

STUDIES ON CERTAIN PHYSIOLOGICAL CHARACTERS OF PHYTOMONAS TUMEFACIENS, PHYTOMONAS RHIZOGENES, AND BACILLUS RADIOBACTER

PART I¹

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INTRODUCTION

A comparison has been made of certain physiological characters of *Phytomonas tumefaciens* (Smith and Town.) Bergey, *et al.*, and *Phytomonas rhizogenes* Riker *et al.*, the cause of crown gall and hairy root respectively, and of *Bacillus radiobacter*, Beij. and Van Deld., a soil saprophyte similar in many physiological characters to *P. tumefaciens*. Since it appeared that the influences of these organisms in plants were doubtless physiological, depending in part upon the utilization of food materials within the tissue, it seemed desirable to determine how they would act on nutrients of known composition.

These studies centered about the following: (1) the utilization of various nitrogen-containing compounds; (2) the use of various carbon sources with certain sources of nitrogen; (3) nitrate reduction in the presence of certain sugars; and (4) the oxidation-reduction potentials induced in certain media.

The early work with these bacteria was primarily concerned with their identification. Descriptions of the crown-gall organ-

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ism may be found in a number of papers including those by Smith and Townsend (1907), Smith, Brown and Townsend (1911), Patel (1929), Muncie and Suit (1930), Riker *et al.*, (1930), and Wright *et al.*, (1930); *B. radiobacter* by Beijerinck and Van Delden (1902), Löhnis (1905), Löhnis and Hansen (1921), Israilsky (1929), Muncie and Suit (1930), Riker *et al.*, (1930); and the hairy-root organism by Muncie and Suit (1930), Riker *et al.* (1930), and Wright, *et al.* (1930). The descriptions of the three bacteria in these and other papers show certain discrepancies omitted here because of the space required for their discussion. It appears that the most valid descriptions are those made with single-cell cultures.

CULTURES OF BACTERIA EMPLOYED

The sources of the cultures employed were diverse. The majority of the strains employed were progenies of single cells. The nine crown-gall cultures used were from raspberry (see T-5, etc., Wright *et al.* 1930). The five hairy-root cultures were obtained: two from a mixed infection on walnut (see T-37-1a and T-37-2b, Riker *et al.*, 1930) and three from apple (see C-1, D-20, and T-38-1a, Wright *et al.*, 1930). The radiobacter cultures were obtained: six from the United States Department of Agriculture and four locally (see R-1, etc., Riker *et al.*, 1930).

The identity of all cultures was determined by differential cultural tests and by reactions on host plants repeated from time to time during the work as described by Riker *et al.* (1930).

UTILIZATION OF NITROGEN

Since no comparisons were found of the utilization by these organisms of nitrogen-containing compounds, representative sources of nitrogen were prepared in liquid media. Their utilization was determined either by macroscopic growth or, in some cases, by stained preparations.

Determinations were made in the following medium: magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.2 gram, sodium chloride (NaCl), 0.2 gram, calcium chloride (CaCl_2), 0.1 gram, dibasic potassium phosphate (K_2HPO_4), 0.2 gram, a nitrogen source (see table 1), 5 grams, and a carbon source, 5.0 grams in 1000 cc. of

water. The nitrogen source, and the salt-carbohydrate were prepared separately in double strength, neutralized, sterilized, mixed and tubed aseptically.

The potato extract was prepared by thoroughly cooking two hundred grams of potato, sliced into sections about one-fourth inch in thickness, in one-half liter of distilled water. This extract, filtered twice through absorbent cotton, neutralized, made up to one-half liter and autoclaved, was added to a sterile double-strength solution of glucose and pipetted into sterile test tubes.

The yeast-water extract, used at the rate of 100 cc. of clear supernatant liquid in each liter of medium, was prepared by autoclaving 100 grams of starch-free yeast in a liter of distilled water.

The amino acids, tyrosine, alanine, glutamic acid and cystine, were used singly and in combination at the rate of one and one-fourth grams each per liter, with and without traces of ferrous sulphate or ferric chloride. Ammonium citrate and ferric ammonium citrate were used in agar shake and agar stab cultures, and also in the liquid media. In order to remove any soluble nitrogen or carbon compounds from the agar it was washed in repeated changes of distilled water so that when the nitrogen source was withheld from the medium, no growth occurred with any of the bacteria. The cultures were seeded from slant cultures forty-eight to seventy-two hours old and incubated at 28°C. for three weeks.

INORGANIC NITROGEN

It may be noted in table 1 that *P. rhizogenes* cultures differed from those of crown-gall and *B. radiobacter* because they produced little or no growth with the inorganic nitrogen compounds, while the other two organisms grew well except with potassium nitrite. With ammonium citrate and ferric ammonium citrate, both in liquid and agar shake cultures, *P. tumefaciens* and *B. radiobacter* grew very well with a browning of the medium in the upper layer. *P. rhizogenes* induced an acid reaction with ammonium citrate, and ferric ammonium citrate which differentiated it from the other two. *B. radiobacter* was differentiated by the alkaline reaction it induced with potassium nitrate.

TABLE 1

Summary of studies on growth and hydrogen-ion concentration produced by *Phytomonas tumefaciens*, *Phytomonas rhizogenes*, and *Bacillus radiobacter* in media containing various sources of nitrogen and carbon*

