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A Bacteriotoxic Substance in Autoclaved Culture Media Containing Glucose and Phosphate[1]

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Received for publication September 24, 1956

Since the early days of bacteriology it has been common knowledge that partial or complete inhibition of growth of some microorganisms may occur when a reducing sugar is autoclaved in certain culture media, particularly those of simple composition. Such inhibition generally is attributed to the production of "toxic caramelization products of glucose" (Doudoroff, 1942; Stanier, 1942; Gould *et al.*, 1944; Corper and Clark, 1946), although Lewis (1930) suggested that products of thermal degradation of reducing sugars may not be toxic *per se*. According to Lewis, growth may be inhibited indirectly due to binding of essential nitrogenous components of the medium by certain products of phosphate-mediated degradation of the sugar, probably carbonyl compounds. Lankford *et al.* (1947), Hill and Patton (1947) and others (Patton and Hill, 1948; Lankford and Lacy, 1949; Lankford, 1950) provided additional evidence for the operation of this mechanism of growth inhibition. They found the total final growth of lactic acid bacteria to be suppressed to a degree related directly to the extent of glucose degradation and inversely to the concentration of the limiting nutrient. No adverse effect upon growth initiation or upon growth rate was observed under the test conditions (Lankford *et al.*, 1947; Lankford and Lacy, 1949). Some recent observations, however, indicate that a substance or substances with primary, intrinsic bacteriotoxicity may be formed when media containing glucose and phosphate are autoclaved.

MATERIALS AND METHODS

Vibrio cholerae (*Vibrio comma*) cultures from several sources were used in certain phases of the investigation, although strains isolated in Calcutta in 1953 by W. Burrows and C. E. Lankford (unpublished) were most intensively studied. Strain CA-381 (Ogawa serotype) was used in most of the tests, but the response of other strains was qualitatively similar. Cultures of other species were selected from the departmental stock culture collection.

The seed cultures usually were grown on peptone-horse meat infusion agar slants, or in a fluid sucrose-glutamate-NH_4^+-salts base (Finkelstein and Lankford, 1955) (table 1), for 15 to 19 hr, then diluted in 0.5 per cent saline for inoculation to the test media. Generally, 10 ml of the defined medium were placed in 7-in tubes and autoclaved for 10 min at 121 C. This defined medium, which with minor modifications was used throughout the study, supports moderately good growth of stationary cultures of *V. cholerae*, even from small inocula, after an initial lag of about 4 hr. Growth response was recorded in terms of periodic visual estimates of turbidity, of Klett-Summerson photoelectrometer scale values and, in certain instances, of viable cell colony counts in agar containing Bacto proteose

TABLE 1. *Composition of chemically defined medium for Vibrio cholerae*

	μg Per ml
NH_4^+ (as NH_4Cl)	200
DL-Glutamic acid	500
Na_2HPO_4*	5000
K_2HPO_4*	5000
S (as Na_2SO_4)	20
Mg^{++} (as $MgCl_2$)	5
Mn^{++} (as $MnCl_2$)	1
Fe^{+++} (as $FeCl_3$)	1
Carbon source† (glucose or sucrose) in "de-ionized" water; final pH 8.0	5000

*Phosphates each 1000 μg in early studies (table 2).

† Sucrose was sterilized in the basal medium; glucose, when used, was sterilized in the medium, or autoclaved separately and added aseptically.

[1] This investigation was supported in part by a contract (AF-18(600)-933) with the USAF School of Aviation Medicine, Randolph Field, Texas, and in part by a grant (E-285-C2) from the U. S. Public Health Service.

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peptone no. 3 (1 per cent), Bacto yeast extract (0.5 per cent) and Bacto agar (1.5 per cent) at pH 7.8.

Results

The pattern of growth inhibition. When glucose was autoclaved in the defined medium containing 0.2 per cent phosphates (table 1) there was a delay in the appearance of visible growth of *V. cholerae*, the duration of which was inversely related to the size of the inoculum (table 2). Relatively large inocula (10^7 to 10^8 cells per ml) produced rather prompt growth whether glucose was autoclaved in the medium, or sterilized separately and added aseptically. As the inoculum was decreased, the "relative lag" differential between the test and the control cultures increased correspondingly. If the test medium contained 0.5 to 1.0 per cent phosphates, growth from smaller inocula usually failed to appear within the time limits of observation. When delayed growth did appear, the subsequent rate of growth and the terminal cell density, as determined by serial Klett readings, were essentially the same as those of the control cultures with glucose sterilized separately (figure 2). Although these results suggest that delayed growth in media autoclaved with glucose may result from a specific inhibitory effect during the lag phase, other possible causes might be incomplete killing of the inoculum with subsequent delayed outgrowth, retarded growth rate, or a combination of these effects. It is possible also that binding of essential nitrogenous components of the medium by glucose degradation products might render them unassimilable, and thus prevent growth, until adaptive enzymes capable of attacking these compounds could be synthesized by the inoculum cells. The possibility of selecting rapidly growing mutant cells out of a slowly growing population of the parent inoculum cells also must be considered.

