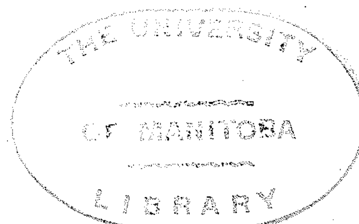
# SOME ASPECTS OF TRANSFORMATIONS

# IN

# THE GENUS AGROBACTERIUM, CONN.

by

Norman E. R. Campbell

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## ABSTRACT

Transformations of virulence between certain members of the genus *Agrobacterium*, Conn were confirmed. The acquisition of virulence by the previously avirulent species did not result in any appreciable changes in cultural or nutritional behaviour in the transformed cells as determined by the methods employed.

The acquisition by transformation of the capacity to utilize nitrate is reported as occurring between two species of this genus. The transformation was concerned uniquely with the capacity to produce the enzyme nitrate reductase; subsequent steps in the assimilatory pathway of the acceptor strain were unaltered by the transformation.

Marked differences in assimilatory response to inorganic nitrogen sources are reported for the strains of *Agrobacterium rubi* and *Agrobacterium radiobacter* used in this investigation. The differences suggest that the strains concerned do not possess the same assimilatory inorganic nitrogen pathways.

## ACKNOWLEDGEMENTS

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The acquisition by transformation of the capacity to utilize nitrate is reported as occurring between two species of this genus. The transformation was concerned uniquely with the capacity to produce the enzyme nitrate reductase; subsequent steps in the assimilatory pathway of the acceptor strain were unaltered by the transformation.

Marked differences in assimilatory response to inorganic nitrogen sources are reported for the strains of *Agrobacterium rubi* and *Agrobacterium radiobacter* used in this investigation. The differences suggest that the strains concerned do not possess the same assimilatory inorganic nitrogen pathways.

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# HISTORICAL

# HISTORICAL

The transformation reaction has been defined by Austrian (1952) as "a hereditary alteration in a susceptible cell resulting from the acquisition from its environment, by other than sexual means, of a genetically active unit directing the inheritable change". As well as conforming to such a definition, the genetically active material should be demonstrable in the progeny of the transformed cell and recoverable from such populations in amounts greater than that required to induce the initial alteration.

Although the phenomenon of bacterial transformations was observed as early as 1928 by Griffith and somewhat later by Dawson and Warbasse (1931), Dawson and Sia (1931) and Alloway (1932) (1933), the chemical nature of the transforming substance was not identified until Avery *et al.* (1944) successfully performed transformations among certain pneumococci using desoxyribonucleic acid fractions. Their experimentation clearly demonstrated the transformation of 'rough' unencapsulated Type III pneumococci to smooth fully encapsulated Type III strains by treatment of a rough strain with DNA obtained from a smooth strain. Their findings indicated that at least one genetic factor was introduced from one strain to another through the agency of the medium, and moreover, this factor or genetic character as it may be called

remained as an integral part of the genetic apparatus in the altered strains during subsequent generations.

Because of the broad biological implications of transformation reactions interest in the precise chemical nature of the transforming substance stimulated extensive research. Experiments by McCarty (1946) and McCarty and Avery (1946) using desoxyribonuclease strongly supported the concept that the active substance was a DNA complex. While this concept was more or less widely accepted by other investigators, Boiven (1947), Taylor (1949) and Hotchkiss (1951), objections had been raised by Mirsky (1947) who pointed out that a small amount of protein associated with the transforming substance might have been responsible for its activity. His objections were based largely on the contention that methods then available were inadequate for the detection of small amounts of protein in the extract residues and on the assumption that the proteolytic enzymes used might not be specific for the residual protein. As well, he criticized the use of desoxyribonuclease as a means of identification of the active substance since it might alter the activity of a nucleoprotein complex whose specificity resided in the protein entity.

Subsequent investigations by Alexander and Leidy (1950) and by Zamenhof et al. (1951) have largely invalidated these objections. Precise separation using electrophoretic methods have produced transforming material active at

concentrations as low as 0.01 μg. while containing less than 1.0% pentose nucleic acid and 0.2% serologically active polysaccharide. As well, investigations by Hotchkiss (1952) have indicated that the only amino acid detectable upon hydrolysis of the transforming substance is glycine; and further that the glycine concentration was at a level to be expected from the breakdown of the adenine portion of DNA.

Additional evidence that transforming principle was a protein-free DNA complex was provided by Hotchkiss (1952) and by Zamenhof _et al._ (1951). These investigators compared transforming principle and calf thymus DNA on the bases of viscosity, stability, composition and electron microscopy. Their findings suggest a marked similarity in molecular structure of the two entities. In his investigation Hotchkiss (1952) prepared an active transforming principle from Type III pneumococci which contained less than 0.02% protein.

Since 1944 there have been a number of reports, McCarty and Avery (1946), MacLeod and Krauss (1947) and Taylor (1949), concerned with transformations of capsular development in pneumococci. Capsular changes reported therein included both quantitative and qualitative variations. As well, capsular antigens of _Escherichia coli_, _Hemophilus influenzae_ and meningococcus have been transformed as reported by Boivin _et al._ (1945), Alexander and Leidy (1951) and Alexander and Redman (1953).

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Beginning in 1948, work was initiated on transformations involving highly selective properties, i.e. drug resistance. Hotchkiss and Marmur (1954) and Hotchkiss (1955) for example reported transformations involving resistance to several drugs including penicillin, streptomycin and sulfanilamide. The techniques developed in these investigations, particularly those concerned with methods of screening mixed populations by use of drug concentrations lethal to the non-transformed cell, permitted a more accurate determination quantitatively of the rate, frequency and extent of transformations.

In general, the greatest emphasis on transformation experiments has been directed to studies of surface properties of cells; with some interest developing in drug resistance transformations during the latter years. The taxonomic area investigated thus far has been largely that of the pneumococci and other pathogens although occasional papers have dealt with species of non-medical interest.

Interest in serological identification of plant pathogenic bacteria begun by Riker _et al_. (1930) was continued by Coleman and Reid (1945) who undertook a serological study of strains of _Alcaligenes radiobacter_ and _Phytomonas tumefaciens_. Again, Coleman and Reid (1949) investigated the conversion of strains of _Alcaligenes radiobacter_ and _Phytomonas tumefaciens_ in the "S" phase to the "R" phase of the heterologous species. Their methods were developed on the bases

of those used by Dawson (1930a) (1930b) and Dawson and Sia (1931). Coleman and Reid converted 'in vitro' Phytomonas tumefaciens to Alcaligenes radiobacter by transferring capsular material from the latter to Phytomonas tumefaciens "S" phase cells.

Klein and Klein (1953) reported transformation reactions involving transmittance of tumor-inducing ability to avirulent crown-gall and related bacteria. In brief, their results indicated that extracts of bacteria-containing crown-gall tumor tissue, a bactrin of virulent crown-gall bacteria, and desoxyribonucleic acid produced by crown-gall bacteria are each capable of transmitting the property of specific host virulence to avirulent strains of Agrobacterium tumefaciens and to Agrobacterium rubi, Agrobacterium radiobacter and Rhizobium leguminosarum. From this study it appeared evident that transformations were to be expected only between closely related species. They were unsuccessful in attempts to transfer tumor inducing capacity from A. tumefaciens to Escherishia coli. All other organisms under consideration in their study were closely related morphologically, serologically and biochemically as outlined by Riker and Berge (1935), Riker et al. (1946) and Stapp (1955). According to Klein and Klein (1953) the acquisition of tumor inducing capacity by a previously avirulent strain does not alter its cultural characteristics; although, the evidence upon which they based this assumption was meager. As well,

they reported that the acquisition of a new tumor inducing capacity by A. rubi, for example, did not entail loss by A. rubi of its activity toward its primary host. This suggests that specific host virulence is controlled in this case at least by multiple genetic factors. A tumor inducing principle present in virulent cells was reported by Klein and Link (1955). This principle only appears in normally avirulent forms after they have undergone transformation with DNA from a virulent strain.

Relatively few publications on transformations have dealt with soil bacteria. Most of these have related to the work of Klein and associates and more recently the report by Corey and Starr (1957) on the genetic transformation of streptomycin resistance in Xanthomonas phaseoli.

Among recent publications on transformations, the reports by Lerman and Tolmach (1957) (1959) have contributed perhaps the most comprehensive addition to our understanding of this phenomenon. Using $P^{32}$ labelled desoxyribonucleic acid in transformation reactions for streptomycin resistance in pneumococci, it was shown that the frequency of transformation is directly proportional to the amount of DNA incorporated. Further, their quantitative studies indicated that only a fraction of the incorporated DNA may be functional in the usual sense. Ribose nucleic acid or severely degraded DNA are not incorporated by the acceptor cell.

# PART I

# VIRULENCE TRANSFORMATIONS

# VIRULENCE TRANSFORMATIONS

## INTRODUCTION

The purpose of Part I of this investigation was to determine whether any marked alterations in cultural or nutritional behaviour were associated with transformation of virulence from virulent to avirulent members of this genus. Klein and Klein (1953) reported that no significant changes of this type occurred in transformed cultures although little experimental evidence was presented to support the statement.

Initially, a survey was made of methods for transformations, in particular with those reported by Klein and his associates. Secondly, cultural and nutritional studies were carried out on parent and transformed cultures to establish if any characters of the donor parent other than virulence accompanied the transformation.

The media and differential tests selected to compare parent and transformed cultures were chosen on the bases of their giving wherever possible purely qualitative answers. The reason for such a selection may be seen by examination of the cultural characterizations of members of this genus as presented in Bergey's Manual of Determinative Bacteriology, 7th ed. A number of these characterizations used in a diagnostic sense, i.e. color response on potato slants, nitrate reduction, etc., are difficult to assess and in many cases

subject to wide variation in interpretation. Others are more definite, i.e. growth responses in media with various carbohydrate or nitrogen sources.

This cultural survey, however, included two media, litmus milk and calcium glycerolphosphate agar, which give results likely to cause difficulty in interpretation. They were included nonetheless since they are considered classical media for differentiating members of the Rhizobiaceae.

