THE INHIBITION OF PHYTOMONAS MALVACEARA IN CULTURE MEDIA CONTAINING SUGARS

I. M. LEWIS

Department of Botany and Bacteriology, The University of Texas

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INTRODUCTION

In some experiments performed recently to determine the various sources of carbon and nitrogen suitable for the growth of *Phytomonas malvaceara*, it was discovered that certain sugars cause complete inhibition of growth under some conditions but form satisfactory sources of carbon under other conditions. Inhibition appears to depend on the composition of the culture medium, the amount of sugar present and the method of sterilization.

The mineral base to which the carbon and nitrogen compounds were added was prepared according to the recent formula of Frazier and Rupp (1928). Two solutions were employed depending on whether ammonia or some other source of nitrogen was to be supplied. The nitrogen free solution contained dibasic potassium phosphate 0.31 per cent, monobasic potassium phosphate 0.08 per cent and 0.02 per cent each of potassium chloride and magnesium sulphate. The salts were dissolved in double distilled water. In the medium used for testing utilization of ammonia, 0.25 per cent of sodium ammonium phosphate was substituted for the potassium phosphates.

Three carbon compounds were used to test for ability to utilize ammonia. These were sucrose, glucose and glycerol. One per cent of each was added separately to the sodium ammonium phosphate solution. The resulting media were then distributed, in about 5 cc. quantities, in pyrex test tubes and sterilized at 122°C. for fifteen minutes.

Fourteen strains of *Phytomonas malvaceara* freshly isolated from infected leaves of cotton were used for inoculation. Each strain was tested for purity, conformation to type description, and pathogenicity before beginning the experiments. All inoculations were made with a single loopful of an aqueous suspension prepared from forty-eight-hour agar slant cultures.

In the medium containing glucose, no growth had occurred at the end of a prolonged incubation period of thirty days while in the media containing sucrose or glycerol growth was prompt and vigorous. It is obvious that these results might be due to a variety of causes. If the organism is not able to use ammonia as a source of nitrogen it might obtain sufficient nitrogenous material from impurities in the sucrose and glycerol but not from the glucose. This appeared to be the most probable explanation but one which was found untenable. Neither sucrose nor glycerol supported growth when added to the nitrogen free mineral solution. Thus, it would appear that the organism is able to use ammonia as a source of nitrogen and either sucrose or glycerol but not glucose as a source of carbon.

It is well known that organisms are variable both as to nitrogen and carbon requirements. Honing (1913) reported growth of a greater number of strains of *B. solanacearum* with either sucrose or glycerol than with glucose. The results of other studies appear to show, however, that glucose is generally more satisfactory as a source of carbon than either sucrose or glycerol and it has been extensively used as the carbon compound for testing utilization of various sources of nitrogen.

That failure of growth in the above experiment was not caused by lack of ability to use glucose was determined by filtering a solution of glucose and then adding it to the sterilized mineral base. In the medium prepared by this method, all of the strains grew promptly and with great vigor. Thus, it is shown that inhibitory substances produced by sterilization at high temperatures rather than inability to assimilate glucose is responsible for the failure of growth.

Since solutions containing sugars undergo changes in reaction when sterilized by heat it seemed possible that the high tem-

perature of sterilization might have raised the hydrogen ion concentration sufficiently to cause inhibition. That other chemical changes also occur was denoted by marked changes in color in the medium containing glucose but not in media containing sucrose or glycerol.

Although many workers have reported on the changes of reaction which occur in various solutions when subjected to heat, it seemed desirable to determine precisely how much change occurs in the media employed here. The two mineral solutions containing 1.0 per cent each of glucose were placed in specially cleaned pyrex flasks and heated at different temperatures for

TABLE 1

Showing hydrogen ion concentration of mineral solutions A and B containing 1.0 per cent glucose

TEMPERATURE		pH OF SOLUTIONS												
		Time in minutes												
		0	5	10	15	20	25	30	40	50	60	70	80	90
°C.														
100	B	7.66	7.56	7.43	7.38	7.31	7.26	7.15	7.08	6.97	6.89	6.72	6.68	6.64
115.5	A	7.15	7.11	7.08	7.03	6.96	6.89	6.73						
	B	7.66	7.42	7.38	7.31	7.28	7.23	7.21						
122	A	7.15	7.06	7.03	6.96	6.86	6.84	6.78						
	B	7.66	7.38	7.35	7.14	6.97	6.94	6.88						