SOURCE OF NITROGEN	SOURCE OF CARBON	GROWTH† OF <i>P.</i> <i>rhizogenes</i> (5 STRAINS)	HYDROGEN-ION CONCENTRATION WITH			
			Control	<i>P. tumefaciens</i> (9 strains)	<i>P. rhizogenes</i> (5 strains)	<i>B. radiobacter</i> (10 strains)
None	Glucose	0	6.8	6.8	6.8	6.8
Inorganic						
Ammonium sulphate	Glucose	+‡	6.4	5.8	5.8	5.8
Ammonium sulphate	Sucrose	—	6.4	5.2		5.2
Ammonium sulphate	Mannitol	—	6.4	4.6		4.8
Ammonium citrate	Glucose	+	6.6	9.0	5.8	9.0
Ferric ammonium citrate	Glucose	+	7.0	7.2	4.7	7.2
Ammonium nitrate	Glucose	0	6.0	5.8	6.0	5.8
Ammonium nitrate	Glycerine	+‡	6.2	6.2	6.0	8.2
Potassium nitrite	Glucose	0	7.4	6.8	7.4	6.8
Potassium nitrate	None	0	6.8	6.8	6.8	6.8
Potassium nitrate	Glucose	0	7.2	7.2	7.2	9.2
Organic						
Methylamine	Glucose	0	7.2	6.0	7.2	6.0
Acetamide	Glucose	+‡	6.4	6.2	6.2	6.4
Oxamide	Glucose	++	6.2	5.0	5.0	5.2
Succinimide	Glucose	+	6.6	7.0	6.6	7.2
Dicyandiamide	Glucose	++	7.0	6.6	6.4	6.6
Asparagine	Glucose	++	6.6	8.4	5.8	8.4
Urea	Glucose	+‡	7.6	9.2	8.2	9.2
Urea	Sucrose	+‡	7.8	9.2	8.2	9.2
Urea	Mannitol	++	8.0	9.0	8.0	9.2
Uric acid	Glucose	+	6.4	6.6	5.4	6.8
Amino acids						
Glycine	Glucose	+	6.4	6.8	6.4	8.0
dl-Alanine	Glucose	+‡	6.6	7.8	6.4	8.1
dl-Alanine	Mannitol	+	7.0	6.6	6.6	6.6
l-Tyrosine	Glucose	+	6.8	4.8	4.8	4.8
l-Tyrosine	Mannitol	+++	6.8	5.4	6.0	5.8
l-Cystine	Glucose	+‡	6.8	4.8	5.2	4.8
l-Aspartic acid	Glucose	++	7.2	9.0	4.8	9.0
l-Aspartic acid	Mannitol	+	7.2	8.8	5.2	8.8
d-Glutamic acid	d-Glutamic acid	—	7.2	8.8		9.2
d-Glutamic acid	Glucose	+++	7.6	7.8	4.6	7.8
d-Glutamic acid	Mannitol	—	7.0	8.0		8.0

TABLE 1—Concluded

SOURCE OF NITROGEN	SOURCE OF CARBON	GROWTH† OF <i>P.</i> <i>rhizogenes</i> (5 STRAINS)	HYDROGEN-ION CONCENTRATION WITH			
			Control	<i>P. tumefaciens</i> (9 strains)§	<i>P. rhizogenes</i> (5 strains)	<i>B. radiobacter</i> (10 strains)§
Amino acids—Concluded						
d-Glutamic acid	Sucrose	—	7.0	7.2		7.2
l-Leucine	Glucose	—	6.8	5.4		5.2
Mixture	Glucose	+‡	7.2	8.0	7.2	8.0
Mixture + FeSO ₄	Glucose	+‡	7.0	7.2	6.6	7.2
Mixture + FeCl ₃	Glucose	+‡	7.0	8.0	7.0	8.0
Miscellaneous						
Peptone	Peptone	++++	7.4	8.2	8.2	8.2
Peptone	Arabinose	+++	6.6	6.3	5.4	6.2
Peptone	Xylose	++++	6.4	6.3	4.4	6.4
Peptone	Glucose	++++	6.8	6.6	4.7	6.6
Peptone	Mannose	+++	6.6	6.6	4.6	6.6
Peptone	Sucrose	++++	6.6	6.6	6.2	6.4
Peptone	Lactose	+++	6.4	6.6	5.2	6.6
Peptone	Raffinose	+++	6.8	6.8	6.8	6.8
Peptone	Salicin	++	6.2	6.1	4.6	6.2
Peptone	Erythritol	+++	6.8	7.4	4.6	7.4
Peptone	Mannitol	+++	6.4	6.4	6.2	6.4
Yeast-water extract	Glucose	++++	6.6	6.5	4.6	6.6
Potato extract	Potato extract	++++	6.6	9.2	9.2	9.2
Potato extract	Glucose	+++	6.8	7.4	4.8	7.6

* The composition of the medium employed is given in the text. The final readings were made after three weeks incubation at 28°C.

† Growth is indicated as follows: 0 = none, + = trace, ++ = slight, +++ = moderate, ++++ = abundant, — = no determinations made.

§ *P. tumefaciens* and *B. radiobacter* each produced moderate to abundant growth in all cases except in the potassium nitrite medium and controls lacking either a carbon or a nitrogen source.

‡ Microscopic examination also made and compared with controls.

AMIDES

The hairy-root cultures gave limited development with nitrogen of simple organic compounds (table 1). No growth was secured with methylamine. Compounds such as acetamide, oxamide, succinimide, dicyandiamide, asparagine, urea, and uric acid were

utilized readily by *P. tumefaciens* and *B. radiobacter* while *P. rhizogenes* produced only a trace to a slight amount of growth. With urea, all three organisms induced an alkaline reaction, whereas with the other compounds there were some differences.

AMINO ACIDS

With the various amino acids, and with glucose or mannitol as a carbon source, the crown-gall and *B. radiobacter* cultures produced good growth, but *P. rhizogenes* produced only a trace, except with aspartic acid, glutamic acid, and tyrosine. Both carbon and nitrogen for *P. tumefaciens* and *B. radiobacter* were provided by glutamic acid.

An alkaline reaction was induced by *B. radiobacter* and *P. tumefaciens* when glycine, alanine, aspartic acid, or glutamic acid were used with glucose, while hairy-root cultures induced an acid reaction. All three organisms induced an acid reaction with tyrosine and cystine. The first two mentioned organisms induced an acid condition with leucine.

Mixtures of amino acids, tyrosine, alanine, glutamic acid, and cystine, with and without the addition of iron salts, were tested. *P. rhizogenes* produced only a trace of growth, while *B. radiobacter* and *P. tumefaciens* produced good growth.

MISCELLANEOUS NITROGENOUS SUBSTANCES

Difco peptone, yeast infusion and potato extract, as seen in table 1, appear to be the most suitable sources of nitrogen for the growth of all three organisms. The hairy-root organism grew better with one or another of these complex substances than with the other nitrogen-containing compounds.

Difco peptone was utilized as the sole source of nitrogen and carbon by all three organisms. Where peptone was used alone, all three organisms induced an alkaline reaction due to the formation of ammonia, as determined by testing with Nessler's solution.

The hairy-root cultures induced a strong acid reaction in media containing peptone and a carbohydrate, except with raffinose, sucrose, and mannitol. It is probable that these carbohydrates

were less readily attacked than the peptone, with the formation of ammonia which neutralized the acids formed.

Yeast-water extract when used with glucose gave results very similar to those secured from peptone with glucose. A decided acid reaction, pH 4.6, was induced by *P. rhizogenes*, while the reaction remained practically unchanged with the others. Crown-gall cultures produced a fairly heavy membrane, but not nearly as heavy as *B. radiobacter* cultures.