## GENERAL CONSIDERATIONS

Reported methods for obtaining transformed cultures are similar in principle. Essentially they consist of the exposure of one culture usually called the acceptor to a cell-free filtrate or to some fraction of it produced by a closely related species referred to as the donor culture. The donor filtrate or appropriate fraction thereof contains biologically active genetic material which effects the transformation. Accordingly, care in handling such material must be exercised to avoid interference with the structural or with the chemical integrity of the genetically active substance.

Avery et al. (1944) and Hotchkiss (1951) reported that age of acceptor culture was an important factor in transformation reactions. They suggested that the acceptor should be exposed to transforming substance only during its logarithmic growth phase when apparently it is most receptive to

the introduction of genetic material. Hotchkiss (1954) found that transformations were aided by subjecting the acceptor culture to cold shock for a short period of time. On return to favourable incubation temperatures such cultures divided synchronously and the likelihood for success in transformations increased.

Preliminary experiments established several points. First, extensive slime formation common to some of the organisms used might interfere with transformations by acting as a mechanical barrier to transforming substance. Accordingly, carbohydrate concentrations in media were held to a level that yielded good growth but discouraged slime formation. Second, some of the methods particularly one described by Klein and Klein (1953) proved to be unreliable. This method involved the use of a U-shaped culture tube whose vertical chambers were separated by a U.F. sintered glass filter. Cross-contamination was found to occur when this type of culture chamber was used.

The selection of suitable cultures was considered carefully. Some acceptor cultures, notably the Löhnis strain of _A. radiobacter_, were exposed repeatedly to extracts from proven virulent donor cultures without success. In this respect the concept of competence reported by Austrian (1952) seems to apply. Only the following cultures gave reasonable uniform results in transformation while possessing

a variety of different nutritional characteristics that could be investigated subsequently.

Donor Cultures--A. tumefaciens P61[i] and A. rubi P60[i]

Acceptor Cultures--A. radiobacter 590[i] and A. radiobacter 1012[ii].

THE TRANSFORMATION REACTION AND DETECTION OF VIRULENCE

METHODS

Methods followed to prepare transformed cultures were generally similar to those reported by Klein and Klein (1953) and by Lerman (1955). Four somewhat different methods were tried. In the first instance, cultures of donors A. tumefaciens P 61 and A. rubi P60 grown in mannitol yeast extract medium for 24 hours were filtered twice in series through Pyrex U.F. sintered glass filters. Filtrates were checked for sterility by culturing in mannitol medium at 25°C. for 96 hours. Ten ml. of the cell-free filtrate were added to young synchronous cultures of A. radiobacter. Incubation was at 25°C. for 48 hours. Following incubation, cultures were centrifuged at 5000 r.p.m., washed twice with physiological saline (0.85% NaCl) and inoculated directly to host plants or carrot tissue for detection of virulence.

To increase the concentration of transforming material available to the acceptor cell, a second method was

[i] Courtesy of the Institute of Microbiology, Science Service, Department of Agriculture, Ottawa.

[ii] Courtesy of Mr. F.D. Cook, Science Service Laboratory, Swift Current, Saskatchewan.

developed. Donor cell filtrates (500 ml.) were collected and lypholyzed partially. After the filtrate volume was reduced to approximately 25 ml., the concentrate was warmed to room temperature and added to young synchronous 25 ml. cultures of _A. radiobacter_.

Third, 48 hour cultures of the donor species were exposed to sonic disintegration using a Mullard ultra sonic generator at 20 kc. for 15 minutes. During treatment the cell container was held in an ice bath to minimize heating effects. The resultant cultural debris was filtered using two Pyrex U.F. sintered glass filters in series to remove whole cells remaining. Filtrates thus prepared were added to young synchronous 25 ml. cultures of _A. radiobacter_.

A fourth method involved the use of DNA fractions derived from donor cultures. The method followed for extracting DNA was essentially that described by Lerman (1955) with the exception that lauryl sulfate (10% aqueous) rather than sodium desoxycholate was used as the lysing solution. After deproteinizing by repeated exposures to octanol-chloroform (10% v/v), the cell extract was treated with ribonuclease. RNA hydrolysis products were removed with Norite A by passing the cell extract through sintered glass filters. The filtrate was examined spectrophotometrically for absorption at 268 mμ.

Cultures prepared for determination of virulence were centrifuged, washed three times with sterile water and the final centrifugate resuspended in 5.0 ml. sterile water. This was used as the inoculum on carrot discs according to the method of Klein and Tennebaum (1955).

Cambium-adjacent secondary phloem removed aseptically from the carrot was transferred to sterile Petri dishes containing a moistened 9.0 cm. filter disc. All inoculations were in quadruplicate. During prolonged incubation, sterile water was added as necessary to ensure a moist atmosphere and to avoid desiccation of the carrot tissue. Discs were examined at weekly intervals for one month. Failure to develop tumor tissue by this time was taken as evidence of non-virulence of the culture.

Hotchkiss (1956) and Zamenhof (1956) have reported that the frequency of transformation was less than 0.1%. If this estimate is accepted, the washed centrifugate forming the inoculum would contain relatively few cells likely to cause tumor development. A series of three consecutive screenings was carried accordingly, in an attempt to isolate only cells with tumor inducing capacity.

In the first screening, tumors were excised at the end of three weeks. The upper half of the extruded tissue was removed aseptically and transferred directly to a sterile Petri dish. A series of six washings in sterile water followed and the tumor material was finally transferred to a second

sterile Petri dish. The washed tumor fragment was macerated thoroughly with a sterile glass rod and the resultant debris suspended in 1.0 ml. sterile water. This suspension was transferred by loop to freshly cut carrot discs for the second screening.

Again, following incubation at 25°C. for 14 days the second carrot series was examined and tumors removed. The washed and macerated tumor fragments, in this instance, formed the inoculum for loop-dilution-plating in mannitol yeast extract agar. After incubation at 25°C. for 48 hours, discrete well-isolated colonies were picked and transferred to mannitol yeast extract broth and to agar slants of the same medium. These isolates were transferred individually to fresh carrot discs for final assessment of virulence.

Detection of virulence of parent and transformed cultures by seedling inoculation was carried out with sunflowers and tomato plants as hosts.

The preparation of cultures for inoculation was essentially the same as in the case of carrot discs with one exception. Washed suspensions of parent and transformed cells were collected by a final centrifugation and the thick cell mass was used as inoculum.

Host plant inoculations were made as uniformly as possible at a point just above the first true leaf emergence. An initial puncture was made by sterile needle. This was followed by introducing visible amounts of inoculum by

needle into the wound. Care was taken to avoid deep penetration which might result in gross damage to the seedling's vascular system.

Seedlings thus inoculated were grown under controlled conditions of light and moisture for a period of six weeks. Failure to develop a tumor response by this time was accepted as a negative result.

Only those isolates which gave significant tumor response plus the parent cultures from which they were derived were selected for further study.

# RESULTS

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When evaluated by the carrot disc technique, only two of the four transformation methods gave reasonably consistent results; i.e. the cell-free filtrate method and secondly, the procedure employing DNA extracts from virulent donor cells. With respect to the latter method, a spectrophotometric comparison of donor cell DNA with purified calf thymus DNA is presented in Fig. I.

A summary of carrot tissue responses to the parent and transformed cultures is presented in Table 1.

Where evident, tumors were of approximately the same size and general appearance; attaining a diameter of from one to two millimeters within three weeks. Prolonged or repeated exposure to light resulted in change of color from creamy white to varying shades of green suggestive of chlorophyll pigmentation. The number of tumor structures per disc varied widely, being greatest in the case of virulent parent inoculations. Virulent transformed cultures produced from one to three tumors per disc in the first screening series. Photographic evidence is presented in Plates I, II, III, and IV. The number of tumors per disc increased at least four-fold in the second series.