Table 2. *Inoculum size in relation to lag in medium autoclaved with glucose**

(*Vibrio cholerae* strain C-402)

Inoculum: Cells per ml (approx)	Time in hr for Culture to Reach Turbidity Equivalent to Klett Scale Value of 50†		
	Glucose separate	Glucose in medium	Increase in "relative lag"‡
10^6	7	10.5	3.5
10^5	10.5	19.5	9.0
10^4	12.5	27.5	15.0
10^2	17	37	20.0

* Medium containing 0.2 per cent Na_2HPO_4 + K_2HPO_4 autoclaved 10 min at 121 C.

† Final turbidity in all cases was about 140 Klett units.

‡ These values represent the resultant of both increase in lag and any reduction in growth rate which may occur, as compared with the medium containing separately sterilized glucose.

Binding of essential nutrients as the mechanism of inhibition was discredited by an experiment in which the inhibitory complete medium was mixed in descending concentrations with the noninhibitory medium (that is, one prepared with glucose sterilized separately). The "toxic" supplement completely inhibited a small inoculum at a concentration of 0.5, and partially inhibited at concentrations as low as 0.01. The apparent lack of stoichiometry weakens any case for binding of nitrogenous components of the medium by a fraction of the partially degraded glucose. However, it has been observed that within a very narrow range of inhibitory effect an increase in the concentration of NH_4^+ and/or glutamate may produce partial reversal of inhibition.

The greatest inhibitory activity was found to be generated only when glucose was autoclaved in the presence of phosphate, and was weak or lacking when glucose was sterilized with other components of the medium, autoclaved separately or when replaced in the medium by sucrose. Thus, phosphate appears to play a rather specific role in the generation of heat-induced glucose toxicity. These observations led to the adoption for most of the succeeding experiments of a toxic solution prepared by autoclaving 10 g each of glucose, Na_2HPO_4 and K_2HPO_4 in 100 ml of water (pH 8) for 15 min at 121 C. This solution was added to a basal medium prepared with sucrose sterilized in the medium as the primary carbon-energy source. Falling concentrations of the autoclaved glucose-phosphate solution (designated hereafter as "GP solution") then were added to the nontoxic sucrose base in order to provide estimates of its inhibitory potency for small inocula. Its concentration is expressed in terms of its original glucose content, as "μg per ml." Under these test conditions 300 μg glucose usually prevented growth during the period of observation. From 10 to 300 μg glucose produced a delay in appearance of visible growth proportional to its concentration within this range.

The initial pH of the GP solution before sterilization, and the time and temperature it is autoclaved also are determining factors in the formation of the inhibitor. Solutions containing equivalent concentrations of glucose and phosphates were adjusted to pH values over the range from 5 to 10, then autoclaved 10 min at 121 C; other solutions at pH 8 were autoclaved at 121 C for periods from 5 to 20 min. The relative inhibitory potency of these solutions was compared in terms of the lowest concentrations which suppressed visible growth of *V. cholerae* in the sucrose base during 20-hr incubation. Very little inhibitor was generated at pH 5 and 6, but as the initial pH of the GP solution was increased from 7 to 10, the inhibitory activity was increased, as might be anticipated from the well known acceleration of glucose degradation by alkali. The inhibitory activity

