent (table 3). Mn^{++}, Mg^{++}, and Fe^{++} were added at concentrations 10 times the normal amounts present in the medium. Cu^{++} and Co^{++} were added at similarly high concentrations. In the one experiment illustrated, Mn^{++} and Mg^{++} were effective in preventing the reduction in count. Fe^{++} was inhibitory at the concentration used, while Cu^{++} and Co^{++} had no effect. No attempt was made to determine what effect other concentrations would have, except Mn^{++}, in which case lower amounts were not as effective as the 0.2 mM. In other experiments, Mn^{++} did not completely eliminate the reduction in viable count, but reduced the magnitude of the drop to one-half or less. This protective effect of Mn^{++} on viability also benefited retention of virulence.

A comparison of growth curves of virulent cultures with those of avirulent strains showed that the latter were able to grow immediately and more rapidly under the defined conditions while the virulent organisms, with two exceptions (Kuma and Yokohama strains), were not. This implied that the organisms which grew following the reduction in count were mainly avirulent cells. This was in consonance with the observa-

TABLE 3

Effect of inorganic ions upon the reduction in viable count of Pasteurella pestis strain Alexander in the presence of glucose

Compound Added	Concentration	Viable Cells per ml × 10^6			
		0 hr	16 hr	24 hr	40 hr
	m moles				
None		1.7	0.38	1.8	5.3
$MnSO_4$	0.2*	1.7	14	43	50
$MgSO_4$	20*	1.7	12	36	58
$FeSO_4$	0.05*	1.7	0.54	0.43	
$CuSO_4$	0.02	1.7	1.0	2.0	
$CoCl_2$	0.025	1.7	0.65	0.71	2.8

* Concentrations given are in addition to those already present in the medium.

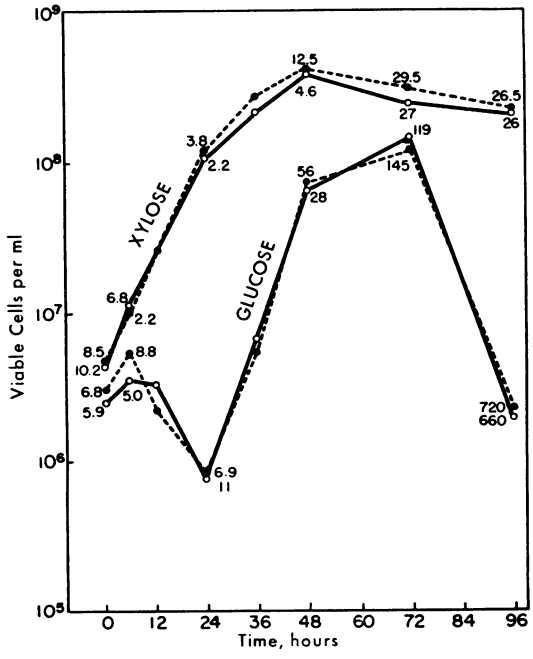

Figure 4. Effect of xylose and glucose on growth and virulence of *Pasteurella pestis* strain Alexander. Figures on curve represent LD_{50} values (mouse, ip).

tions of Fukui *et al.* (1957) which indicated that cultures of *P. pestis* in complex media lose virulence when grown with aeration at 37 C because avirulent cells become the predominant type in time. To confirm this, virulence titrations were conducted at various intervals during the growth of virulent cultures to determine whether they did become increasingly avirulent. Figure 4 shows the results of duplicate experiments of this type. With glucose as the energy source, no loss in virulence was detected until the curve had reached its lowest level and then started to rise again. Then a rather rapid loss occurred as apparently less virulent organisms predominated. The further loss occurring during the death phase of the organisms was probably influenced by factors other than the glucose effect. The loss in virulence in the presence of xylose in a single transfer under these conditions was too small to show a significant shift in the LD_{50} value, although it undoubtedly caused some change in the avirulent:virulent cell ratio.

A number of energy sources were tested to determine whether the resulting growth curves corresponded to that obtained with glucose or with xylose. The following compounds supported

TABLE 4

Effect of serial transfer in the presence of various energy sources upon the virulence of Pasteurella pestis strain Alexander