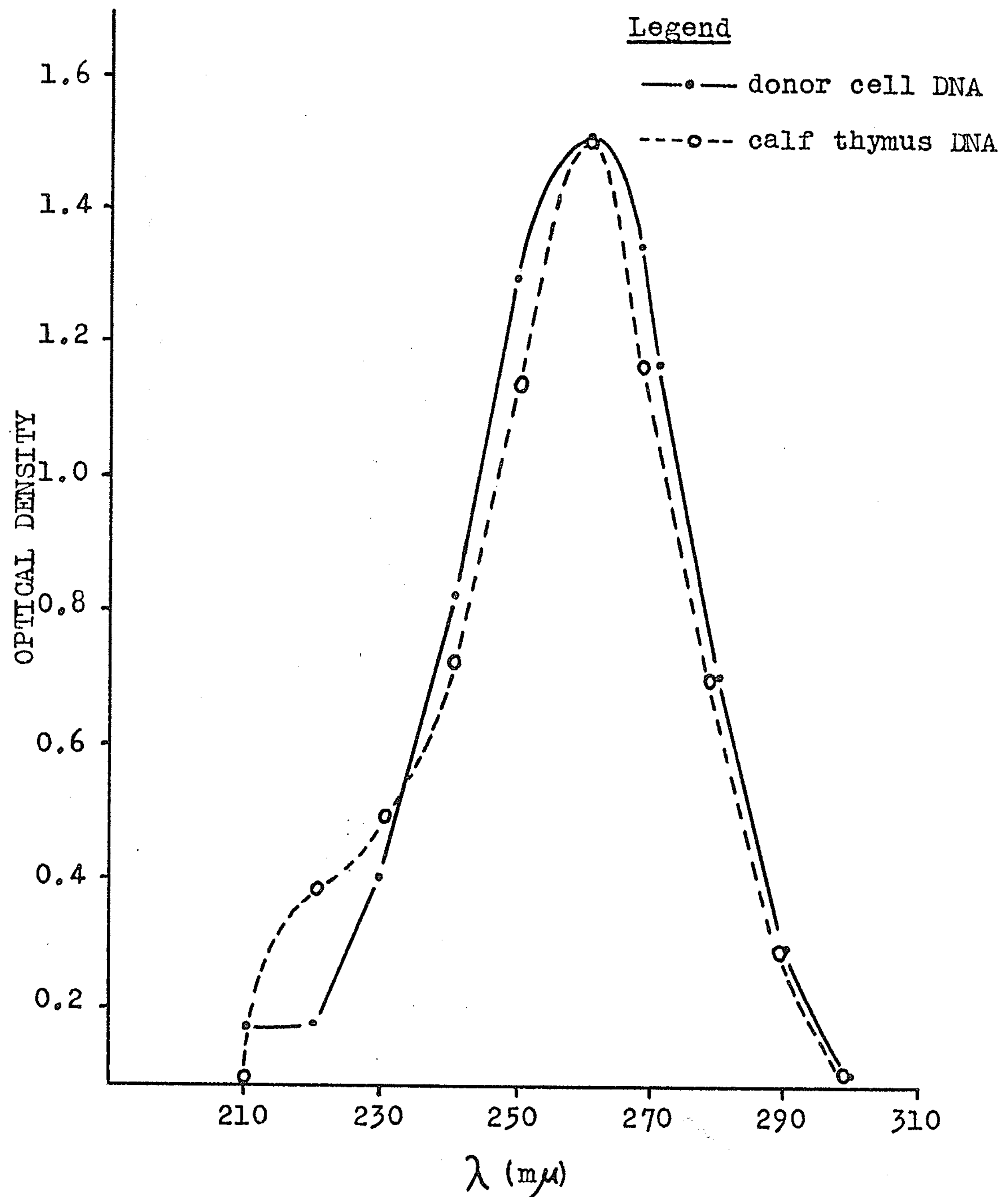


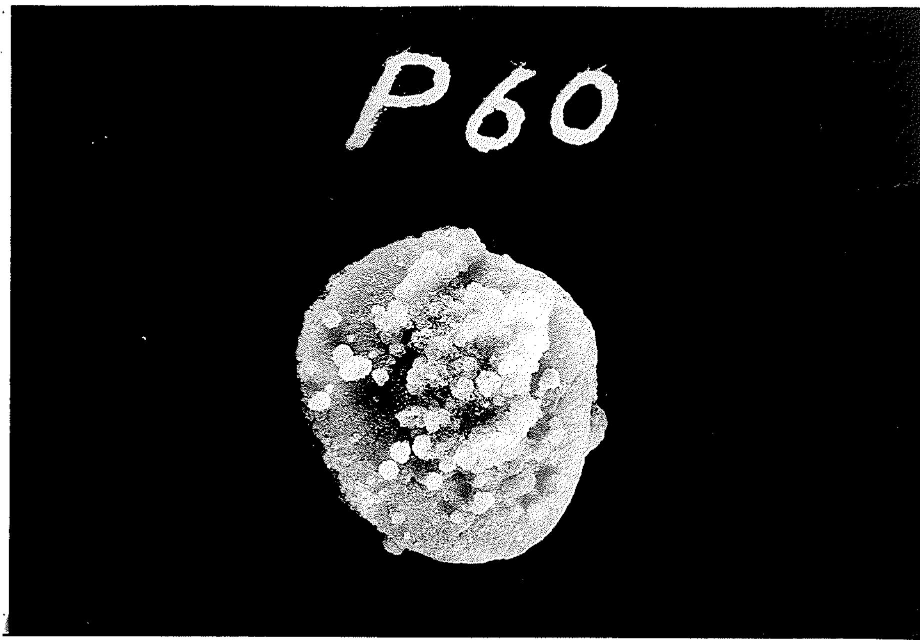

Fig. 1. Comparison of optical densities of donor cell DNA extract with purified calf thymus DNA.

Table 1. Carrot tissue response to parent and transformed cultures

| Treatments | Positive tumor responses per 20 carrot discs by | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *A. tumefaciens* B61 | *A. rubi* r60 | *A. radiobacter* 1012 | *A. radiobacter* 590 | 1012 (r60) | 1012 (r61) | 590 (r60) | 590 (r61) |
| Parent Controls | 20 | 20 | 0 | 0 | | | | |
| Cell-free filtrate | | | | | 9 | 13 | 11 | 10 |
| IMA extract | | | | | 4 | | | 6 |

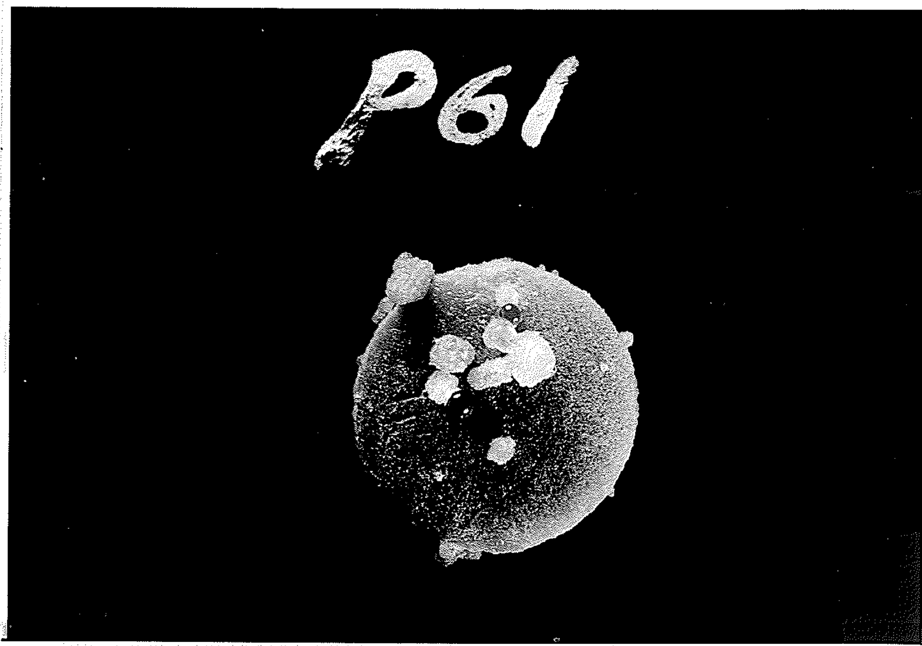
Plate I

Illustration 1.




Heavy tumor response by parent A. *rubi* P60 at three weeks.

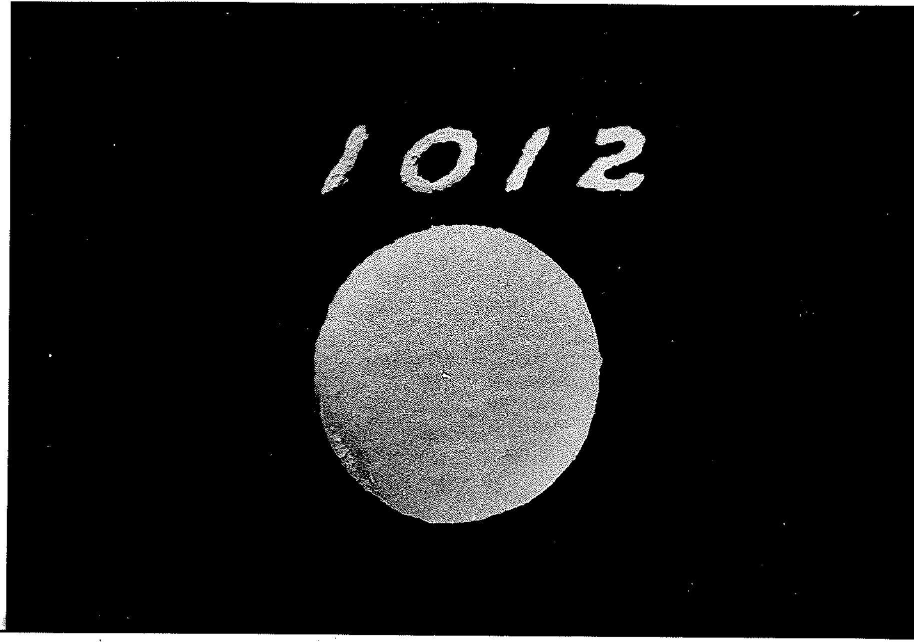
Illustration 2.




Moderate tumor response by parent A. *tumefaciens* P61 at three weeks.

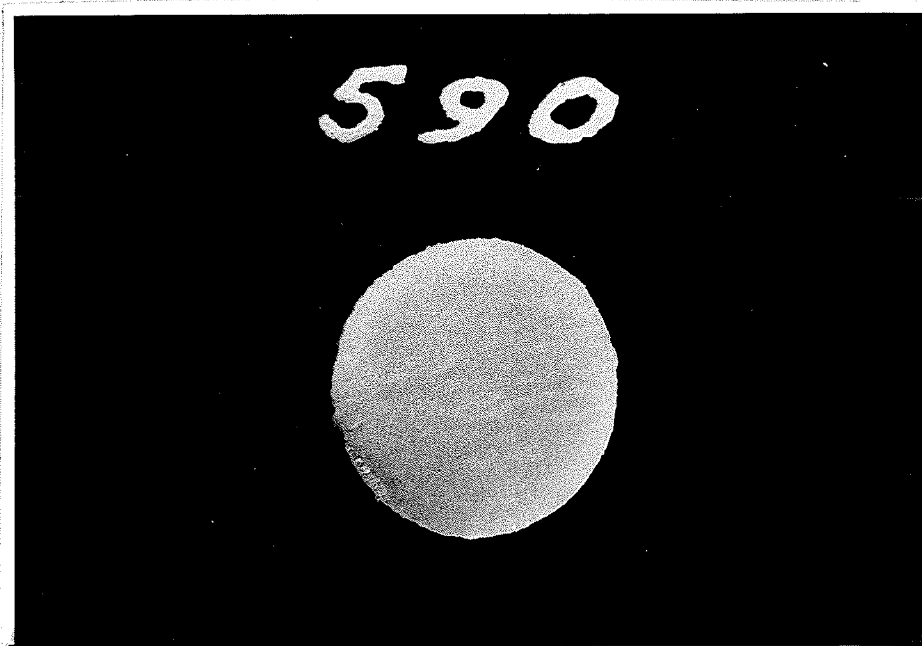
Plate II

Illustration 1.