With the potato extract alone, i.e., with no further salts or carbohydrates, the three groups of organisms each produced good growth with an alkaline reaction. When potato extract was used with glucose, the hairy-root cultures induced a strongly acid reaction, pH 4.8, while the other two types of organisms induced a slightly alkaline reaction, pH 7.4 and 7.6.

The type of growth of the three organisms in the various media was in many instances quite different. The crown-gall organism produced a heavy membrane in most cases, accompanied by a veil, with an otherwise clear liquid. *P. rhizogenes* usually produced a rather uniform turbidity in the broth, with slight if any membrane or veil. *B. radiobacter* produced a heavy membrane in most cases with an accompanying uniform turbidity of the medium, and a moderate-to-abundant sediment.

P. rhizogenes seems to lack the ability of *P. tumefaciens* and *B. radiobacter* to utilize the simpler nitrogenous compounds. The hairy-root organism thrived best with the more complex sources of nitrogen. With the simpler nitrogen-containing compounds, growth was either absent or very meager and seldom increased after the third or fourth day, while the other two organisms grew well in almost every case. This might result in part from the inability of the organism to use the ingredients of the medium, but other factors may be influential.

NITRATE NITROGEN WITH A REDUCING AGENT

Since *P. rhizogenes* failed to produce growth in a medium containing 0.5 per cent potassium nitrate, it was considered that this might result from an unfavorable oxidation-reduction potential. By the addition of sterile sand, sterile divided filter paper, or

sterile agar to tubes of liquid culture medium, which was ordinarily too highly oxidized to permit growth of root-nodule bacteria, Allyn and Baldwin (1930) induced growth which, they considered, was due to trapping of small volumes of the medium in which the organisms were able to make the necessary adjustments. Similar trials, with sterile washed sand and sterile agar, were made with *P. rhizogenes* in mannitol and glucose media containing potassium nitrate at the rate of 0.5, 1.0, and 5.0 grams per liter. The carbohydrate was autoclaved separately from the other constituents.

The inocula were 0.2 cc. per 10 cc. of medium of suspensions from two-day-old cultures prepared in sterile distilled water and standardized to a turbidity which would conceal a two millimeter loop, made from No. 26 B. and S. gauge wire, at twenty millimeters depth. After seeding, sterile washed sand or sterile agar was added under aseptic conditions to give a column of ten millimeters in the bottom of the tubes. With the use of the higher concentration of potassium nitrate no growth was initiated, while growth occurred with the two lower concentrations.

Reducing agents were used by Allyn and Baldwin (1930) to facilitate the growth of *Rhizobium* cultures in media which would otherwise be too highly oxidized. Similar trials, in which fresh potato blocks were used as the reducing agent in Petri dishes containing the glycerophosphate medium employed by Riker *et al.* (1930), were made. The growth of *P. rhizogenes* was favored. There is, however, a possibility that diffusion of the nutrients from the potato block into the surrounding medium favored growth, rather than merely the lower oxidation-reduction potential.

Thioglycollic acid was also used as a reducing agent with the mannitol and glucose nitrate media. The potassium nitrate was used in varying amounts, namely, 0.5, 1.0, and 5 grams per liter, while the thioglycollic acid was used at the rate of 0.025 to 0.15 gram per liter. The sugar and salts were prepared separately, autoclaved, mixed aseptically and pipetted in 8-cc. portions to sterile test tubes. One cubic centimeter of the bacterial suspension as earlier described and 1 cc. of the diluted and neutralized thioglycollic acid were added to each tube. The thioglycollic acid was found to be sterile, and was prepared by dilution in

sterile distilled water and neutralized with sterile N/14 NaOH. Brom-thymol blue was added to check the hydrogen-ion concentration. The use of the nonnitrogenous reducing agent, thioglycollic acid, gave results similar to those with sand. Growth was initiated with the lower concentrations of potassium nitrate, and with all the concentrations of thioglycollic acid used, but did not increase after the third day. The total growth was meager.

No attempts have thus far been made to stimulate growth of *P. rhizogenes*, in the medium with nitrate as the source of nitrogen, by the addition of small amounts of what might be growth promoting substances as accomplished by Reader (1928), Farries and Bell (1930), and others working with various microorganisms.

UTILIZATION OF CARBON SOURCES

The availability of various carbon sources was examined because of certain earlier mentioned discrepancies in previous work. In that work, sources of nitrogen such as peptone were commonly used which might serve also as sources of carbon. In the present studies potassium nitrate was employed, which was readily available for *P. tumefaciens* and *B. radiobacter*. Because it was not used by *P. rhizogenes*, results from this organism were negative and are omitted.

The composition and preparation of the medium, inoculation, and incubation were the same as described under the heading "Utilization of Nitrogen." Carbohydrates, glucosides, alcohols, and organic acids or their salts were employed as sources of carbon, and the results obtained are given in table 2.

SUGARS

The saccharides arabinose, glucose, lactose, sucrose, raffinose, and dextrin were utilized to about the same extent by *P. tumefaciens* and *B. radiobacter*. *P. tumefaciens*, however, was slower in starting growth where lactose was used. *P. tumefaciens* induced no decided change in the hydrogen-ion concentration, but *B. radiobacter* induced an alkaline reaction (pH 7.6 to pH 9.4), probably due to the reduction of the nitrate and corresponding

TABLE 2

Summary of studies on growth and hydrogen-ion concentration produced by *Phytophthora tumefaciens* and *Bacillus radiobacter* in media with various sources of carbon, containing potassium nitrate as a nitrogen source*