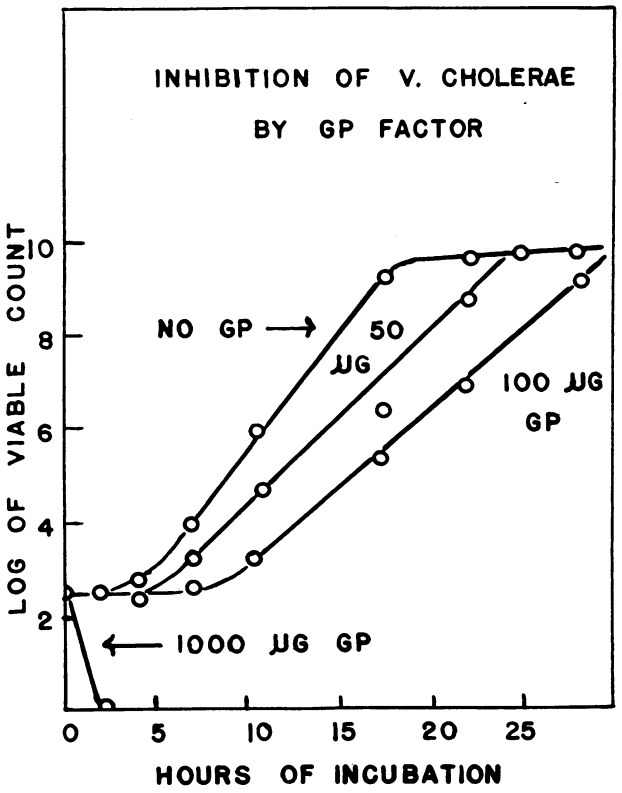

FIG. 1. Growth curves of *Vibrio cholerae*, strain CA-381, in NH_4^+-glutamate-sucrose medium in presence of supplements of glucose-phosphate solution (GP). Stationary cultures.

also was found to increase to a maximum as the time of autoclaving was increased from 5 to 20 min.

The nature of the inhibition produced by autoclaving GP solution was investigated by making periodic viable cell colony counts of cultures exposed to several concentrations of the mixture (figure 1). The cultures were sampled at regular intervals and appropriate dilutions, as well as the undiluted cultures, were plated in proteose no. 3-yeast extract agar. High concentrations of GP solution (1000 µg per ml) rapidly killed an inoculum of about 200 cells; lower concentrations only prolonged lag, without killing, to a degree related to the concentration. The viable cell counts also suggested that a slight prolongation of the division time in the exponential phase may account in part for the delay in appearance of visible growth. Such an effect usually was not detected in the small segment of the growth curve which can be examined turbidimetrically (figure 2).

The possibility of selection of rapidly growing mutant cells, or of specific enzyme adaptation, as an explanation for delayed growth has been investigated by experiments involving transfer of inocula from tubes in which growth appeared after prolonged delay caused by the GP inhibitor. If delayed growth should occur as a consequence of synthesis of adaptive enzymes for utilization of "bound" nutrient unassimilable by the unadapted inoculum cells, it might be expected that such adapted cells would produce relatively prompt growth when transferred to fresh inhibitory medium. Similarly, if delayed growth in an inhibitory medium were the result of selection of rapidly growing mutants, such mutants might be expected to grow promptly when transferred to inhibitory media. However, repeated transfers of dilutions from partially inhibited cultures to a new series of GP concentrations has on each occasion very nearly reproduced the qualitative and the quantitative pattern of growth inhibition of the primary culture. Thus, there seems little likelihood that mutation and selection, or enzyme adaptation, can be the determining factor in delayed growth. However, some degree of physiologic adjustment or adaptation to the toxic environment probably accounts for late initiation of growth.

Characteristics of the GP inhibitor. A series of tests was made to determine what classes of compounds might reverse the toxicity of autoclaved GP solution, with the expectation that the results might suggest something of the nature of the inhibitor. Certain reducing agents first were tested for reversing effect when added to the sucrose base which had been toxified by the addition of 500 µg of autoclaved GP solution (table 3). Both cysteine and glutathione were effective in counteracting this GP toxicity within the range from 10 to 100 µg per ml. $Na_2S_2O_5$ was about 3 to 10 times as effective but produced a zonal pattern of response, possibly as a result of its own toxicity at 1000 µg per ml. Sodium thioglycolate, at 3 to 30 µg, eliminated the toxicity, although its effect was apparent only after prolonged incubation. $NaHSO_3$ also reversed the GP toxicity, even at bactericidal concentrations of the latter (figure 2).