No tumor response by parent A. <u>radiobacter</u> 1012 at three weeks.

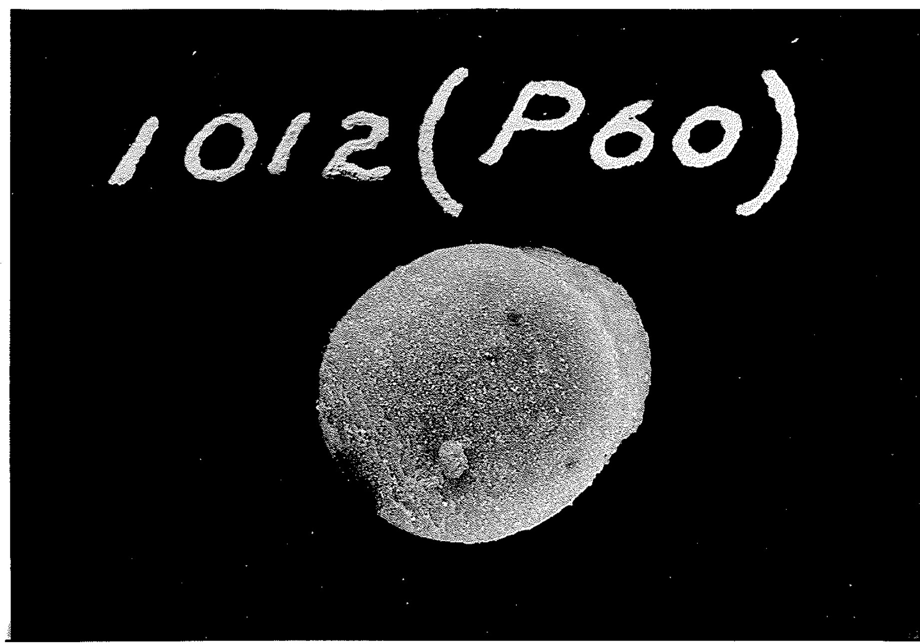
Illustration 2.




No tumor response by parent A. <u>radiobacter</u> 590 at three weeks.

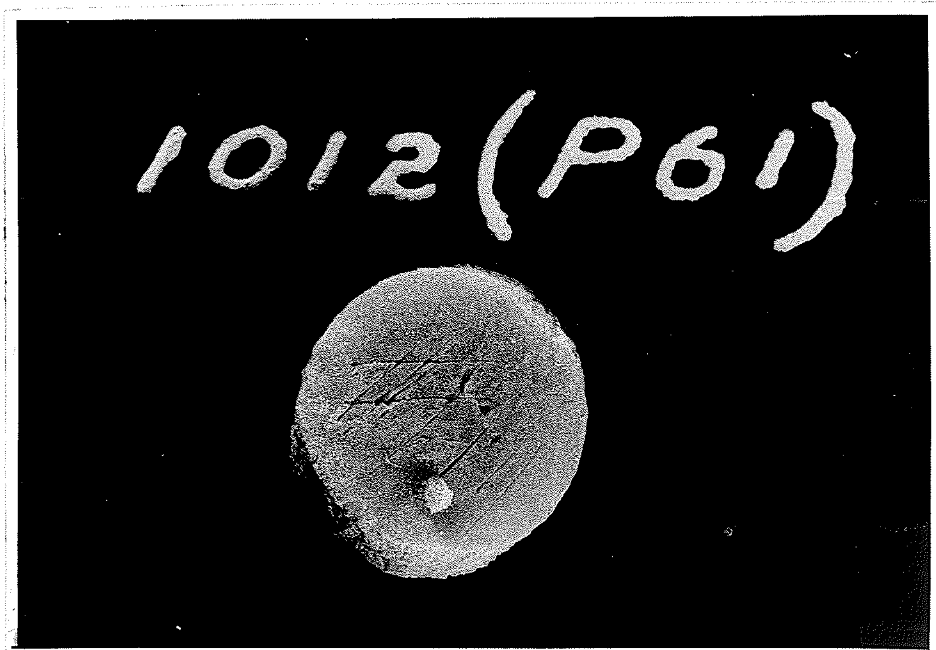
Plate III

Illustration 1.




Single well developed tumor by A. radiobacter 1012 (P60), first carrot series at three weeks.

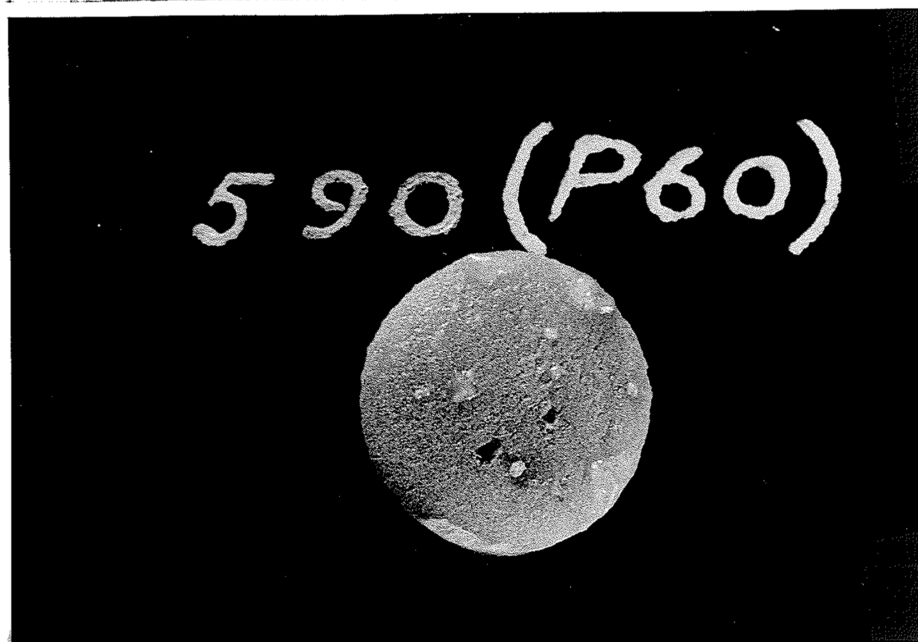
Illustration 2.




Single well developed tumor by A. radiobacter 1012 (P61), first carrot series at three weeks.

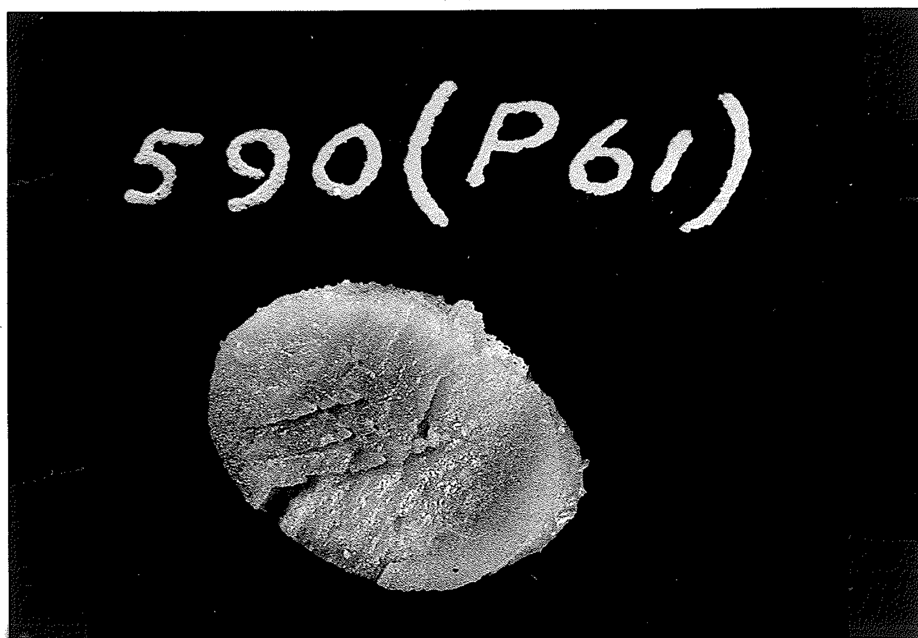
Plate IV

Illustration 1.




Small tumors by A. radiobacter 590 (P60), first carrot series at three weeks.

Illustration 2.



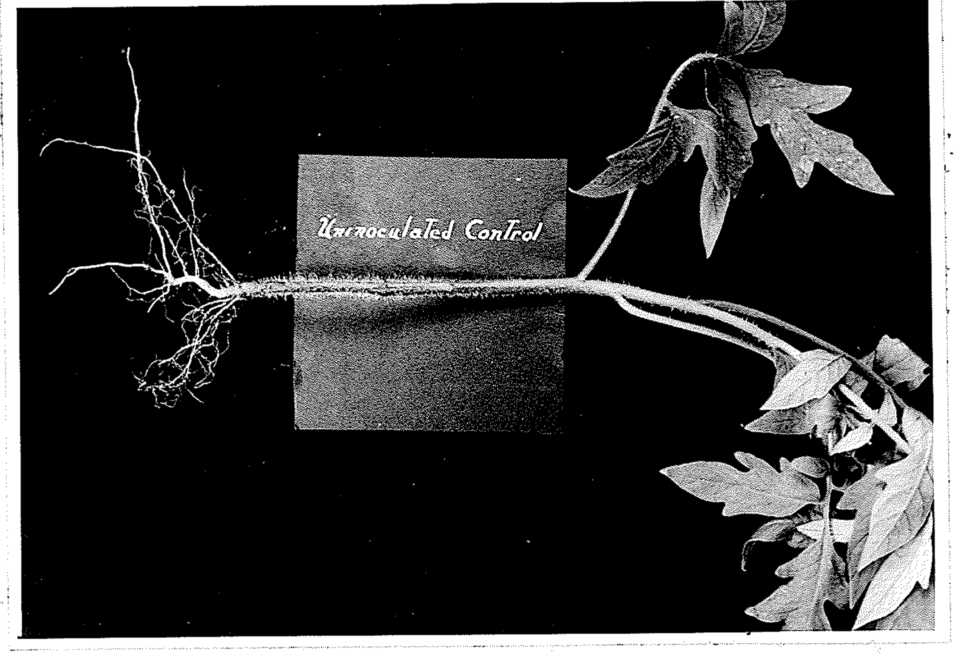

Spreading tumor growth on side of carrot disc by A. radiobacter 590 (P61), first carrot series at three weeks.