SOURCE OF CARBON	GROWTH† OF		HYDROGEN-ION CONCENTRATION WITH		
	<i>P. tumefaciens</i> (9 strains)	<i>B. radiobacter</i> (10 strains)	Control	<i>P. tumefaciens</i> (9 strains)	<i>B. radiobacter</i> (10 strains)
Arabinose.....	++++	++++	6.8	7.2	9.2
Glucose.....	++++	++++	7.2	7.2	9.4
Lactose.....	+++	+++	7.0	7.0	8.0‡
Sucrose.....	++++	++++	7.0	7.0	7.6‡
Raffinose.....	++++	++++	7.0	7.2	9.4
Dextrin.....	++++	++++	7.2	7.4	9.2
Salicin.....	++++	++++	7.0	6.8	7.6‡
Methyl alcohol.....	+§	+§	7.2	7.2	7.4
Ethyl alcohol.....	+++	+++	7.2	4.8	9.2
Propyl alcohol.....	+++	+++	7.2	5.6	5.4
Butyl alcohol.....	+§	+§	7.2	7.2	7.2
Glycerol.....	++++	++++	6.8	7.0	9.2
Mannitol.....	++++	++++	7.0	7.0	9.2
Adonitol.....	++	++++	7.4	7.4	8.8
Quercit.....	+§	++**	7.2	7.2	7.2
Dulcitol.....	+++	++++	7.0	7.0	7.4‡
Lactositol.....	+++	++++	7.2	7.2	7.6‡
Arabitol.....	++++	++++	7.2	7.2	9.2
Sodium formate.....	+§	+§	7.2	8.4††	8.5††
Sodium acetate.....	+++	+++	7.0	8.4‡	9.0
Sodium propionate.....	++++	++++	7.0	9.0	9.0
Butyric acid.....	++§	++§	6.8	6.8	7.2
Lactic acid.....	++++	++++	6.8	8.6	9.2
Glycollic acid.....	++§	++§	6.8	7.8††	7.4††
Calcium gluconate.....	++++	++++	7.0	7.2	8.6
Stearic acid.....	++§	++§	7.4	7.4	7.4
Sodium oxalate.....	0§	0§	7.2	7.2	7.2
Sodium malonate.....	++§	++§	7.0	7.2	7.2
Sodium succinate.....	++++	++++	6.8	9.0	9.0
Malic acid.....	++++	++++	7.0	9.0	9.2
Tartaric acid.....	++§	++§	7.0	7.2	7.2
Sodium citrate.....	++++	++++	6.8	9.4	9.6
None.....	0§	0§	6.8	6.8	6.8

* The composition of the media employed is given in the text. The final readings were made after three weeks incubation at 28°C.

† Growth is indicated as follows: 0 = none, + = trace, ++ = slight, +++ = moderate, ++++ = abundant.

‡ Reaction decidedly alkaline after six weeks.

§ Microscopic examination made.

** Slight brownish-black color.

†† Tested at six weeks.

accumulations of basic ions. With the glucoside, salicin, similar results were induced, with the exception that at three weeks a reaction of pH 7.6 was induced by *B. radiobacter* and one of pH 9.0 at nine weeks.

ALCOHOLS

When ethyl or propyl alcohol was used, equal growth was produced by *P. tumefaciens* and *B. radiobacter*. The crown-gall organism induced an acid reaction, but *B. radiobacter* induced an alkaline reaction with ethyl alcohol and an acid reaction with propyl alcohol. Nitrates disappeared with many of the *B. radiobacter* cultures after nine weeks. With methyl or butyl alcohol no macroscopic evidence of growth was visible, but stained preparations indicated that the organisms were able to start development, but were unable to continue.

With the poly-hydroxy alcohols, mannitol, glycerol, and arabitol, very good growth was produced. The reaction again was decidedly alkaline with *B. radiobacter* but remained unchanged with *P. tumefaciens*. When adonitol, lactositol, and dulcitol were used, considerably more growth was produced by *B. radiobacter* than by *P. tumefaciens*, but not as alkaline a reaction as with the other carbon sources. With quercit, a slight brownish to black color was produced by *B. radiobacter* with slight growth. The crown-gall organism produced only a trace of growth and no discoloration of the medium or change in reaction.

SALTS OF ORGANIC ACIDS

When sodium formate, butyric acid, glycollic acid, stearic acid, sodium malonate, or tartaric acid were used in place of the carbohydrate, neither *P. tumefaciens* nor *B. radiobacter* produced more than slight growth. Only with the use of sodium formate and glycollic acid was there any great change in hydrogen-ion concentration, where growth was slight, and this only after six weeks incubation.

With sodium acetate only moderate growth took place with either of the organisms, but abundant growth appeared with sodium propionate, lactic acid, calcium gluconate, sodium suc-

ciate, malic acid, and sodium citrate. The medium became decidedly alkaline in reaction. Where sodium oxalate was used no growth was produced by either organism.

It may be noted that a wide range of carbonaceous materials was utilized by *P. tumefaciens* and *B. radiobacter* (table 2). Both types of microorganisms utilized the compounds to about the same extent. They were, however, differentiated on the basis of final reaction with many of the compounds, especially upon incubation for a long time. *B. radiobacter* induced, in each case where good growth occurred, a strongly alkaline condition, probably due to the reduction of the nitrate. The acid reaction, induced by *P. tumefaciens* when ethyl or propyl alcohol was used, may result from the rapid rate of oxidation of the alcohol to acid in comparison with the slow release of the basic ions as the nitrate nitrogen was utilized. This may likewise account for the difference in reaction induced by *B. radiobacter* where these two alcohols were employed. The alkaline reaction induced with sodium formate, sodium acetate, and sodium succinate by *P. tumefaciens* may come through the accumulation of basic ions upon utilization of the organic radical, or upon absorption of the nitrate ion. As a general rule, the sugars and a few of the poly-hydroxy alcohols were better suited than the other materials to the growth of those two organisms, although some of the organic acids or their salts seemed readily available. The differences noted are worthy of further study in relation to the pathogenicity and nonpathogenicity of the two organisms.

NITRATE REDUCTION IN RELATION TO CARBON SOURCE

The nitrate reduction studies were made, using various sources of carbon in the nitrate medium previously described. Inoculation was made from slant cultures by means of a one millimeter wire loop and incubated at 28°C. Qualitative tests were made for nitrites with Trommsdorf's reagent, and for nitrates with a diphenylamine reagent. Results after three and six weeks incubation are reported, although observations in many cases were also made up to fourteen weeks. Table 3 shows a summary of these qualitative tests.

TABLE 3

Summary of results of qualitative tests for nitrate and nitrite in media containing potassium nitrate with various carbon sources, and acted upon by *Phytomonas tumefaciens* or *Bacillus radiobacter**

SOURCE OF CARBON	<i>P. tumefaciens</i> (9 STRAINS)		<i>B. radiobacter</i> (10 STRAINS)	
	Nitrate†	Nitrite‡	Nitrate†	Nitrite‡
Arabinose.....	+++	0	+++	0
Glucose.....	+++	0	+++ 0‡	0
Lactose.....	+++	0	+++	0
Sucrose.....	+++	0	+++	0
Raffinose.....	+++	0	+++ 0‡	0
Dextrin.....	+++	+	+++ (0)	0
Salicin.....	+++	0	+++	0
Methyl.....	+++	0	+++	0
Ethyl.....	+++	+	+++§	0
Propyl.....	+++	0	+++	0
Butyl.....	+++	0	+++	0
Glycerol.....	+++	+	0	0
Mannitol.....	+++	+(0)	0‡	0
Adonitol.....	+++	+(0)	+++	0
Quercit.....	+++	0	+++	0
Dulcitol.....	+++	0	+++	0
Lactositol.....	+++	0	+++	0
Arabitol.....	+++	0	+++ 0‡	0
Sodium formate.....	+++	0	+++	0
Sodium acetate.....	+++	0	+++	+(0)
Sodium propionate.....	+++	++(+)	+++ (+++)§	++(0)
Butyric acid.....	+++	0	+++	0
Lactic acid.....	+++	++(+)	+++ (0)	++(0)
Glycollic acid.....	+++	0	+++	0
Calcium gluconate.....	+++	+(0)	0‡	0
Stearic acid.....	+++	0	+++	0
Sodium oxalate.....	+++	0	+++	0
Sodium malonate.....	+++	0	+++	0
Sodium succinate.....	+++	+(0)	+++	0(++)
Malic acid.....	+++	+	-	+++
Tartaric acid.....	+++	0(++)**	+++	0(++)**
Sodium citrate.....	+++	0	+++	++(++++)