It was considered of importance to determine whether substances in complex culture media might counteract any GP inhibitor which might be formed if such a me-

TABLE 3. *Reversal of GP inhibition of growth by reducing reagents*

Concentration of Supplement	Growth after Addition of Supplement to Inhibitory Medium*							
	$Na_2S_2O_5$†		Cysteine		Glutathione		Thioglycolate	
	18	33	18	33	18	33	18	33
µg/ml								
0	−	−	−	−	−	−	−	−
1	−	−	−	−	−	−	−	−
3	−	+	−	−	−	−	−	+
10	+	+	−	+	−	+	−	−
30	+	+	−	+	−	+	−	+
100	+	+	+	+	+	+	−	+
300	+	+	+	+	+	+	−	+
1000	−	−	+	+	+	+	+	+

* Sucrose base toxified by addition of 500 µg GP solution; reversing agents added prior to about 10^4 cells of *Vibrio cholerae* CA-381. Growth recorded after 18- and 33-hr incubation.

† Toxic at 1000 µg per ml in inhibitor-free control.

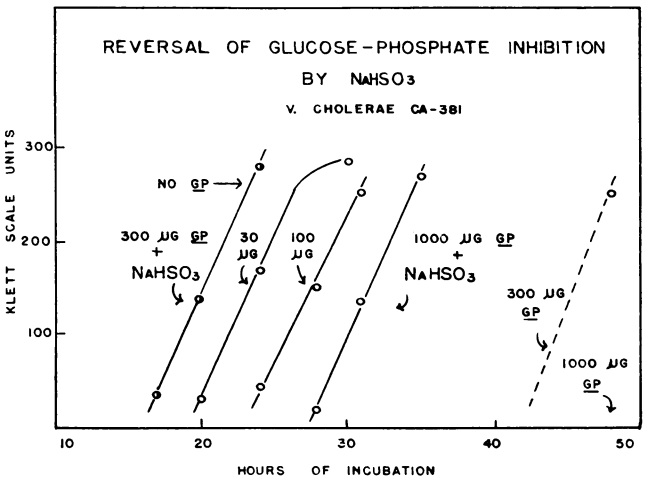

FIG. 2. Reversal of GP inhibition of *Vibria cholerae* strain CA-381 by $NaHSO_3$. The basal medium, NH_4^+-glutamate-sucrose base was toxified by the addition of supplements of GP solution. $NaHSO_3$ (100 µg per ml) was added at the time of inoculation with about 5000 cells. Stationary cultures.

dium contained glucose. It was determined that GP solution, added to nutrient agar in concentrations as high as 2560 µg per ml, did not reduce the apparent time of growth initiation nor the number of colonies arising from a small surface inoculum of *V. cholerae*. Moreover, Bacto peptone and yeast extract were highly effective in counteracting the inhibitory activity of GP when they were added as supplements to the liquid defined medium. As little as 0.1 to 1 µg of yeast extract or peptone produced definite reversal of 500 µg of GP at 16 and 13 hr, respectively; a "vitamin free" casein hydrolyzate was somewhat less effective, and a mixture of vitamins (biotin, B_{12}, folic acid, nicotinic acid, pyridoxal, pantothenic acid, pantethine, *p*-aminobenzoic acid and thiamin) was inactive. The soluble ash from peptone and yeast extract also was inactive. It would appear, therefore, that some specific organic nutrilite in peptone and yeast extract is antagonistic to the inhibitor. It cannot be cysteine or glutathione, since the crude peptone and yeast extract are more effective than these compounds.

The effectiveness of reducing agents in reversing GP inhibition suggested that its activity may be associated with carbonyl compounds. This possibility was tested by treating 15 ml of GP solution with 5 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 50 per cent H_2SO_4. After allowing sufficient time for reaction the mixture was extracted repeatedly with ethyl acetate to remove excess reagent and any hydrazones formed. The controls were aliquots of GP solution, one untreated and one treated with H_2SO_4 and extracted with ethyl acetate alone. The ethyl acetate was removed from both solutions, and each was adjusted to pH 8, filter sterilized and assayed for inhibitory activity. Only about 10 per cent of its original activity remained in the hydrazine-treated sample, as compared with the controls. Therefore it is apparent that at least the major portion of the inhibitory activity is associated with one or more carbonyl compounds.