The inoculation of growing seedlings as a means of virulence detection produced varied results. The virulent parent A. tumefaciens P61 and transformed cultures produced from it as the donor species gave reasonably good results in tomato seedlings. Photographic evidence in this respect is presented in Plates V, VI, VII, VIII, IX, X and XI.

No tumor response in seedlings by A. rubi P60 parent or transformed cultures was detected despite repeated trials. This species is reported in Bergey's Manual of Determinative Bacteriology, 7th ed., to be pathogenic only to members of the genus Rubus and closely related plants.

In the interests of brevity, particularly in presentation of tabular results, a form of culture notation was followed. Parent cultures A. rubi, A. tumefaciens and the two A. radiobacter strains are designated as P60, P61, 1012 and 590 respectively. Transformed isolates are designated by a double number; the first number designates the acceptor culture and the second number in parenthesis denotes the donor parent, i.e. 1012 (P61) represents A. radiobacter 1012 transformed by A. tumefaciens P61.

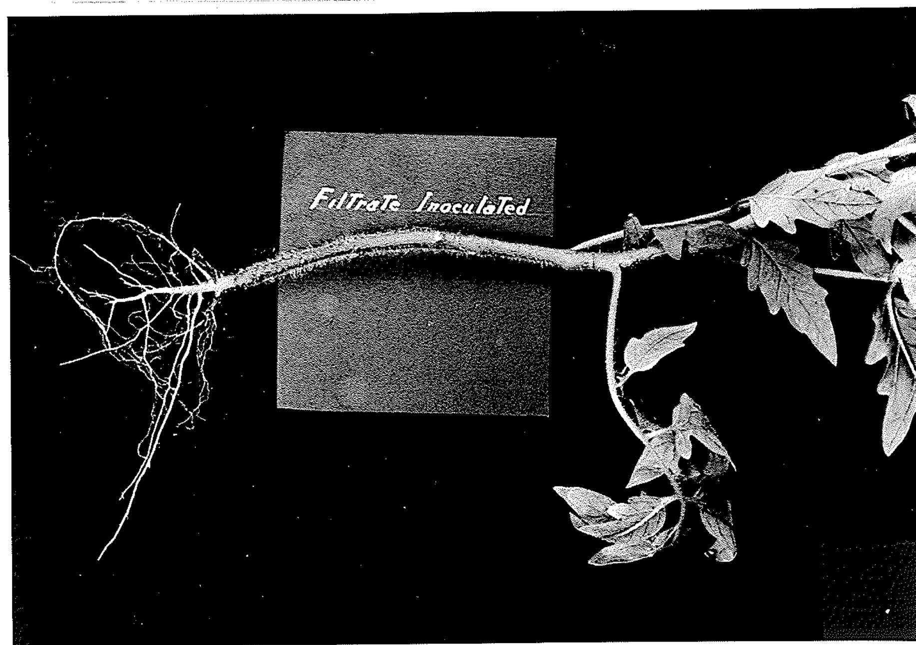
PLATE V




Uninoculated tomato seedling

These represent one form of control used throughout the seedling inoculation series. At least four uninoculated controls were included in every 24 seedling set. None showed evidence of tumor formation.

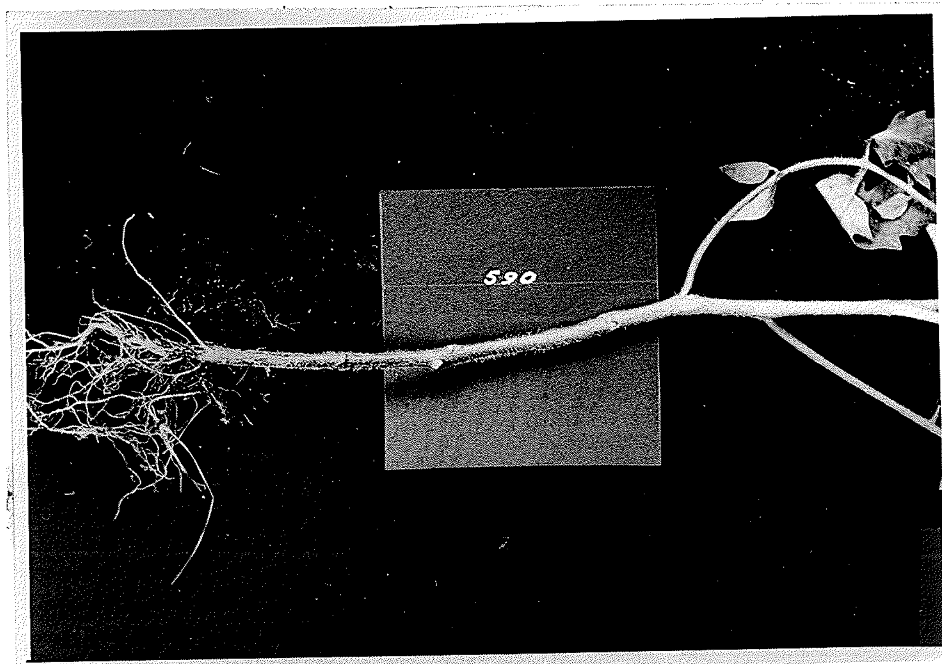
PLATE VI




Filtrate inoculated control

This represents the second form of control used throughout the seedling inoculation series. Cell-free filtrate was introduced at a point immediately above the first leaf node in each case. No evidence of tumor development was noted in any seedling treated in this way. At least three such treatments were included in every 24 seedling set.

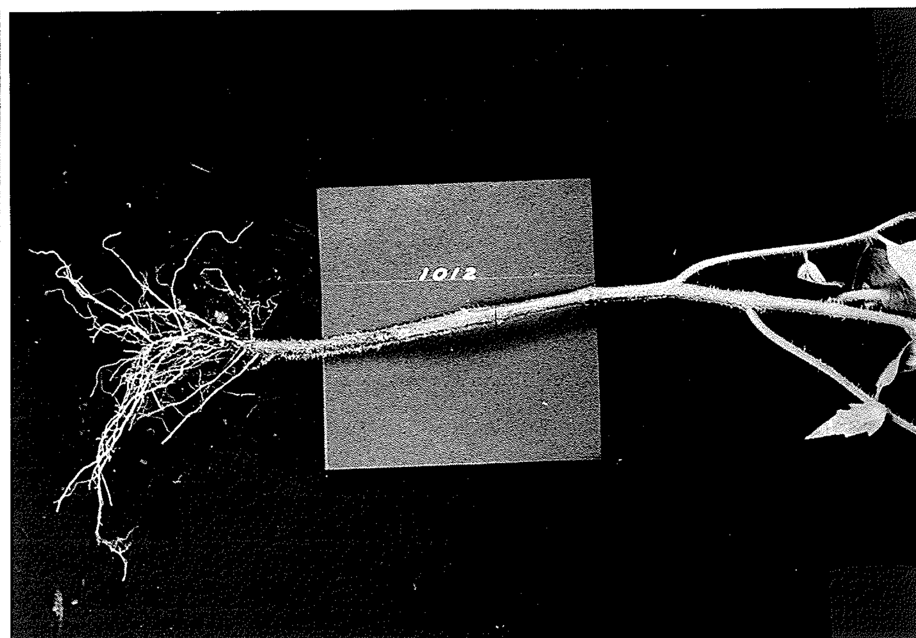
## PLATE VII




Seedling inoculated with parent A. radiobacter 590 culture

This represents one of a number of seedlings thus inoculated. In no instance was there evidence of tumor development. A scar-like callus developed at the site of inoculation in some cases but this could be readily distinguished from tumor development.

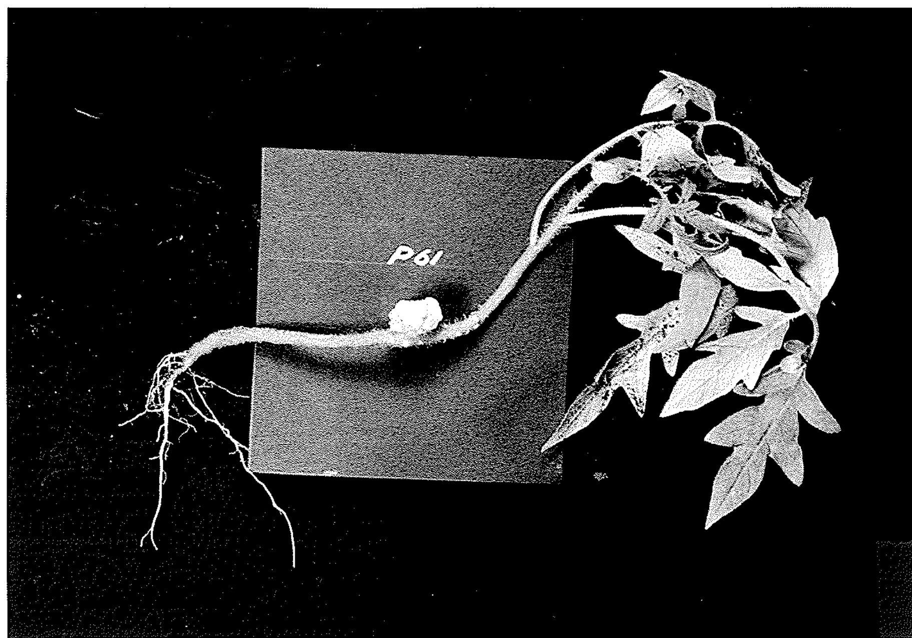
PLATE VIII




Seedling inoculated with parent A. radiobacter 1012

One of a number of seedlings thus inoculated. In no instance was there evidence of tumor development. Callus tissue developed occasionally at the site of inoculation.

