ATTENUATION OF CROWN GALL BACTERIA BY CULTIVATION IN MEDIA CONTAINING GLYCINE

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The mechanism by which the crown gall bacterium, *Agrobacterium tumefaciens*, and related microorganisms stimulate atypical and pathological growth in host plants is a question of basic biological importance. One of the approaches to this problem is through the production of attenuated cultures and through a study of the chemical and physical chemical factors associated with inducing this attenuated state.

The virulence of *A. tumefaciens* is a remarkably stable character when compared with that of most plant and animal pathogens. All early attempts to attenuate cultures were unsuccessful. They included cultivation in media varying widely in oxidation-reduction potential, pH range, and containing toxic additives such as phenol and crystal violet. One avirulent strain (A6-6) was derived as a variant from a virulent culture (A6) by Hendrickson *et al.* (1934). Berge *et al.* (1934) noted that growth was inhibited and complete attenuation followed when a virulent culture of *A. tumefaciens* was maintained throughout a series of transfers on media containing glycine. This observation, confirmed subsequently by Longley *et al.* (1937), provided a consistent and reproducible means for obtaining avirulent organisms of this species. It, likewise, made possible a comparative study of the physiology and cultural characteristics of attenuated and virulent cultures. Here an amino acid has been reported specifically to influence virulence.

A number of reports have described the inhibition of microorganisms by amino acids and by media rich in amino nitrogen. For example, Whitehead (1924) found that certain streptococci were retarded by the butyl alcohol-soluble fraction of protein hydrolysates which contained principally monoamino, monocarboxylic amino acids. Gordon and M'Leod (1926) also noted that amino acids of the same group, particularly glycine, tryptophan, cystine, and phenylalanine inhibited meningococci and pneumococci. Likewise, Pohlman (1931) found glycine to retard the growth of certain rhizobia. Wolf and Baldwin (1940) observed that rhizobia, after continued cultivation in glycine media, lost their infectivity for appropriate host plants. As synthetic media containing amino acids came into more extensive use, the inhibitory action of amino acids has been noted with much greater frequency (Hutchings and Peterson, 1943; Greene, 1945; Evans, 1948). Quite commonly, the toxicity of an amino acid was overcome

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by one or more amino acids of closely related chemical structure (Gladstone, 1939; Pelczar and Porter, 1943; Kobayashi *et al.*, 1948), suggesting that such compounds metabolically interfere with one another.

The study reported here deals with the influence of various culture conditions upon the action of glycine. A note which summarized a part of this investigation has already appeared (Van Lanen, Baldwin, and Riker, 1940).

MATERIALS AND METHODS

The following cultures were employed: virulent crown gall strains A6 and A1 which were described by Riker *et al.* (1930); a strain of attenuated virulence, A6-6, described by Hendrickson *et al.* (1934); Gly 15D, which was a strain of A6 attenuated by Berge *et al.* (1934) through cultivation in glycine media; and H100, a virulent strain derived from an infected hop plant. Crown gall strains A6, A6-6, H-100, and Gly 15D were derived from mechanically-picked single-cell isolations. The virulence of each culture was determined by repeated inoculation into plants.

The stock medium (medium no. 79 of Fred and Waksman, 1928) for carrying cultures of crown gall, rhizobia, and *Agrobacterium radiobacter* contained 5.0 g of mannitol, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of K_2HPO_4, 1.0 g of $CaCO_3$, 100 ml of 2 per cent starch-free yeast extract, 15 g of agar, and water to make 1 liter.

To investigate the effect of amino acids and related compounds upon growth and virulence, a synthetic medium of the following composition was used: mannitol, 5.0 g; KNO_3, 5.0 g; $CaCl_2$, 0.19 g; NaCl, 0.2 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2 g; and distilled water to make 1 liter. Amino acids and other nitrogenous adjuncts were added to this basal medium as indicated. All media, unless otherwise stated, were adjusted to pH 6.8 and were sterilized by autoclaving at 15 pounds for 30 minutes.