various periods of time. The reactions were determined electrometrically, both before and after heating. The results are shown in table 1. The nitrogen free solution is designated in the table as solution A, the sodium ammonium phosphate solution as solution B. It is seen that the hydrogen ion concentration increases in all cases but that the final reaction even after a period of thirty minutes at 122°C. is only slightly acid, about pH 6.8. *Phytomonas malvaceara* grows very well in peptone beef extract broth adjusted to a reaction of pH 5.4 with hydrochloric acid. There seems then no reason to believe that failure of growth in the synthetic medium containing glucose is due to the

reaction of the sterilized medium. It appears rather that sterilization of glucose at high temperature, in the mineral solution, causes some other chemical change which renders it unsuitable for utilization or detrimental to the growth of the organism.

Before continuing experimental investigation of this problem a search was made to determine whether such a phenomenon had been reported previously. Two instances only which seem to be comparable have come to my attention. Fisher and Bunte (1928) studied the effect of overheated media containing lactose. Milk was heated for a period of forty to sixty minutes at a temperature of 125° to 127°C. and then mixed with an equal volume of sterile 4.0 per cent agar dissolved in water. On this medium, *Salmonella schotmülleri* failed to grow while *Salmonella enteritidis* grew normally. The same result was obtained in a synthetic medium containing lactose 2.0 per cent, sodium citrate 0.2 per cent, ammonium sulphate 0.01 per cent and sodium phosphate 0.05 per cent.

Uyeda (1905) reported that *B. nicotiana* grew in a synthetic medium containing asparagin as the sole source of nitrogen and carbon but failed to grow in the same medium when glucose was added. Erwin F. Smith (1914) comments on this finding of Uyeda as follows: "That the latter which with asparagin made a weak growth, made none whatever when 1.0 per cent of glucose was added, should have indicated to Uyeda that bacteria are variable like other things, because, in the first place 1.0 per cent glucose is not a poison. . . . . " Smith's comment fails to shed any light on the nature of the phenomenon for it is obviously not a case of variability in the utilization of a carbon compound but inhibition caused by it in a culture medium otherwise suitable for growth. In other words 1.0 per cent of glucose, whether it is a poison or not, suppressed growth completely. That *Phytomonas malvaceara* is similarly inhibited by 1.0 per cent of glucose and other sugars with various sources of nitrogen and in culture media which, without the sugars, support abundant growth will be shown in subsequent sections.

EFFECT OF DIFFERENT METHODS OF STERILIZATION

Since glucose sterilized by filtration proved to be suitable for the growth of *Phytomonas malvaceara*, while no growth occurred in the medium sterilized at 122°C., additional experiments were performed to test the effect of other methods of sterilization. The sodium ammonium phosphate mineral solution plus 1.0 per cent of glucose was placed in pyrex tubes and sterilized either by heating in an Arnold sterilizer for thirty-minute periods on three successive days or by heating in an autoclave at 10 pounds pressure for a period of twenty minutes. There were no signs of inhibition in media sterilized by either of these methods. Thus, it appears that the temperature reached is of more importance than the total period of heating. It was also observed that the solutions sterilized at lower temperatures suffered much less marked changes in color. The medium was likewise suitable for growth when glucose was sterilized at 122°C. in distilled water or the mineral solution minus phosphate and then added to the sterilized mineral solution. No growth occurred when glucose and phosphate were sterilized together for fifteen minutes at 122°C. and then added to the solution containing potassium chloride and magnesium phosphate. Thus it appears that glucose and ammonium phosphate react together at high temperatures to form compounds which are inhibitory.

EFFECT OF THE SOURCE OF NITROGEN

In the light of Uyeda's results with *B. nicotiana* it seemed desirable to test other sources of nitrogen to determine whether or not inhibition occurs in media which, without glucose, are suitable for growth. The list of compounds tested included asparagin, glutamic acid, alanine, glycine, beef extract 0.3 per cent each, tyrosine 0.1 per cent, and peptone 0.1 to 1.0 per cent. These were dissolved in the nitrogen free phosphate mineral solution which contained 1.0 per cent glucose and were then sterilized at 122°C. for fifteen minutes. Similar media without additional carbon and media containing sucrose or glycerol were prepared.