Energy Source	Transfer Number	Minimum Viable Count × 10^6	Maximum Viable Count × 10^6	Approximate Number of Generations	Age of Culture	Mouse LD_{50} (95% Confidence Limits)
					hrs	
Glucose	1	3.7	110	4.9	72	234 (90–608)
	2	0.77	240	8.2	48	4300 (1410–13,100)
	3	9.3	480	5.7	32	4.8×10^5 (N.C.*)
	4	11.4	1500	7.1	32	4×10^5 (N.C.*)
Xylose	1	6.5	580	6.5	48	10.8 (4.6–25)
	2	4.7	340	6.2	48	12 (5–29)
	3	8.4	330	5.3	48	46 (17.7–120)
	4	3.5	220	6.0	48	62 (24–161)
Galactose	1	6.5	380	5.9	48	10 (4.4–23)
	2	3.7	150	5.3	48	5 (2–12.5)
	3	3.7	260	6.1	48	18 (6–54)
	4	3.3	170	5.7	48	35 (14.4–138)
Mannose	1	6.5	110	4.1	48	9.2 (3.7–23)
	2	0.26	190	9.3	48	45 (17.6–115)
	3	48.5	350	6.2	32	78 (29–211)
	4	11.1	1200	6.8	32	485 (187–1260)

* Non-calculable.

TABLE 5

Effect of pH upon growth and virulence of Pasteurella pestis strain Alexander

Energy Source (Concentration 0.25%)	Viable Cells per ml (ct × 10^6)/pH					Percentage of Virulent Colonies (48 hr Culture)
	0 hr	16 hr	24 hr	48 hr	72 hr	
						%
Glucose	7.59	1.65	4.18	143	2.24	0
	6.6	6.9	6.8	6.2	6.0	
	7.59	1.09	1.29	337	513	0
	7.0	6.9	6.9	6.7	6.5	
	7.59	4.63	11.2	330	387	10
	7.4	7.2	7.1	6.9	6.8	
	7.59	3.53	9.06	57	110	100
	7.8	7.4	7.6	7.0	6.8	
	7.59	1.08	5.6	210	73	100
	8.0	8.0	7.6	7.0	6.8	
Xylose	7.59	40.6	77	106	341	100
	6.6	7.0	6.9	7.0	7.0	
	7.59	30	36.5	52	71.3	90
	7.0	7.0	6.9	7.0	7.0	
	7.59	38.1	57	147	124	100
	7.4	7.2	7.1	7.2	7.2	
	7.59	50.7	71.3	39.5	7	100
	7.8	7.6	7.6	7.6	7.6	
	7.59	10.1	19.8	174	4.05	100
	8.0	8.0	8.0	8.0	7.6	

good growth of strain Alexander: galactose, ribose, maltose, mannose, L-arabinose, and ammonium gluconate. No drop in viable count was observed. The organism did not grow in the presence of ammonium lactate, and grew poorly with D-arabinose. Growth with mannitol was erratic in four trials. Since mannitol is normally considered a good energy source (Higuchi and Carlin, 1957) it is possible that a response was obtained similar to that with glucose, but of a lesser degree.

Additional experiments were carried out to magnify the loss of virulence with glucose by carrying the organism through several serial transfers. A comparison of the effect of cultivation in the presence of glucose, galactose, xylose or mannose was made with strain Alexander as the test organism. The organism was grown, with shaking, at 37 C, in the synthetic medium to which was added 0.25 per cent of the appropriate sugar. Virulence titrations were carried out when the cells were approaching the end of the logarithmic phase of growth, and a transfer of the culture to fresh media was made at this time. Since loss of virulence in strains of *P. pestis* grown in shaken cultures at 37 C in deacidified casein partial hydrolyzate or heart infusion broth (Difco) had been shown to be dependent upon the number of generations produced (Fukui *et al.*, 1957), it was important to compare cultures of essentially the same physiological age, even though the actual ages of the cultures differed.

Table 4 summarizes these experiments. The total number of generations produced in four serial transfers was approximately the same for each carbohydrate. After a single transfer, loss of virulence in the culture containing glucose was noted, and after three transfers the culture was avirulent. The culture retained virulence best

in the presence of xylose or galactose; the effect of mannose was intermediate between that of glucose and galactose or xylose.

Recent experiments have shown that the effect produced by glucose cannot be considered the primary cause of attenuation of the culture. The effect, however, accelerates the rate of loss of virulence by decreasing the number of virulent cells under conditions which favor the growth of avirulent organisms. Ogg *et al.* (*unpublished data*) have shown that virulence can be maintained by manipulation of the initial pH. This effect was confirmed in our system (table 5). The typical reduction in count was observed with glucose at the pH values tested; however, there was no loss of virulence at pH 7.8 or 8.0. Xylose produced no loss in virulence under any of the above conditions.