Liquid media were inoculated with a suspension prepared by washing the cells from a 24-hour culture grown on stock agar. For attenuation studies the growth was homogenized in 5 ml of sterile water, and 0.2 ml of this suspension was added to each 5 ml of medium. Thereafter, each transfer consisted of 1 ml of culture. For certain experiments, i.e., growth curves in which repeated observations of turbidity were necessary, media were prepared in Evelyn tubes or in 16 by 150 mm test tubes which had been selected previously for similarity in size and light absorption. When only 1 ml of medium was available, the micro attachment of the Evelyn apparatus was used. The Evelyn colorimeter (no. 540 filter) was employed in all determinations of turbidity.

Virulence was commonly measured by puncture inoculations on Bonny Best tomato plants. They were grown in 4-inch pots in a greenhouse maintained at about 24 C and were used when 4 to 6 inches high. Inoculations were made by puncturing the upper stem at 3 points about 1 cm apart with 24 gauge nichrome wire. For routine tests the needle was touched to bacterial growth on an agar slant, and for close comparison of strains, the needle was dipped into a standardized suspension which delivered about two million cells into each puncture. Plants were always inoculated in duplicate or triplicate with suitable controls.

The galls from virulent cultures appeared conspicuously within 15 days. Preliminary records were made then, and final records were made after 30 days. Attenuated cultures produced no apparent change during this period while pathogenic cultures caused gall formation that varied in size according to their virulence.

EXPERIMENTAL RESULTS

The attempt to find chemicals that would attenuate crown gall bacteria involved many trials that gave negative results. The details are omitted. However, attenuation was achieved by a number of successive transfers in a medium containing glycine. Effective concentrations ranged between 0.1 and 0.3 per cent. When less glycine was used, growth eventually approached that in the basal medium and virulence was not altered despite the number of transfers. On the other hand, concentrations greater than 0.3 per cent were bacteriostatic and, likewise, failed to affect virulence. All secondary colonies, which developed from giant colonies following transfer of a virulent culture on glycine agar medium, retained normal virulence.

Attenuation by glycine appeared to be a gradually induced change associated with growth processes. It seemed entirely possible, therefore, that culture conditions which influenced growth might alter the rate at which virulence was lost. The descriptions of some representative experiments follow which were designed to determine the effect of these particular conditions, first upon growth and second upon virulence, when glycine was used in the medium.

Growth in glycine media. Media with varying concentrations, respectively, of glycine in 0.5 per cent peptone were seeded with strain A6. Turbidity measurements were made periodically throughout a 48-hour period of incubation. Stained preparations also were made for a comparison of the morphology of cells grown in the presence and absence of glycine. Figure 1 shows that growth was initiated about equally well in media with and without glycine. However, after a brief period of development growth became retarded in all media containing glycine, the curves levelling off more rapidly with the higher concentrations. After 48 hours, turbidity in the presence of 0.2 per cent glycine was approximately one third of that obtained in the control medium. Continued incubation failed to reveal any marked increase in turbidity such as might result from a rapid development of resistance toward this compound.

Differences were observed in both morphology and staining characteristics. Whereas cells from the control medium without glycine occurred singly or in pairs of equal size, those from glycine media were either abnormally small or large and globular. Quite commonly one cell of a pair would be smaller and the other larger than normal resulting in tadpole or comma shapes. Stains were absorbed irregularly giving a granular appearance in contrast to the homogenous appearance of normal cells. In other experiments in which the glycine concentration was sufficiently high to prevent any noticeable growth these morphological changes did not take place. Abnormal cells cultivated in glycine media again became normal when returned to media free of this amino acid.

Transfers required to attenuate. Strain A6 was transferred at five-day intervals in mannitol-nitrate medium supplemented with glycine. The initial glycine level was reduced to 0.025 per cent so as to permit gradual acclimatization, and after each five transfers the concentration was raised 0.05 per cent. At intervals of a few transfers and after each transfer following the appearance of organisms with lowered virulence, dilution plates were poured from stock agar and about 50 colonies were selected from each series for plant inoculations. The relative percentages of virulent, intermediate (inducing only a slight swelling), and attenuated colonies obtained during 31 transfers are recorded in table 1.