* The composition of the medium employed is given in the text. Tests for nitrate were made with diphenylamine reagent, and for nitrite with Trommsdorff's reagent. Controls tested showed nitrate but no nitrite. The results are given of tests made after three weeks. Similar tests were made after six weeks, but the results are omitted when they showed no change. Results after six weeks are shown in parenthesis when they differed from the set taken at three weeks.

† Tests recorded as follows: 0 = none, + = trace, ++ = moderate, +++ = strong, - = no determination made.

‡ Trace in a few cultures.

§ Nitrates disappeared from a few cultures at nine weeks.

** Test at nine weeks.

The nitrate completely disappeared from *B. radiobacter* cultures in three weeks when glucose, raffinose, glycerol, mannitol, arabinol, and calcium gluconate were employed as the carbon source. With dextrin and lactic acid the nitrate had completely disappeared in six weeks, and with sodium propionate or ethyl alcohol in nine weeks.

Nitrite was present at one period or another with *B. radiobacter* when sodium acetate, sodium propionate, lactic acid, sodium succinate, malic acid, tartaric acid and sodium citrate were used. Nitrites appeared with *B. radiobacter* more freely when the organic acids or their salts were used than with other sources of carbon. Nitrite was found with the crown-gall cultures where dextrin, ethyl alcohol, glycerol, mannitol, adonitol, sodium propionate, lactic acid, calcium gluconate, sodium succinate and malic acid were used as carbon sources. Where nitrite was present after three weeks it was not always present after six weeks, and vice versa. As a general rule the presence of nitrite with *P. tumefaciens* cultures was detected most often with the alcohols or the organic acids.

Daily qualitative tests for nitrate reduction were made upon media containing glycerol as the carbon source, and inoculated with *P. tumefaciens* or *B. radiobacter*. Either phenol red or metacresol purple was added as an indicator. In these trials, both with and without the indicator present, it was found that a crown-gall culture showing the presence of nitrite one day might not necessarily show it the following day. With *B. radiobacter* no nitrite was observed in the daily tests except in one instance where one culture showed a trace of nitrite on the sixth day, but none thereafter. In the majority of cases, with this medium, the nitrate disappeared between the seventeenth and nineteenth days from cultures of *B. radiobacter*. A change in reaction of the *B. radiobacter* cultures as shown by the indicator was first noticeable at nine days. The disappearance of nitrate without the appearance of nitrite is discussed following the quantitative determinations. The cultures incubated at 30°C. showed a more uniform disappearance of the nitrates than at room temperature.

QUANTITATIVE STUDIES ON NITRATE REDUCTION

Without peptone

Quantitative studies of nitrate reduction were also made. Arabinose, glucose, sucrose, and mannitol were used as carbon sources. Preparation of the media and inoculations were made as described earlier. The medium contained approximately 0.7 gram of nitrate nitrogen per liter. The cultures were incubated at two different temperatures, 25° to 27°C. and at 32°C. These two temperatures were used because Riker (1926) found that gall formation in tomato plants followed inoculations with the crown-gall organism at the lower but not at the higher temperature. Only the results obtained at 32°C. are reported as there was no material difference between these results and those obtained at 25° to 27°C. The analysis in certain cases was made in terms of both total nitrogen and nitrate nitrogen. The total nitrogen was determined by the Davisson-Parsons method (1919), and the nitrate nitrogen by the Gunning-Arnold method (1907). The organisms were grown in culture tubes containing 10 cc. of medium, and were introduced into a Kjeldahl flask, along with 90 cc. of distilled water for analysis. The results show (table 4) that nitrate had not completely disappeared in three to six weeks from the *B. radiobacter* cultures, but that much more had been lost than from the *P. tumefaciens* cultures. The crown-gall cultures, perhaps utilized the nitrate only in building bacterial protoplasm. The macroscopic evidence of growth with the two organisms was about the same.

With peptone

Further quantitative studies were made upon the disappearance of nitrate where 0.1 per cent Difco peptone was added to the medium previously used. This contained approximately 6.8 mgm. of nitrate nitrogen and approximately 8.4 mgm. of total nitrogen per 10 cc. *P. rhizogenes* was used along with the other two organisms. Only the single temperature of 32°C. was used.

In the presence of peptone, the nitrate was reduced more rapidly by *B. radiobacter* than where potassium nitrate was the

only source of nitrogen. Nitrates disappeared (table 5) from the nitrate-peptone medium after six weeks incubation. A comparison of the total nitrogen lost from the tubes at six weeks, with that of the nitrate nitrogen at the start, showed that the lost nitrogen

TABLE 4

*Loss of nitrate nitrogen from cultures of Phytomonas tumefaciens and Bacillus radiobacter, using media containing potassium nitrate and different sources of carbon**

CULTURE	WEEKS	LOSS OF NITRATE NITROGEN FROM 10 CC. WITH			
		Arabinose	Glucose	Sucrose	Mannitol
		mgm.	mgm.	mgm.	mgm.
<i>P. tumefaciens</i>	3	0.54	0.16		0.09
<i>P. tumefaciens</i>	6	0.62	0.30	0.53	0.89
<i>B. radiobacter</i>	3	0.63	0.73		1.03
<i>B. radiobacter</i>	6	3.03	3.60	2.74	1.79

* The composition of the media is given in the text.