Four of the compounds, peptone, beef extract, glutamic acid and alanine supported growth in the absence of additional carbon. All of the sources of nitrogen proved suitable in the presence of either sucrose or glycerol. In the media containing glucose, no growth occurred except with peptone and, here, only when the amount was greater than 0.2 per cent. The time of observation extended over a period of thirty days. In peptone glucose media containing either 0.1 or 0.2 per cent peptone and 1.0 per cent glucose but without phosphate, growth occurred promptly. Faint turbidity was evident within twenty-four hours, the growth becoming abundant within forty-eight to seventy-two hours.

These results show that in the presence of glucose and phosphate sterilized together at 122°C. for fifteen minutes, growth is completely inhibited in culture media which without glucose are suitable for growth. Similar results were obtained by adding 1.0 per cent each of glucose and sucrose to the sodium ammonium phosphate solution. With sucrose alone, growth was abundant but when glucose also was present no growth occurred.

EFFECT OF THE AMOUNT OF GLUCOSE AND PHOSPHATE

Since inhibition appears to be due to products formed by the reaction of phosphate with glucose the degree of inhibition should be proportional to the amount of these compounds present in the medium. The effect of the amount of glucose was tested in a solution containing peptone 0.1 per cent, dibasic potassium phosphate 0.5 per cent and glucose 0.1 to 1.0 per cent at intervals of one-tenth. Through the range from 0.1 to 0.4 per cent the organism grows as well as in the control tubes without phosphate. Above 0.6 per cent no growth ever occurred. It is evident, therefore, that inhibition is proportional to the amount of glucose present.

In a similar manner the effect of different amounts of phosphate was determined. In this case the basic medium contained peptone 0.1 per cent, glucose 1.0 per cent and dibasic potassium phosphate 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, and 0.50. No inhibition occurred when phosphate was present in amounts of less than 0.2 per cent. Above this amount no growth occurred.

EFFECT OF HYDROGEN ION CONCENTRATION DURING STERILIZATION

It has been shown that failure of growth is not due to the hydrogen ion concentration reached by the media during sterilization. Throughout the experiments it has proved impossible to distinguish between inhibitory and non-inhibitory media by differences in hydrogen ion concentration. It remains to be determined whether or not inhibitory substances are formed in acid media, sterilized and then adjusted to a point within the required range of the organism.

The effect of hydrogen ion concentration during sterilization has been determined. The medium contained peptone 0.1 per cent, dibasic potassium phosphate 0.5 per cent, and glucose 1.0 per cent. The reaction was adjusted to about pH 5.4 before sterilization at 122°C. for fifteen minutes. After sterilization the reaction was readjusted to pH 7.2. No change of color occurred during the heating period and all strains of the organism grew promptly and vigorously. In a lot of the same medium sterilized without adjustment to acid reaction no growth occurred.

In a medium containing 0.1 per cent phosphate, 0.1 peptone and 1.0 per cent glucose sterilized for fifteen minutes at 122°C. growth occurred promptly. In a lot of the same medium adjusted to about pH 8.8 before sterilization the reaction remained alkaline throughout but inhibitory substances were not formed. Similar results were obtained when alkalinity was maintained by calcium carbonate. Thus, it appears that inhibitory substances are not formed in alkaline solutions in the absence of phosphate. All of the evidence indicates that the inhibitory substance is produced by specific reactions between glucose nitrogen compound, and phosphate in alkaline media.

THE EFFECT OF CARAMELIZATION

Since, in all alkaline solutions containing glucose, chemical changes were denoted by the production of a red-brown color, it seemed desirable to determine definitely whether caramelization of glucose alone by heat results in the formation of inhibitory substances. In this experiment 5.0 grams of glucose were

heated in an evaporating dish until the solution became very strongly caramelized. The resulting dark red-brown syrup was then diluted with 500 cc. of distilled water to which was added 0.1 per cent peptone and 0.1 dibasic potassium phosphate. The resulting medium brought to a reaction of pH 7.2 was then sterilized at 122°C. for fifteen minutes. All strains of *Phytomonas malvaceara* grew as well as in the control medium containing uncaramelized glucose.

EFFECT OF OTHER SUGARS

Four additional sugars, maltose, lactose, galactose and levulose have been tested. The medium contained peptone 0.1 per cent and dibasic potassium phosphate 0.5 per cent. The sugars were added in concentrations of from 0.1 to 1.0 per cent. The resulting media were then sterilized at 122°C. for fifteen minutes. None of the media supported growth in concentrations greater than 0.6 per cent of the sugar while levulose proved to be inhibitory at a concentration of 0.4 per cent. Levulose was found to be inhibitory when sterilized at 10 pounds pressure for twenty minutes.