While organisms of intermediate virulence were obtained during the first nine transfers, no fully attenuated strains appeared during this period. Thereafter, attenuated organisms gradually increased in ratio to virulent until by the end

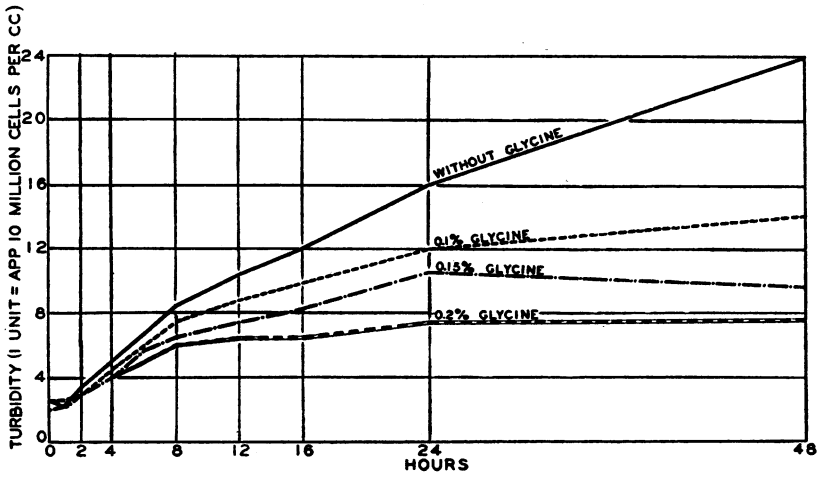

Figure 1. The effect of varying concentrations of glycine on the growth of *Agrobacterium tumefaciens* in the peptone medium.

of the thirty-first transfer, none of the isolates was virulent and 85 per cent were attenuated. The remaining 15 per cent were of intermediate virulence, those falling into the intermediate group ranged from 10 to 22 per cent of all isolates sampled. Strain A6 was originally a mechanically picked single cell isolate. These results (confirmed subsequently with other single celled cultures) support the conclusion that the attenuation process involves a gradual loss of virulence of all organisms subjected to glycine. It was not a selection of organisms lacking virulence from an original mixture.

Influence of the basal media. Attenuation rates were determined in the following media each with added glycine: (1) mannitol-nitrate; (2) mannitol-nitrate containing 0.5 per cent peptone; and (3) mineral salts only, in the amounts used in the mannitol-nitrate medium, i.e., with glycine as the sole carbon source. Glycine was supplied initially at 0.05 per cent and was increased by 0.05 per cent after each five transfers. Virulence was tested periodically as before.

The results, summarized in table 2, show that the most rapid and complete attenuation occurred in the medium supplemented with peptone. Attentuation took longer in mannitol-nitrate. No detectable reduction in virulence appeared with glycine alone. Growth was meager in the latter medium, fair in the mannitol-nitrate medium, and good in the presence of peptone.

The ability of peptone to enhance the rate and extent of attenuation was of special interest. Since growth was actually improved by peptone, it was con-

TABLE 1

The effect of the number of transfers in glycine-mannitol-nitrate media on the activity of attenuated strains of Agrobacterium tumefaciens

TRANSFERS IN GLYCINE MANNITOL-NITRATE MEDIUM	VIRULENT STRAINS	INTERMEDIATE STRAINS	ATTENUATED STRAINS
	Per cent	*Per cent*	*Per cent*
9	88	12	0
11	57	16	26
13	49	19	32
15	44	22	35
17	43	16	41
19	50	10	41
27	22	11	68
31	0	15	85

TABLE 2

The effect of different basal media on attenuation of Agrobacterium tumefaciens by glycine

MEDIUM	GROWTH AND VIRULENCE					
	20 Transfers		30 Transfers		40 Transfers	
	Growth*	Gall size	Growth	Gall size	Growth	Gall size
Peptone + glycine	++	Small	++	None	++	None
Mannitol-nitrate + glycine	+	Medium	+	Medium	+	None
Glycine	±	Large	±	Large	±	Large
Peptone	++++	Large	++++	Large	++++	Large

* Growth indicated as follows: ++++ excellent, ++ fair, + poor, ± questionable.

sidered unlikely that its free amino acid content contributed substantially to the action of glycine. It was observed, however, that the final reaction in peptone medium was alkaline in contrast to a slightly acid or neutral reaction in the other media. Consequently, the effect of pH on growth and attenuation was investigated.