The active factor was found to be nonvolatile, since vacuum distillation of GP solutions to one-half the original volume at pH 7 and pH 3 yielded all of their original activity in the residues and none in the distillates. Nearly all (95 to 99 per cent) of the inhibitory activity was removed from the GP solution by adsorption with acid-washed norite charcoal, from which part of the inhibitor was recovered by elution with methanol, ethanol, *n*-butanol, acetone or pyridine, but none with diethyl ether, chloroform, HCl (pH 2), or weak NaOH (pH 10.5). However, direct extraction of the GP solution with diethyl ether appears to offer a more promising method for concentration of the inhibitor. Solutions of autoclaved GP were adjusted to pH 7 and aliquots were extracted by shaking with 3 portions of ether, and by continuous ether extraction for 24 hr. Continuous extraction yielded a dark brown, water-soluble, syrupy residue containing about 50 per cent of the original activity, but the degree of concentration was only about 20- to 50-fold. Fractional extraction produced a similar ether residue having 400- to 500-fold greater activity than the original solution, although the yield was poor (20 to 25 per cent). Only 0.12 and 0.72 µg of this fraction were required to suppress visible growth of about 1000 cells of *V. cholerae* for 13 and 23 hr, respectively.

Paper chromatograms of the water-soluble residue from the continuous ether extract were prepared with *n*-butanol-acetic acid-water (4:1:1) solvent. These yielded two distinct but overlapping areas of strong reducing activity in the region of Rf 0.8 to 0.9, as determined by tests with ammoniac $AgNO_3$ and triphenyl-tetrazolium chloride. This area also was acid to bromcresol green, and nearly all of the inhibitory activity appeared to be concentrated here, as indicated in tests for activity in 1-in segments cut from parallel strips and added to inoculated tubes containing the sucrose base. Most of the brown color of the extract was streaked over the portion of the strip below the active area. It seems likely, therefore, that the inhibitory activity may be associated with at least two related, acidic, carbonyl compounds, probably aldehydes.

A limited survey has been made to determine whether gram negative bacteria other than *V. cholerae* are sensitive to inhibition by autoclaved GP solution. The tests were performed in the *V. cholerae* basal medium, modified by substitution of glucose, sterilized separately, for sucrose, and the addition of filter-sterilized nicotinamide (1 µg), L-cystine (10 µg) and L-tryptophan (10 µg) (table 4). *Salmonella paratyphi*, *Escherichia coli* and *Serratia marcescens* were inhibited by GP solution or by an ether extract at concentrations somewhat greater than that required to inhibit *V. cholerae* in this medium.

Strains of *Aerobacter aerogenes*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*, on the other hand, eventually grew in the highest test concentration after temporary inhibition. Although general conclusions concerning the relative sensitivity of *V. cholerae* and other species are not warranted from these data, it is apparent that the inhibitory activity of autoclaved GP solutions is not limited to *V. cholerae*.

TABLE 4. *Inhibition of gram negative bacteria by GP factor*

Test Species	Inhibitory Concentration (μg/ml)			
	Ether extract* of autoclaved GP		Autoclaved GP	
	Hr incubation†			
	12	21	12	21
Vibrio cholerae, CA-381	8	16	320	640
Salmonella paratyphi	16	64	320	640
Escherichia coli (Texas)	32	64	640	1280
Salmonella typhimurium	32	>128	1280	>2560
Serratia marcescens	32	128		1280
Aerobacter aerogenes	128	>128	2560	>2560
Pseudomonas aeruginosa	128	>128	640	>2560

* Residue from continuous ether extraction.

† No visible growth at concentrations and times of incubation shown; growth in control at 10 hr.