TABLE 5

*Loss of nitrogen from cultures of Phytomonas tumefaciens, Phytomonas rhizogenes, and Bacillus radiobacter in media containing different sources of carbon, and with potassium nitrate and peptone present**

CULTURE	LOSS OF NITROGEN FROM 10 CC. WITH							
	Arabinose		Glucose		Sucrose		Mannitol	
	Nitrate	Total	Nitrate	Total	Nitrate	Total	Nitrate	Total
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
<i>P. tumefaciens</i>	0.20	0.30	0.72	0.05	0.62	0.10	0.06	0.15
<i>P. rhizogenes</i>	0.00	0.07	0.00	0.02	0.19	0.00	0.00	0.12
<i>B. radiobacter</i>	6.85	6.89	6.85	6.74	6.85	6.64	6.75	6.69

* Composition of the media is given in the text. Analysis was made after forty-two days incubation at 32°C. Nitrate was determined by Gunning-Arnold method, and total nitrogen was determined by the Davisson-Parsons method.

is approximately the same as that contained in the nitrate. A loss of nitrate with *P. rhizogenes* was shown in one instance (table 5), but is hardly beyond the limits of experimental error, so that one cannot consider that *P. rhizogenes* reduced nitrate. With *P. tumefaciens* there was some nitrate nitrogen loss, but the total

nitrogen loss in all cases but one, where arabinose was used as the carbohydrate, was very small. This indicates that the nitrate was not reduced to any extent beyond the point where nitrogen was utilized by the microorganism for bacterial protoplasm.

Qualitative tests showing nitrate present and nitrate absent in short time incubation periods cannot be taken as conclusive evidence that no nitrate reduction has occurred. The organism may reduce only a few molecules of the nitrate at any one time, but reduce them completely, in which case a qualitative test for nitrite would be of no value. However, when quantitative analysis was used as a measure of the loss of nitrate nitrogen and the loss of total nitrogen was obtained, such analyses showed that considerable nitrate nitrogen had disappeared with the cultures of *B. radiobacter*. If the nitrate was reduced to free nitrogen, it would have been lost from the solution rapidly. If the reduction proceeded to ammonia, this might have been lost as a gas from cultures which became alkaline, as with *B. radiobacter* in certain media.

TIME-POTENTIAL MEASUREMENTS

In view of the fact that in certain instances *B. radiobacter* reduced nitrates completely, which was not the case with *P. tumefaciens*, it was desirable to see if there was any difference between the oxidation-reduction potentials induced by the different organisms. This electrometric determination of the oxidation-reduction potentials of the systems studied was made with the use of the standard calomel half-cell and a vacuum-tube potentiometer as described by Allyn and Baldwin (1932). The culture tubes used were constructed with an enlarged end, in order to prevent the electrodes contacting the walls of the tube. These tubes were blown from eight-inch test tubes.

The electrodes used were made from platinum wire of No. 32 B. and S. gauge. Two electrodes, together with a saturated potassium-chloride agar bridge, were used per tube, and were sterilized by autoclaving. The two electrodes, together with a tube for the potassium-chloride agar bridge, and a larger glass tube drawn to a fine bore, through which the inoculum was added, were held

together by wrapping with cotton which served as the cotton plug. These were suspended in the large test tube with the lower end of the tube for the potassium-chloride agar bridge placed in a smaller tube of potassium-chloride agar, while the lower ends of the electrodes were immersed in water between the walls of the two tubes.

The media used were (1) the glucose nitrate medium, (2) the glucose nitrate peptone medium used in the study of nitrate reduction, and (3) the glucose potato-extract medium used in the study of utilization of carbon sources. Fifty cubic centimeters of the medium were placed in the large test tubes, autoclaved for one hour at 15 pounds steam pressure and cooled at room temperature. Then the potassium-chloride agar bridge and electrodes were transferred to the tubes of culture medium.

Potentiometer readings were taken on the sterile media as soon as possible, usually at about one to one-and-one-half hours after the material was removed from the autoclave. Inoculations were made with 1 cc. of a suspension of the organism, standardized to a turbidity as previously mentioned. A control tube open to the air was used in addition to a control sealed from the air by means of a 3-inch vaseline plug. The cultures were incubated at approximately 25°C. Potentiometer readings were taken at quite frequent intervals for the first two days, but later were made two or three times per day.

The organisms commonly lowered the oxidation-reduction potential during their growth, as manifested by the potential-time measurements obtained (figs. 1, 2, and 3). The potential in the glucose nitrate medium was lowered very rapidly by *B. radiobacter*, as shown in figure 1. The medium, however, became more oxidized again. Two such trends were observed at about the eighth and sixteenth day, after which there was a gradual decrease in the potential to about the twenty-fifth day, to a value which was then maintained for approximately two weeks. In one instance with the glucose nitrate medium, it was noted that the potential again dropped suddenly after the fortieth day to $E_h - 0.214$, where it was stabilized. These observations correlate with the nitrate reduction data where it was noted that in many

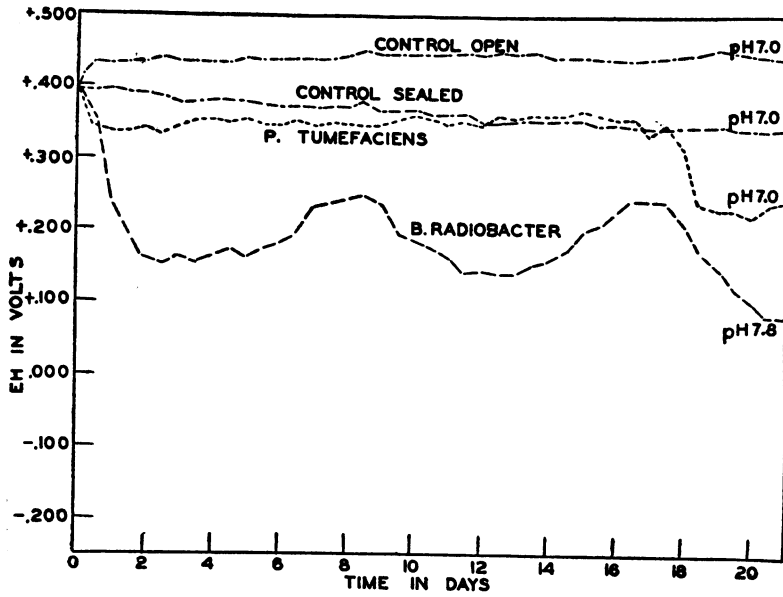


FIG. 1. POTENTIAL-TIME CURVES OF GLUCOSE NITRATE BASAL-SALT MEDIUM INOCULATED WITH PHYTOMONAS TUMEFACIENS AND BACILLUS RADIOBACTER