Reaction of the medium. Several experiments were conducted using mannitol-nitrate medium with 1 per cent peptone and with the phosphate raised to 1.3 per cent to provide added buffer. Results of one typical trial in which strain A6 was grown in this medium and the same with glycine added within the pH range 4.0 to 8.0 are shown in figure 2. Here the growth response of *A. tumefaciens* in

the presence of glycine does not parallel that in the basal medium adjusted to the same pH levels. Instead, glycine caused no inhibition at pH 4.0, a mild inhibition at pH 5.0, and sharply decreased growth as the medium approached neutrality. The greatest differential in growth between glycine and nonglycine media occurred at pH 7.0, although it likewise was pronounced at pH 6.0 and 8.0. This observation, combined with the preceding experiment in which peptone was used, indicated that the rate of attenuation might be accelerated by a neutral or alkaline reaction. This possibility was investigated further.

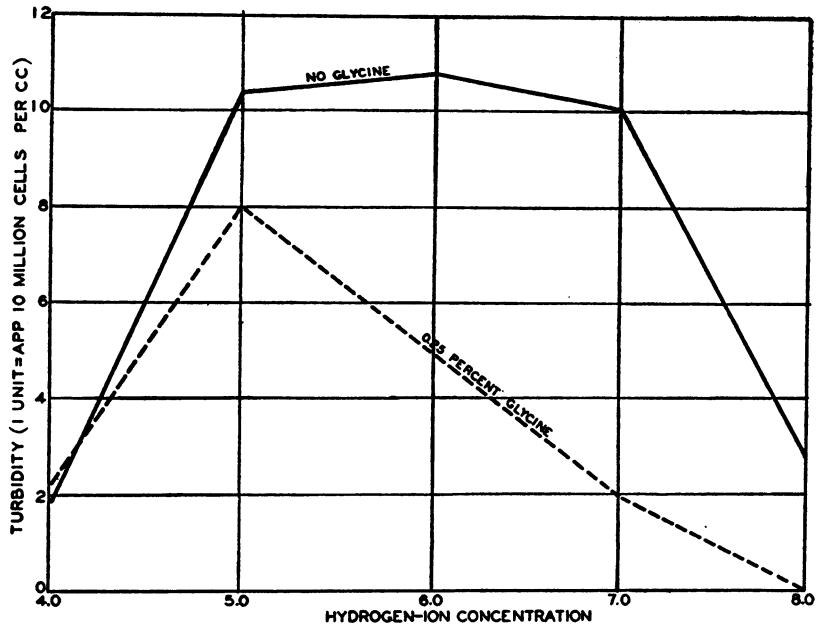

Figure 2. The effect of pH and of glycine on the growth of *Agrobacterium tumefaciens* in mannitol-nitrate-peptone medium after 48 hours' incubation.

Mannitol-nitrate medium containing 1.3 per cent phosphate was supplemented with 0.025 per cent glycine and adjusted from pH 5.0 to 9.0. Strain A6 was seeded into these media and transferred at five-day intervals with the glycine being increased 0.05 after each five transfers. It was found that growth was not decreased appreciably over the control medium adjusted to pH 5.0 but decreased markedly as in the preceding experiment as the pH was raised. Very poor to negligible development occurred at pH 9.0, and it was necessary to discontinue this particular series. Virulence tests of isolated colonies from the series set at pH 8.0 showed that attenuation was complete after 20 transfers whereas at pH 7.0 only about 60 per cent and at pH 6.0 and below none of the isolates was attenuated. These representative findings indicated that the inhibition of growth by glycine in alkaline media was directly associated with the attenuation process. In addition the data suggested that the accelerating action of peptone was probably attributable largely to the alkaline reaction created by its use.

SUMMARY

The experiments described herein confirm and amplify earlier observations with regard to the action of glycine on the growth and virulence of crown gall bacteria.

Glycine inhibited the growth of crown gall organisms in concentrations of 0.1 per cent and above. It affected immediately the initiation of growth, the total amount of growth, and the morphology of the cells. Inhibition occurred in both synthetic and natural media, either of which provided abundant growth when glycine was omitted.

After continued cultivation in media containing glycine in concentrations which suppressed growth, complete loss of virulence invariably resulted with cultures of both single colony and single cell origin. The rate of attenuation, as measured by the number of transfers to render cultures incapable of gall production, was enhanced by utilizing small inocula, peptone in the medium, and by maintaining a neutral or slightly alkaline reaction. Attenuation did not occur in glycine media adjusted to pH 5.5 or more acid, and growth was not appreciably inhibited in this range.

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