INHIBITION OF OTHER SPECIES

Since *Phytomonas malvaceara* is so markedly inhibited by the products formed by sterilization of sugars in media containing phosphates, other species were tested for comparison. Six species capable of utilizing ammonia were tested in the sodium ammonium phosphate solution. *Serratia marcescens, Salmonella enteritidis, E. coli, Aerobacter aerogenes, Ps. fluorescens* and *Ps. aeruginosa* were not inhibited. In a medium containing peptone 0.1 per cent, beef extract 0.3 per cent, dipotassium phosphate 2.0 per cent, and glucose 5.0 per cent sterilized for thirty minutes at 122°C. some additional species were inhibited while others grew as promptly and vigorously as in the control medium which contained no glucose. The inhibited species included *Staph. albus, Staph. aureus, Sarcina lutea, B. mycoides* and *B. anthracis.* The non-inhibited species included *E. coli, B. subtilis, Aerobacter aerogenes, Serratia marcescens,* and *Ps. fluorescens.*

THE RÔLE OF PHOSPHATE IN TRANSFORMATIONS OF HEXOSE SUGARS

The transformations which occur in hexose sugars due to the influence of caustic alkalies, lead hydroxide, calcium hydroxide, or sodium carbonate have been studied by Lobry de Bruyn and Van Ekenstein (1895) and by Nef (1914). These observers found that when either d-glucose, d-mannose or d-fructose was treated with these solutions a mixture containing all of these sugars resulted and in addition a 3-ketohexose was formed which they designated as d-glutose.

More recently Spoehr and Wilbur (1926) have shown that similar transformations occur when d-glucose or d-fructose is treated with dibasic or neutral sodium phosphate. They question the nature of the so-called d-glutose and offer the suggestion that it is probably a mixture of the nature of formose, acrose, and the condensation products of glyceric aldehyde and dihydroxyacetone. They regard it as a significant fact that "solutions of both d-glucose and d-fructose in the presence of disodium phosphate, in time become colored through the formation of tar." They found also that tar formation does not occur if there is present in the mixture an oxidizing or reducing agent. When the glucose-disodium phosphate mixture was reduced with aluminium amalgam, no tar resulted but acetone was formed as a product of reduction. This was offered as evidence of the splitting of the hexose into a molecule containing three carbon atoms, presumably glyceric aldehyde, which is converted into dihydroxyacetone or acetal. Either of these two compounds yields acetone on reduction. In the absence of a reducing agent or an oxidizing agent the splitting products polymerize, resulting in tar formation or condense to form an optically inactive mixture. It would appear then that the chief action of phosphate is due to the dissociating influence of the salt. This also explains the catalytic effect of disodium phosphate on the oxidation of hexoses.

To explain the transformation of glucose to methyl glyoxaline due to the action of strongly dissociated zinc hydroxide ammonia, Windaus and Koop (1905) assumed that glyceric aldehyde is first formed. Windaus (1907) found also that the reaction is

not confined to glucose but that the same methyl glyoxaline is yielded by mannose, fructose, sorbose, arabinose, xylose, rhamnose, and lactose.

The reactions which are known to occur at high temperatures in solutions containing phosphate, sugars, and nitrogen bearing compounds appear to afford an explanation for all of the phenomena observed in the present investigation. The phosphate acts as a buffer to maintain alkalinity during the heating period and influences dissociation of the sugar. The resulting aldehyde reacts with the nitrogen compound to form a new substance which is suitable as a source of nitrogen for some species but not for others. The original source of nitrogen is completely exhausted only when the amount is relatively small and in the presence of sufficient sugar and phosphate. The exact proportions of nitrogen bearing compound, sugar and phosphate vary with the temperature and period of sterilization.

SUMMARY AND CONCLUSIONS

1. *Phytomonas malvaceara* fails to grow in culture media containing glucose, maltose, lactose, galactose or levulose and various nitrogenous compounds when sterilized at 122°C. for fifteen minutes. This failure of growth is not due to lack of ability to assimilate the various sugars but to chemical changes which are caused by the high temperature of sterilization.

2. Inhibition depends on the amount of peptone, sugar, and phosphate present; the reaction of the solution during the heating period; and the temperature and length of the period of sterilization. It is not due to changes in hydrogen ion concentration during sterilization.

3. Inhibition occurs in culture media which, in the absence of the sugars, are suitable for growth.

4. The evidence seems to warrant the conclusion that inhibition is due to conversion of the nitrogen compound into a form which is not suitable for assimilation by some species of bacteria.

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