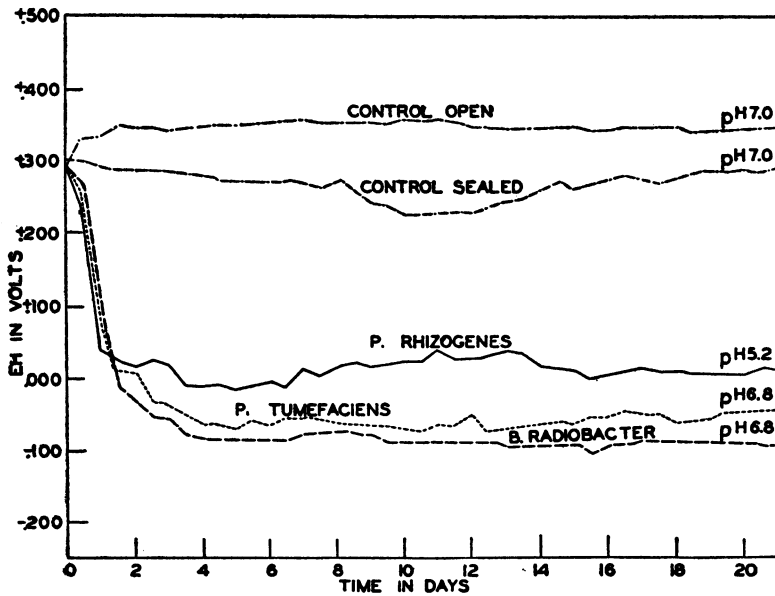


FIG. 2. POTENTIAL-TIME CURVES OF GLUCOSE PEPTONE BASAL-SALT MEDIUM INOCULATED WITH PHYTOMONAS TUMEFACIENS, P. RHIZOGENES AND BACILLUS RADIOBACTER

cases the nitrates disappeared at about the seventeenth to nineteenth day while in other cases this required a longer period. The reaction at twenty-one days was pH 7.8, and at forty-two days it was pH 8.8. The control tube was at pH 7.0. The potential was not lowered to any extent by *P. tumefaciens* in the same medium, figure 1. There occurred a period of a few days in which the oxidation-reduction potential was somewhat reduced, but as a

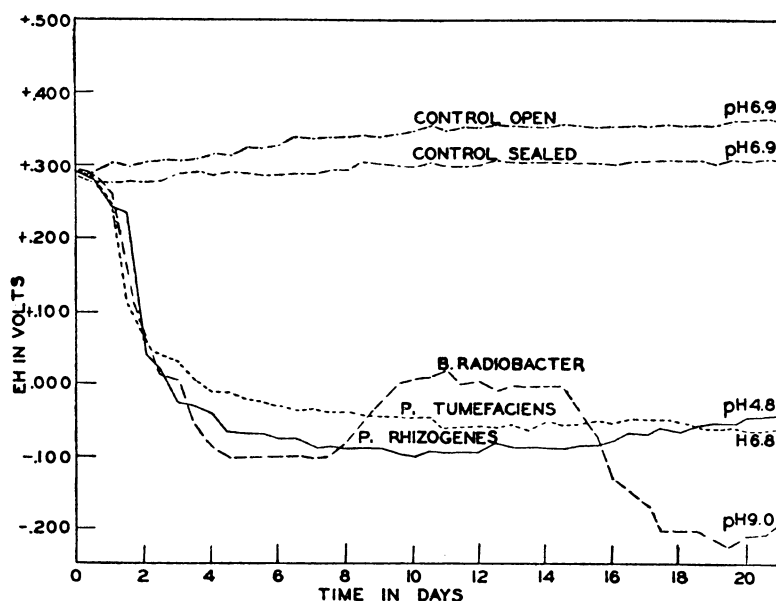


FIG. 3. POTENTIAL-TIME CURVES OF GLUCOSE PEPTONE NITRATE BASAL MEDIUM INOCULATED WITH PHYTOMONAS TUMEFACIENS, P. RHIZOGENES AND BACILLUS RADIOBACTER

general rule the potential was not much lower than with the uninoculated and sealed tube. On the forty-fourth day the potential dropped to Eh +0.228, which is approximately the same potential reached on the eighteenth day, but which had been maintained only for a few days. The pH was at 7.0.

With the glucose peptone medium the lowest potential was again produced by *B. radiobacter*. It may be noted, figure 2, that *P. tumefaciens* and *P. rhizogenes* although not producing

quite as low a potential as *B. radiobacter*, held the potential rather uniformly parallel to that of *B. radiobacter*.

A decided drop in potential was produced by each of three organisms in the glucose nitrate medium to which 0.2 per cent peptone had been added (fig. 3), with *B. radiobacter* producing the lowest potential. After a few more days the potential produced by *B. radiobacter* was higher than before and again after another week dropped suddenly to Eh -0.200 . This potential was not reached in the nitrate medium until the fortieth day. Qualitative tests for nitrates, made on the thirtieth day, showed nitrates to be present with the crown-gall and hairy-root cultures, but absent with the *B. radiobacter* cultures. This again helps to explain the findings in the quantitative nitrate reduction studies where it was shown, tables 4 and 5, that the addition of peptone markedly increased the rate of nitrate reduction. In this medium *P. tumefaciens* and *P. rhizogenes* did not produce as low a potential as *B. radiobacter*. *P. rhizogenes* produced a slightly lower potential than the crown-gall organism for about a two-week period, after which it became less reduced.

The potential in the glucose potato extract medium was lowered to about the same extent by all three organisms. It was noted, however (fig. 4) that the potential of the controls, both open and closed, was much lower at the start of the experiment than with the other media used. The potential of the open tube decreased for about one day; after which it increased gradually for about ten days. The closed control, however, showed a rather rapid drop in potential on the first day which was about the same as for the open control. The potential then decreased slowly to about the sixth day; after which it seemed to be stabilized at about an Eh 0.0. All of the cultures grew more quickly in the glucose potato-extract medium than in any of the other media. This was especially noticeable in connection with *P. rhizogenes*.

If one compares the potential of the controls in the various media, it is noted that there is no great difference between the medium exposed to the air and the same medium sealed from the air, except in the case of the glucose potato-extract medium. In this instance, as noted previously, the potentials were about the same for the first day, after which they became more separated.