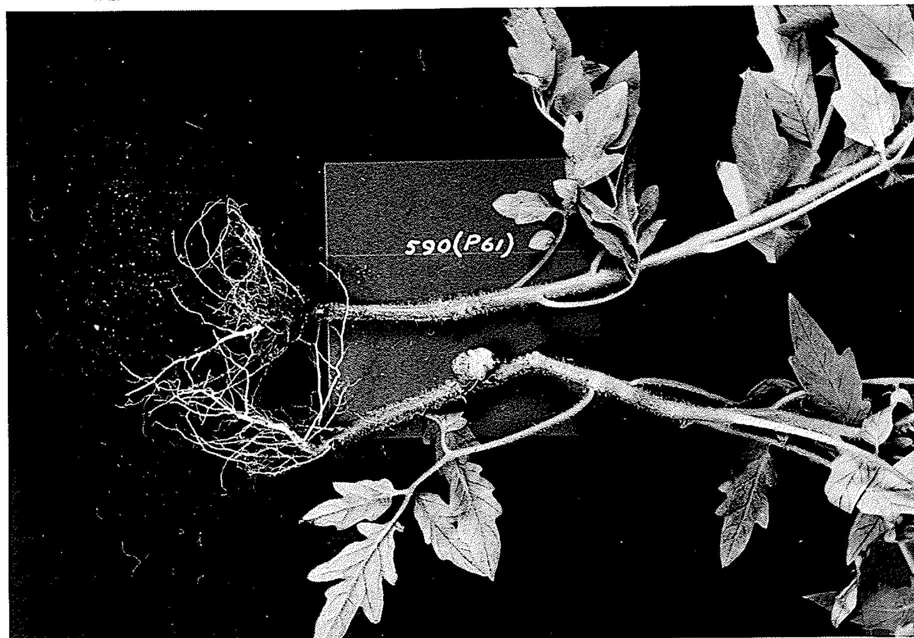
PLATE IX




Seedling inoculated with parent A. tumefaciens P61

One of a number of seedlings inoculated with the virulent parent culture. Note the very large extruded tumor at the site of inoculation. The example shown above represented one of the largest tumor observed in the entire series. Tumors were uniformly white in color, form and having a markedly corrugate surface. No evidence of secondary metastatic tumor.

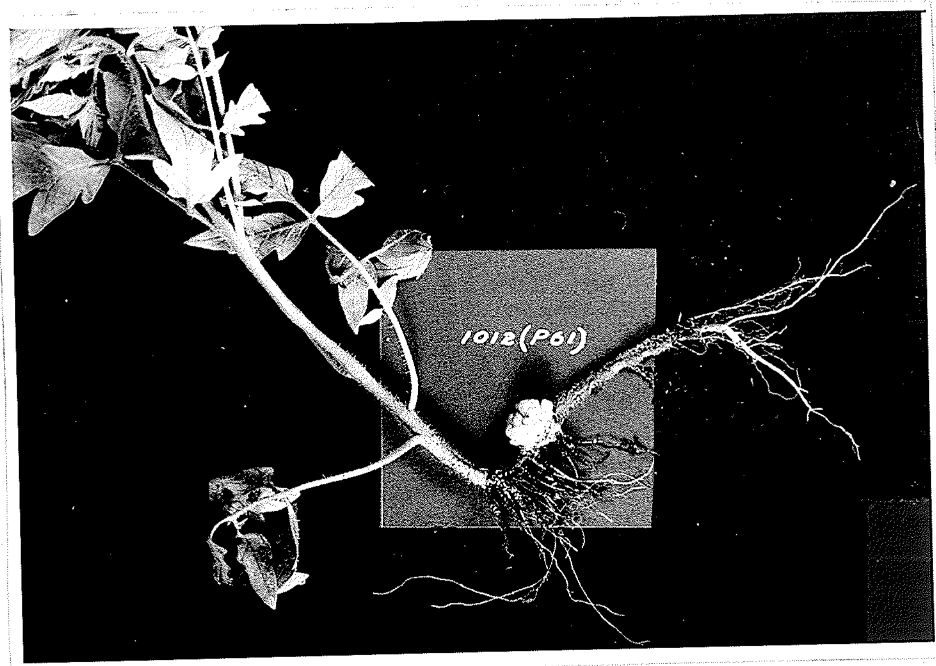
## PLATE X




Seedling inoculated with transformed A. radiobacter 590(P61)

The lower seedling shows a single large tumor at the site of inoculation. This example represented the more usual response.
The upper seedling developed several quite small tumors at and around the inoculation site. These can be seen on the upper surface immediately opposite the lower seedling tumor. This response was noted in several instances.

PLATE XI




Seedling inoculated with transformed *A. radiobacter* 1012(P61)

Large well-developed tumor at the site of inoculation. The inoculation was at a point above the first true leaves but the stem weakened at the point of emergence and permitted the development of extensive rooting below the tumor. The corrugate tumor surface is clearly seen in this case. Inoculum in this case was from the second carrot series with *A. radiobacter* 1012(P61).

# DISCUSSION

Of the two methods that gave evidence of transformation of virulence, the cell-free filtrate technique is, undoubtedly, the easier to carry out. Questions arise with respect to the validity of transformation results obtained by the filtration technique. The possibility that an occasional whole cell might pass through a U.F. sintered glass filter should not be ignored. The probability, however, that whole cells might pass two such filters in series seems extremely small. Incubation at optimum temperatures of donor cell filtrates for periods as long as seven days failed to show detectable growth.

The DNA extraction method, while somewhat more time consuming, almost entirely eliminates the chance for whole donor cell contamination. If by some unlikely circumstance a few donor cells remain viable after exposure to the lauryl sulfate lysing solution, subsequent treatments with sodium perchlorate, octanol-chloroform and ethanol at the concentrations employed would almost certainly ensure their destruction.

From results presented in Fig. 1, it is evident that the donor cell DNA preparation closely approximates purified calf thymus DNA as far as its absorption spectrum is concerned. According to Lerman (1956) depolymerization of DNA during extraction by the method followed is slight.

Although some DNA hydrolysis products may have escaped adsorption to Norite during the extractive process, the likelihood that they would account for the spectrophotometric results observed is considered rather small.

After several trials, the lypholized filtrate method and the preparation of donor cell extracts by sonic disintegration were abandoned. Although lypholized donor filtrate seemed a likely means for introduction of larger amounts of excreted DNA to acceptor cultures, the concentration of slime during lypholization increased to a point where handling of the material was most difficult.

Local heating at the probe surface combined with a relatively low efficiency of cell destruction led to the abandonment of sonic disintegration as a means of preparing donor cell DNA extracts.

The detection of virulence by carrot disc inoculation proved to be considerably more rapid than detection by seedling inoculation. Moreover, the carrot disc assay lends itself readily to the usual laboratory restrictions of time and space. The results presented in Plates I to IV, particularly with reference to the low number of tumors appearing from transformed culture inoculation as compared to the number from virulent parent inoculation, lend support to the generally accepted contention that transformations occur at a low rate. In this case only a very small proportion of acceptor cells were transformed with respect to virulence.

A surprising development as far as carrot disc assays were concerned was the tumor response to A. rubi inoculations. In a preliminary series of carrot disc inoculations conducted to test the efficicy of the method, A. rubi as well as all A. tumefaciens cultures at our disposal produced tumors within one week. Since A. rubi is considered to be pathogenic only to members of the genus Rubus and related plants, one would not expect it to produce tumors on carrot tissue.

Tomato seedling inoculations by parent and transformed cultures, Plates V to XI, were less practical for our purposes than the carrot assay. Adequate growth of seedlings was possible only under growth chamber conditions for a greater part of the year and such facilities were not always available for the necessary number of replications.

Inoculations with A. rubi F60 and with cultures transformed by this donor parent did not produce tumors in any instance on seedlings; probably for the reason cited previously. Indeed, not all A. tumefaciens inoculations gave positive results. Some variation in response is expected through faulty inoculation, or through development of necrotic lesions at the site of inoculation resulting in secondary infection and death of the seedling. As well, some seedlings may have been more resistant than others and as a result dominant in the host-parasite relationship. If, as Klein and Klein (1956) contend, virulence transformations

occur step-wise from an initial low order of virulence to a higher order by repeated transformations, it is not surprising that apparently negative results occur frequently as a result of a single transformation exposure.

# CULTURAL

# AND

# NUTRITIONAL STUDIES

## CULTURAL AND NUTRITIONAL STUDIES

Washed suspensions of cells of the various isolates were used in the differential media listed below. One loopful of a turbid suspension formed the inoculum in the case of liquid media. Where streak plates were used, a cross-hatch inoculation by needle dipped twice in the washed suspension was employed. Sterilization of media unless otherwise indicated was by autoclave at 120°C. for 15 minutes. All incubations were at 25°C. for 72 hours unless otherwise noted.

### CARBOHYDRATE MEDIA[1]

Following are listed the sugars used in the preparation of carbohydrate broth media: maltose, sucrose, arabinose, dextrose, lactose, galactose, mannitol and xylose.

Response by the isolates to various carbohydrate sources is reported as growth (detectable turbidity), indicated by a positive sign; and no growth by a negative sign. Changes in acidity are noted in the tabular results.

### MISCELLANEOUS DIFFERENTIAL MEDIA[1]

Differential media used in this aspect of the study are listed below. Responses of cultures to these media were

[1] A description of the composition and preparation of these media is presented in the Appendix.

interpreted on the bases of routine methods, i.e. those appearing in the Manual of Methods for Pure Culture Study of Bacteria, 1946.

Nitrate Broth, Difco--for nitrate reduction.

Tryptophane Broth--for indole production

Asparagine Broth--for ability to utilize asparagine as the sole carbon and nitrogen source.