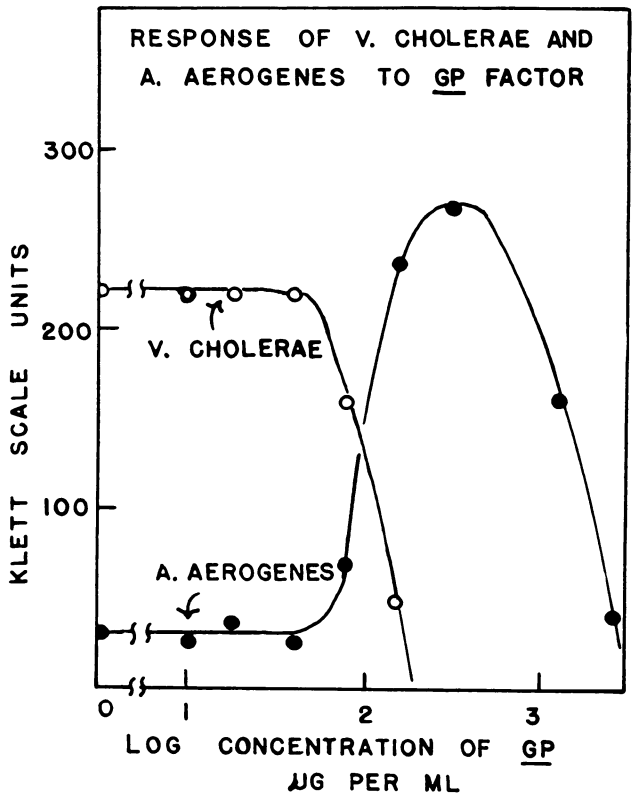

FIG. 3. Zones of stimulation and inhibition of *Aerobacter aerogenes* (UT stock) by GP solution in enriched NH_4^+-glutamate-sucrose base. GP concentration stated in terms of original glucose content. Stationary cultures.

An interesting side observation in the test for inhibition of *E. coli* (Texas) and *A. aerogenes* (U. T. stock) was a zone of growth stimulation produced by subinhibitory concentrations of the GP solution (figure 3). This effect was not obtained with the ether extract and was not observed with certain other strains of the same species. It seems likely that an ether-insoluble growth stimulant was generated simultaneously with the inhibitor. This stimulatory effect probably would be masked by the temporary inhibition produced by autoclaving glucose (0.25 to 0.5 per cent) in the type of chemically defined media ordinarily used for growing these species.

DISCUSSION

Culture media may contain certain components which retard growth initiation without exerting a substantial effect upon subsequent growth rate or final cell density. These effects are likely to be most pronounced with small inocula and are therefore of interest for their potential influence in media designed for detecting or enumerating small numbers of microorganisms. Their possible influence on growth responses and their interpretation in studies of nutritional or metabolic behavior of bacteria also justifies more thorough investigation of these effects.

It now seems established that one mechanism for growth inhibition in autoclaved media containing glucose and phosphate is that caused by glucose degradation products which react with growth-limiting nutrients to form new, unassimilable compounds. As Lewis (1930) points out, however, the more common presumption for the formation of "toxic caramelization products" having direct toxicity for the cell lacks experimental substantiation for previously reported growth inhibitions. The observations of Baumgartner (1938), for example, on delayed growth of *E. coli* in glucose-phosphate broth do not offer sufficient evidence for his presumption of a factor with primary toxicity. Ether extraction of autoclaved GP solution was found by Ramsey (1953) to increase the sensitivity of response of *Lactobacterium fermentum* (*Lactobacillus fermenti*) to its growth stimulatory effect, presumably through elimination of a "toxic" substance, which could not be removed by norite adsorption. However, the nature of this factor remains in doubt, since Ramsey failed to retoxify his medium by adding back the ether extract residue. Recently, McKeen (1956) reported the formation of fungistatic Maillard-type products of heat-induced interaction of glucose and amino acids, particularly glycine, the latter forming a condensation product found by Rogers *et al.* (1953) to stimulate growth of *Lactobacillus gayonii*.

The rapid bactericidal effect of autoclaved GP solution for *V. cholerae* cells is evidence for a direct antibacterial effect. Moreover, its activity in low concentrations

which preclude appreciable binding of the "bulk" nutrients of the medium (that is, NH_4^+ and glutamate) also indicates that its primary action is on the cell, although secondary effects through reactions with medium components are not excluded. No clue as to the specific site of its antibacterial effect can be deduced, since a multitude of cellular constituents react with carbonyl compounds. It is possible that identification of these components which contribute to the highly effective reversing action of yeast extract might provide a lead toward the more sensitive and reactive cellular materials.

Summary

A bacteriotoxic factor (or factors) is formed in a simple, chemically defined culture medium for *Vibrio cholerae* (*Vibrio comma*) when glucose and phosphate are present during heat sterilization. Depending upon its concentration and the size of the inoculum, the factor may be rapidly bactericidal, or merely bacteriostatic; if delayed initiation of growth occurs in its presence, there is only a minor influence upon the growth rate and total cell crop.

The same or a similar factor is present in autoclaved neutral solutions of glucose and phosphate, from which it can be removed by absorption with norite, or partly extracted with ether. A nonvolatile residue of an ether extract, less than 1 μg of which inhibits growth of small inocula of *V. cholerae*, contains carbonyl compounds upon which its inhibitory activity appears to depend. The factor also inhibits certain other gram negative species. The inhibition produced by the factor is counteracted by reducing agents, by certain carbonyl reagents, and by small quantities (0.1 to 10 μg) of peptone and yeast extract.

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