DISCUSSION

The results of work on the utilization of nitrogen from different sources showed that considerable difference existed between *P. rhizogenes* on the one hand, and *P. tumefaciens* and *B. radiobacter* on the other. This was more marked in the basal-salt media with various carbohydrates, where an inorganic source was supplied as nitrate nitrogen. Under the conditions used in this study,

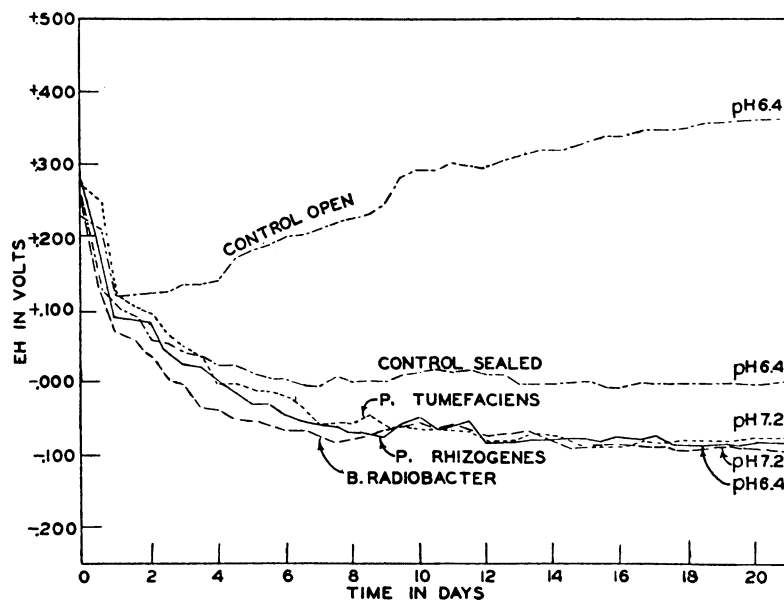


FIG. 4. POTENTIAL-TIME CURVES OF GLUCOSE POTATO-EXTRACT MEDIUM INOCULATED WITH PHYTOMONAS TUMEFACIENS, *P. RHIZOGENES* AND *BACILLUS RADIOBACTER*

P. rhizogenes was much more limited in its utilization of nitrogenous compounds than was either *P. tumefaciens* or *B. radiobacter*.

P. rhizogenes was unable to utilize to any extent the inorganic sources of nitrogen. The inability of *P. rhizogenes* to produce growth in a nitrate medium might come, not alone from the inability to utilize nitrates, but from other factors among which should be mentioned (1) an unfavorable oxidation-reduction potential, and (2) the lack of growth-stimulating substances.

Work by Allyn and Baldwin (1932), Dubos (1929), Webster (1925), Wright (1929), and others, in which it has been shown that organisms have an optimum oxidation-reduction potential for growth, indicates that the somewhat similar results obtained in this study may be concerned with the ability of the organism to utilize nitrate nitrogen. The failure of *P. rhizogenes* to utilize nitrate nitrogen in a 0.5 per cent nitrate medium, and its ability to utilize nitrate nitrogen to a slight extent where a reducing agent was added, gives further strength to this suggestion. In none of our studies with the use of a reducing agent has good growth been obtained with *P. rhizogenes* where nitrate nitrogen has been used as the only source of nitrogen.

The fact that *P. rhizogenes* produced its best growth in a glucose, potato-extract medium may result from the lower oxidation-reduction potential of the medium at the beginning. It may, on the other hand, be explained by the presence of some growth-stimulating substance. Other workers have found that the addition of very small amounts of certain complex substances to the medium enabled certain microorganisms to utilize otherwise unavailable nitrogen sources. This likewise may be the reason for the good growth produced by the organisms in media containing peptone.

In the study of the utilization of nitrogen sources as shown by growth, *P. tumefaciens* and *B. radiobacter* each utilized nitrogen-containing compounds to about the same extent. The difference in nitrate reduction is worthy of further consideration in relation to the pathogenicity and nonpathogenicity respectively of the two organisms.

When the various sugars, glucosides, and alcohols were used as a carbon source, *B. radiobacter* produced an alkaline reaction in all cases where it made satisfactory growth except with propyl alcohol. The alkaline reaction was undoubtedly because of the fact that potassium nitrate was reduced and the basic ions remained in excess. With butyl alcohol, only a trace of growth resulted and no change in reaction was produced. When propyl alcohol was employed *B. radiobacter* and *P. tumefaciens* both produced good growth and an acid reaction. This alcohol may be oxidized

to an acid rapidly enough to overcome the alkalinity resulting from nitrate reduction. The utilization of ethyl alcohol with an acid reaction by *P. tumefaciens* and an alkaline reaction by *B. radiobacter* is noteworthy.

The basic reaction resulting from the utilization of the salts of the organic acids may have been in part because of the more or less complete utilization of the acid radical with the basic ions remaining. An acid reaction resulted in most instances where carbohydrates were utilized by *P. rhizogenes*, while in the same instances very little if any acid was formed by *P. tumefaciens*.

The reduction of nitrate by the various organisms, where nitrate was used in the media as a nitrogen source, is in part correlated with the potentials obtained. *B. radiobacter* induced the lowest potential. Likewise *B. radiobacter* reduced nitrates to the greater extent. The time of nitrate reduction by *B. radiobacter* also corresponded closely to the periods of rapid drop in potential.

SUMMARY

A comparison has been made of certain physiological characters of *P. tumefaciens*, *P. rhizogenes*, and *B. radiobacter* with emphasis on their nitrogen and carbon metabolism.

In these studies cultures obtained from both colony isolation and single-cell origin were employed.

Organic and inorganic compounds containing nitrogen were utilized, as shown by growth, as sources of nitrogen by *P. tumefaciens* and *B. radiobacter*. Amino acids were utilized to about the same extent by *P. tumefaciens* and *B. radiobacter*. Amino acids singly or in a mixture, and inorganic sources of nitrogen were utilized little if at all by *P. rhizogenes*. Nitrate nitrogen was utilized by *P. rhizogenes* to a very limited extent where a reducing agent was employed.

Nitrates were reduced to a very small extent by *P. tumefaciens*, but completely by *B. radiobacter* when certain carbon sources were employed. They were reduced more quickly by *B. radiobacter* in a medium containing both nitrate and peptone, than in a medium containing nitrate but no organic nitrogen compounds.

Glucose potato-extract was one of the most favorable media for the growth of *P. rhizogenes*.

Where various carbohydrates, glucosides, alcohols, and organic acids or their salts were utilized by *P. tumefaciens* and *B. radiobacter* they were used to about the same extent as indicated by growth, but with some differences in final reaction.

All three types of organisms when grown in a potato-extract glucose medium reduced the potential of the medium to about the same extent, whereas when grown in a glucose nitrate medium, in a glucose nitrate peptone medium, and a glucose peptone medium, they showed considerable difference with respect to the potentials induced.

Oxidation-reduction potentials determined over a period of time help to account for quicker reduction of nitrate when peptone was added to the medium. Literature will be cited at the end of Part II.