Under the conditions in which rapid loss of virulence did occur, the avirulent cells which appeared were believed to be the result of mutation and selection during growth, or possibly of the selective growth of avirulent organisms present initially in the inoculum. An attempt was made to determine the ratio of avirulent to virulent cells by plating an aliquot of the inoculum on agar, and titrating the progeny of 20 colonies chosen at random as described in the section on methods. All 20 colonies generally proved to be virulent for mice when strains Alexander and Saka were tested in this manner. The fact that this method disclosed few or no avirulent cells in the original culture does not eliminate the possibility of the presence of a few avirulent organisms in the inoculum. The difference in behavior of strains Kuma and Yokohama might be ascribed to their genetic instability and a possibly greater percentage of avirulent mutants in the inoculum.

DISCUSSION

The reduction in viable count upon culturing *P. pestis* at 37 C with glucose in the medium did not appear to be the result of the inability of the organism to attack glucose. If the failure of virulent strains to utilize glucose under these conditions were the explanation, it would be expected that the reduction in viable count in the absence of any added energy source would parallel that occurring with glucose. Since the decrease in viability was considerably less rapid when no energy source was added, the evidence favors the hypothesis that a toxic condition existed which caused the cells to die and lyse. Similar evidence and interpretation were afforded by the fact that incubation in the presence of both glucose and xylose produced a growth curve similar to that obtained with glucose alone.

The literature records instances of the depression of bacterial growth by the inclusion of glucose in the medium. Glucose has usually been shown to exert its effect by interfering with amino acid metabolism (Kligler and Grossowicz, 1941; Kligler *et al.*, 1943; Shaposhnikov and Tauson, 1948; Forbes and Sevag, 1951). Jordan (1952) believed the inhibition of growth of *Pasteurella septica*, when cultured with glucose in the medium, was due to the accumulation of inhibitory amounts of H_2O_2. The possibility of such a mechanism involving *P. pestis* in the present case should be considered since Herbert (1949) suggested that H_2O_2 produced by this organism might accumulate under certain aerobic conditions, thus inhibiting growth. The case discussed in the present paper would appear to differ from others reported in that it is closely controlled by temperature and that the effect is selective for most virulent strains of *P. pestis*.

SUMMARY

Five virulent strains of *Pasteurella pestis* cultivated at 37 C in a chemically defined medium containing glucose as the energy source, underwent a reduction in viable count, then recovered and grew in a logarithmic manner. Two glycerol-fermenting virulent strains and seven avirulent strains produced normal growth curves. The reduction in count appeared to be caused by death and lysis of the cells. It was not apparent when other energy sources were used, and did not occur at temperatures below 37 C. The addition of relatively large amounts of Mn^{++} or Mg^{++} to the medium prevented or reduced the fall in viable count.

When the organisms which grew after the culture had undergone a reduction in count were titrated for virulence at various stages of growth, they were found to become progressively less virulent, except when the initial pH was above 7.8. Cultures of the Alexander strain lost virulence for mice rapidly upon serial transfer in a synthetic medium containing glucose, but showed a markedly smaller shift in virulence if the energy source was xylose, galactose, or mannose.

REFERENCES

FOLIN, O. AND MALMROS, H. 1929 An improved form of Folin's micromethod for blood sugar determinations. J. Biol. Chem., **83,** 115–120.

FORBES, M. AND SEVAG, M. G. 1951 Inhibition by glucose of the synthesis of proline from glutamic acid. Arch. Biochem. Biophys., **31,** 406–415.

FUKUI, G. M., OGG, J. E., WESSMAN, G. E., AND SURGALLA, M. J. 1957 Studies on the relation of cultural conditions and the virulence of *Pasteurella pestis*. J. Bacteriol., **72,** 714–717.

HERBERT, D. 1949 Studies on the nutrition of *Pasteurella pestis*, and factors affecting the growth of isolated cells on an agar surface. Brit. J. Exptl. Pathol., **30,** 509–519.

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.

JORDAN, R. M. M. 1952 The nutrition of *Pasteurella septica*. II. The formation of hydrogen peroxide in a chemically-defined medium. Brit. J. Exptl. Pathol., **33,** 36–45.

KLIGLER, I. J. AND GROSSOWICZ, N. 1941 The function of nicotinic acid in bacterial metabolism. J. Bacteriol., **42,** 173–192.

KLIGLER, I. J., GROSSOWICZ, N., AND BERGNER, S. 1943 Inhibition of bacterial growth by glucose in media devoid of nicotinic acid. Proc. Soc. Exptl. Biol. Med., **52,** 332–334.

LITCHFIELD, J. T., JR. AND WILCOXON, F. W. 1949 A simplified method of evaluating dose-effect experiments. J. Pharm. Exptl. Therap., **96,** 99–113.

SHAPOSHNIKOV, V. N. AND TAUSON, T. A. 1948 Observations on the biochemical properties of proteolytic bacteria. Mikrobiologiya, **17,** 419–424.