Ferric Ammonium Citrate Broth--for citrate utilization.

Inorganic Basal Salts Broth, with mannitol--for dependence on amino acid and growth factor sources.

Lead Acetate Agar, Difco--for $H_2S$ production.

Litmus Milk--for changes in reaction and consistency.

Mannitol Calcium Glycerolphosphate Agar--for growth characteristics, particularly by *A. radiobacter* strains.

Preliminary experiments had indicated that *A. rubi* P60 failed to grow in a mannitol inorganic salts medium with nitrate as the source of nitrogen. Moreover, in a similar medium with an ammonium salt as the nitrogen source growth was weak even after prolonged incubation.

To assess whether the growth habit or lack of it in these media was due to due to deficiency in amino acid or growth factor synthesis, the following nutritional study was undertaken.

The parent *A. rubi* P60 culture together with a representative group of its transformed *A. radiobacter* isolates were used. Cultures were washed three times in sterile saline (0.85% NaCl), respired in saline for 24 hours, then inoculated by loop to tubes of mannitol mineral salts medium, containing various amino acid or growth factor treatments.

Amino acids included in the survey were as follows: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine.

Amino acid solutions (0.05 M of the dl form) were made up separately, sterilized by autoclave, and added at the rate of 0.1 ml. per 9.0 ml. inorganic basal salts medium. Amino acid combinations were as follows: one set of two tubes per culture received no amino acids and served as controls. A second set received all amino acids. Further sets were made up with all amino acids less one; i.e. all amino acids less alanine, all amino acids less arginine, etc.

Growth factors considered in the study included biotin, calcium pantothenate, niacin, p-amino benzoic acid, pyridoxine HCl, riboflavin and thiamine HCl. Solutions at concentrations of 100 µg./ml. were sterilized by filtration and added to the basal medium at the rate of 0.1 ml./9.0 ml. of medium to yield an approximate concentration of 1.0 µg./ml.

Growth factor combinations were as follows: one set of two tubes contained only basal medium as controls. One set contained all growth factors listed. Further sets contained all growth factors less one; i.e. all growth factors less biotin, all growth factors less calcium pantothenate, etc.

# RESULTS

# RESULTS

The form of culture notation followed was the same as indicated in Virulence Transformation Results.

Cultural responses to different carbohydrate sources are reported in Tables 2, 3, 4 and 5. Although slight acid production accompanied growth in some cases and is designated in tabular results by the notation 'sl. ac', no visible gas formation accompanied it.

The results of inoculations in the various differential media used are presented in Tables 6 to 13 inclusive. Where doubtful results were obtained or where cultures failed to grow, the tests were repeated. The tests for reduction of nitrates were made at 24 hour intervals for three days by methods outlined in the Manual of Methods for Pure Culture Study of Bacteria, care being exercised to observe aseptic precautions.

Ferric ammonium citrate broth, while not considered a differential medium for members of this group, yielded interesting quantitative results among the cultures tested.

The results of amino acid and growth factor surveys with A. rubi F60 and transformed isolates are presented in Tables 14 and 15.

Table 2. Cultural responses in carbohydrate media by parent and transformed cultures

| Cultures | Carbohydrate Sources | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | maltose | sucrose | arabinose | dextrose | lactose | galactose | mannitol | xylose |
| 590 | + | + | + | + | + | + | + | + |
| F61 | + | + | + | +sl.ac | + | +sl.ac | +sl.ac | + |
| 590(F61)1 | + | + | + | + | + | + | + | + |
| " 2 | + | + | + | + | + | + | + | + |
| " 3 | + | + | + | + | + | + | + | + |
| " 4 | + | + | + | + | + | + | + | + |
| " 5 | + | + | + | +sl.ac | + | + | + | + |
| " 6 | + | + | + | + | + | + | + | + |
| " 7 | + | + | + | + | + | + | + | + |
| " 8 | + | + | + | + | + | + | + | + |
| " 9 | + | + | + | + | + | + | + | + |
| " 10 | + | + | + | + | + | + | + | + |
| " 11 | + | + | + | + | + | + | + | + |

Table 3. Cultural responses in carbohydrate media by parent and transformed cultures

| Cultures | Carbohydrate Sources | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | maltose | sucrose | arabinose | dextrose | lactose | galactose | mannitol | xylose |
| 1012 | + | + | + | + | + | + | + | + |
| P61 | + | + | + | +sl.ac | + | +sl.ac | +sl.ac | + |
| 1012(P61)1 | + | + | + | + | + | + | + | + |
| " 2 | + | + | + | + | + | + | + | + |
| " 3 | + | + | + | + | + | + | + | + |
| " 4 | + | + | + | + | + | + | + | + |
| " 5 | + | + | + | + | + | + | + | + |
| " 6 | + | + | + | + | + | + | + | + |
| " 7 | + | + | + | + | + | + | + | + |
| " 8 | + | + | + | + | + | + | + | + |
| " 9 | + | + | + | + | + | + | + | + |

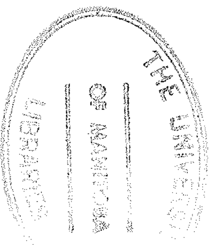

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Table 4. Cultural responses in carbohydrate media by parent and transformed cultures

| Cultures | Carbohydrate Sources | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | maltose | sucrose | arabinose | dextrose | lactose | galactose | mannitol | xylose |
| 590 | + | + | + | + | + | + | + | + |
| r60 | + | +sl.ac | +sl.ac | +sl.ac | - | +sl.ac | +sl.ac | + |
| 590(r60) 1 | + | + | + | + | + | +sl.ac | + | + |
| " 2 | + | + | + | + | + | +sl.ac | + | + |
| " 3 | + | + | + | + | + | + | + | + |
| " 4 | + | + | + | + | + | + | + | + |
| " 5 | + | + | + | +sl.ac | + | +sl.ac | + | + |
| " 6 | + | + | + | +sl.ac | + | +sl.ac | + | + |
| " 7 | + | + | + | +sl.ac | + | +sl.ac | + | + |
| " 8 | + | + | + | +sl.ac | + | +sl.ac | + | + |
| " 9 | + | + | + | +sl.ac | + | +sl.ac | + | + |
| " 10 | + | +sl.ac | + | +sl.ac | + | +sl.ac | + | + |
| " 11 | + | + | + | + | + | +sl.ac | + | + |

Table 5. Cultural responses in carbohydrate media by parent and transformed cultures

| Cultures | | Carbohydrate Sources | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | maltose | sucrose | arabinose | dextrose | lactose | galactose | mannitol | xylose |
| 1012 | + | + | + | + | + | + | + | + |
| F60 | + | +sl.ac | +sl.ac | +sl.ac | - | +sl.ac | +sl.ac | + |
| 1012(F60)1 | + | + | + | +sl.ac | + | + | + | + |
| " 2 | + | + | + | + | + | + | + | + |
| " 3 | + | + | + | + | + | + | + | + |
| " 4 | + | + | + | + | + | + | + | + |
| " 5 | + | + | + | + | + | + | + | + |
| " 6 | + | + | + | + | + | +sl.ac | + | + |
| " 7 | + | + | + | + | + | + | + | + |
| " 8 | + | + | + | + | + | + | + | + |
| " 9 | + | + | + | + | + | + | + | + |

Table 6. Cultural responses in differential media by parent and transformed cultures

| Culture | Differential Tests | | | | | |
|---|---|---|---|---|---|---|
| | Nitrate reduction | Indole production | Asparagine utilization | Citrate utilization | $H_2S$ production | Inorganic Salts Medium |
| 1012 | + | - | + | + | + | + |
| F61 | +weak | +weak | + | +weak | + | + |
| 1012(F61)1 | - | - | + | + | + | + |
| " 2 | +weak | - | +weak | + | + | + |
| " 3 | - | - | + | +weak | + | + |
| " 4 | - | - | + | + | - | + |
| " 5 | +weak | - | + | + | + | + |
| " 6 | + | - | + | + | + | + |
| " 7 | +weak | - | + | +weak | + | + |
| " 8 | +weak | - | + | + | + | + |
| " 9 | - | - | + | + | - | + |

Table 7. Cultural responses in differential media by parent and transformed cultures

| Culture | Differential Tests: Nitrate reduction | Indole production | Asparagine utilization | Citrate utilization | $H_2S$ production | Inorganic Salts Medium |
|---|---|---|---|---|---|---|
| 1012 | + | - | + | + | + | + |
| F60 | - | - | - | - | - | - |
| 1012(F60)1 | - | - | + | + | + | - |
| " 2 | +weak | - | + | +weak | + | + |
| " 3 | + | - | + | +weak | + | + |
| " 4 | +weak | - | +weak | +weak | + | - |
| " 5 | + | - | + | + | + | + |
| " 6 | +weak | - | +weak | +weak | + | - |
| " 7 | - | - | + | + | + | + |
| " 8 | +weak | - | + | +weak | + | + |
| " 9 | - | - | + | +weak | + | + |

Table 8. Cultural responses in differential media by parent and transformed cultures

| Culture | Differential Tests | | | | | |
|---|---|---|---|---|---|---|
| | Nitrate reduction | Indole production | Asparagine utilization | Citrate utilization | $H_2S$ production | Inorganic Salts Medium |
| 590 | +weak | - | + | + | +weak | + |
| F61 | +weak | +weak | + | +weak | + | + |
| 590(F61)1 | - | - | + | +weak | +weak | + |
| " 2 | - | - | + | + | - | + |
| " 3 | +weak | - | + | + | - | + |
| " 4 | - | - | + | + | - | + |
| " 5 | - | - | + | + | + | + |
| " 6 | - | - | + | + | - | + |
| " 7 | +weak | - | +weak | +weak | +weak | + |
| " 8 | - | - | + | + | +weak | + |
| " 9 | - | - | + | + | +weak | + |
| " 10 | +weak | - | + | + | - | + |
| " 11 | +weak | - | + | + | +weak | + |

Table 9. Cultural responses in differential media by parent and transformed cultures

| Culture | Differential Tests | | | | | |
|---|---|---|---|---|---|---|
| | Nitrate reduction | Indole production | Asparagine utilization | Citrate utilization | $H_2S$ production | Inorganic Salts Medium |
| 590 | +weak | - | + | + | +weak | + |
| P60 | - | - | - | - | - | - |
| 590(P60)1 | - | - | +weak | + | +weak | + |
| " 2 | - | - | + | +weak | +weak | + |
| " 3 | +weak | - | + | + | - | +weak |
| " 4 | - | - | +weak | +weak | - | + |
| " 5 | - | - | + | + | - | + |
| " 6 | +weak | - | +weak | + | - | + |
| " 7 | - | - | + | + | - | + |
| " 8 | - | - | + | + | + | + |
| " 9 | +weak | - | +weak | +weak | +weak | - |
| " 10 | +weak | - | + | + | - | +weak |
| " 11 | +weak | - | + | + | - | + |

Table 10. Cultural responses in differential media by parent and transformed cultures

| Cultures | Differential Media | |
|---|---|---|
| | Litmus Milk | Mannitol glycerol phosphate agar |
| 1012 | browning, with deep serum zone no coagulation | heavy mucoid growth, browning |
| P60 | acid reaction, soft curd no coagulation | moderate growth, no browning |
| 1012(P60) 1 | dark brown, with deep serum zone no coagulation | heavy mucoid growth, browning |
| " 2 | | |
| " 4 | | weak growth, no browning |
| " 5 | | moderate growth, browning |
| " 6 | | |
| " 7 | | |
| " 8 | | |
| " 9 | | |

Table 11. Cultural responses in differential media by parent and transformed cultures

| Cultures | | Differential Media | |
| --- | --- | --- | --- |
| | | Litmus Milk | Mannitol glycerol phosphate agar |
| 590 | | dark chocolate brown moderate serum zone. No coagulation | heavy mucoid growth, browning |
| P60 | | acid reaction, soft curd | moderate growth, no browning |
| 590(P60) | 1 | chocolate brown, moderate serum zone no coagulation | heavy mucoid growth, browning |
| " | 2 | " | |
| " | 3 | deep | |
| " | 4 | moderate | |
| " | 5 | " | |
| " | 6 | " | |
| " | 7 | " | |
| " | 8 | slight | |
| " | 9 | moderate | |
| " | 10 | " | |
| " | 11 | " | |

Table 12. Cultural responses in differential media by parent and transformed cultures

| Cultures | | Differential Media: Litmus Milk | Differential Media: Mannitol glycerol phosphate agar |
|---|---|---|---|
| 1012 | | browning, deep serum zone no coagulation | heavy mucoid growth, browning |
| P61 | | litmus reduced, no serum zone no coagulation | heavy mucoid growth, no browning |
| 1012(P61) | 1 | slight browning, deep serum zone no coagulation | heavy mucoid growth, browning |
| " | 2 | | ↓ |
| " | 3 | dark brown, deep serum zone no coagulation | ↓ |
| " | 4 | ↓ | ↓ |
| " | 5 | ↓ | ↓ |
| " | 6 | ↓ | ↓ |
| " | 7 | slight browning, moderate serum zone no coagulation | ↓ |
| " | 8 | dark brown, deep serum zone no coagulation | ↓ |
| " | 9 | ↓ | ↓ |

Table 13. Cultural responses in differential media by parent and transformed cultures

| Cultures | Differential Media: Litmus Milk | Differential Media: Mannitol glycerol phosphate agar |
|---|---|---|
| 590 | dark chocolate brown, moderate serum zone. No coagulation | heavy mucoid growth, browning |
| F61 | litmus reduced, no serum zone. No coagulation. | heavy mucoid growth, no browning |
| 590(F61) 1 | chocolate brown, moderate serum zone. No coagulation | heavy moderate growth, browning |
| " 2 | ↓ | ↓ |
| " 3 | slight browning, slight serum zone, No coagulation | slight growth, no browning |
| " 4 | chocolate brown, moderate serum zone. No coagulation | heavy mucoid growth, browning |
| " 5 | ↓ | ↓ |
| " 6 | ↓ | ↓ |
| " 7 | ↓ | ↓ |
| " 8 | moderate browning, slight serum zone. No coagulation. | ↓ |
| " 9 | chocolate brown, moderate serum zone. No coagulation. | ↓ |
| " 10 | ↓ | ↓ |
| " 11 | ↓ | ↓ |

Table 14. Responses of A. rubi P60 and some transformed isolates in a basal nitrate medium (BNM) containing different combinations of growth factors.

| | Cultures | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatments | *A. rubi* P60 | 590(P60)9 | 590(P60)10 | 1012(P60)1 | 1012(P60)4 | 1012(P60)6 | 590(P60)3 |
| BNM | - | - | ± | - | - | - | ± |
| BNM + all growth factors (BNMG) | - | - | ± | - | - | - | ± |
| BNMG - biotin | - | - | ± | - | - | - | ± |
| " - niacin | - | - | ± | - | - | - | ± |
| " - pantothenate | - | - | ± | - | - | - | ± |
| " - pyridoxine | - | - | ± | - | - | - | ± |
| " - PABA | - | - | ± | - | - | - | ± |
| " - riboflavin | - | - | ± | - | - | - | ± |
| " - thiamine | - | - | ± | - | - | - | ± |

+ growth; ± weak to doubtful; - no growth.

Table 15. Responses of A. rubi P60 and some transformed isolates in a basal nitrate medium (BNM) containing different combinations of amino acids

| Treatments | cultures | | | | | | |
|---|---|---|---|---|---|---|---|
| | A. rubi P60 | 590(P60)-9 | 590(P60)-10 | 1012 (P60)1 | 1012 (P60)4 | 1012 (P60)6 | 590 (P60)3 |
| BNM | - | - | ± | - | - | - | ± |
| BNM + all amino acids (BNMA) | + | + | + | + | + | + | + |
| BNMA - alanine | + | + | + | + | + | + | + |
| " - argenine | + | + | + | + | + | + | + |
| " - aspartic | - | - | + | - | - | - | + |
| " - cysteine | + | + | + | + | + | + | + |
| " - glutamic | + | + | + | + | + | + | + |
| " - glycine | + | + | + | + | + | + | + |
| " - histidine | + | + | + | + | + | + | + |
| " - isoleucine | - | - | + | - | - | - | + |
| " - leucine | - | - | + | - | - | - | + |
| " - lysine | + | + | + | + | + | + | + |
| " - methionine | + | + | + | + | + | + | + |
| " - phenylalanine | - | - | + | - | - | - | + |
| " - proline | - | - | + | - | - | - | + |
| " - serine | + | + | + | + | + | + | + |
| " - threonine | + | + | + | + | + | + | + |
| " - tryptophane | - | - | + | - | - | - | + |
| " - tyrosine | + | + | + | + | + | + | + |
| " - valine | - | - | + | - | - | - | + |

+ growth; ± weak growth; - no growth.

# DISCUSSION

## DISCUSSION

The cultural survey of carbohydrate sources yielded very little evidence of variation in response by transformed isolates when compared with their acceptor parent cultures. The exceptions noted in the case of *A. radiobacter* 590 (P61) and *A. radiobacter* 1012 (P60) isolates occurred so infrequently that little significance can be attached to them. Such low frequency of variation could reasonably be expected between isolates from the same culture.

Variations in response by *A. radiobacter* 590 (P60) isolates were considerably more numerous although the distinctions were still only a matter of degree. The failure of the donor parent *A. rubi* P60 to grow with lactose as the carbohydrate source did not appear in any of the virulent transformed isolates.

Whether such quantitative differences could be considered significant in terms of a transformation is doubtful even in the case of the *A. radiobacter* 590 (P60) isolates.

Differential tests reported in Tables 6 to 13 yielded considerably more variation in response by virulent transformed isolates. A number of these variations were of a quantitative nature, i.e. a weakly positive response in the case of asparagine utilization by *A. radiobacter* 1012 (P61) -2. On the other hand, some variations were purely qualitative, i.e. no hydrogen sulphide production by a number of the *A. radiobacter* 590 (P61) isolates.

Only three of the various differential tests gave a range of results with transformed isolates that can reasonably be considered for discussion. The first of these, the nitrate reduction test yielded some quite interesting variations between transformed isolates. Some A. radiobacter 1012 (F61) isolates, for example, gave negative tests for nitrite although both parents gave positive results. Since the parent acceptor A. radiobacter 1012 strongly reduced nitrate within 24 hours, the possibility arises that the negative tests displayed by the isolates concerned may have occurred through a complete assimilation of nitrite to some further reduction product. Before such an evaluation can be made, more information regarding nitrate assimilatory pathways in A. radiobacter 1012 must be available. For the same reason the negative results for nitrate reduction shown by three of the A. radiobacter 1012 (F60) isolates is unexplained.

The second differential test of interest was citrate utilization. Marked differences in growth habit between parent cultures were noted. A. radiobacter 1012 gave strong growth and a heavy ring with some pellicle formation. A similar heavy growth response was noted for A. radiobacter 590, whereas, A. tumefaciens F61 gave weak growth with no ring and A. rubi F60 gave no growth response. Six transformed isolates of the A. radiobacter 1012 (F60) group gave cultural responses in this medium that were most noticeably weaker than those of the parent A. radiobacter 1012.

As well, weak growth responses in this medium by isolates from A. radiobacter 1012 (P61) and A. radiobacter 590 (P61) were indistinguishable from those displayed by the virulent donor parent. Whether such variations in response by the transformed isolates were related to the virulence transformation or whether they occurred by chance must remain a matter for conjecture until a more extensive study is undertaken.

Hydrogen sulphide production by transformed isolates varied quite noticeably, particularly in the instances where A. tumefaciens P61 was the donor parent. One isolate, 590 (P61) -5, showed strong $H_2S$ formation although production by the parent acceptor A. radiobacter 590 was weak. As well, several instances of complete lack of $H_2S$ production by isolates was demonstrated despite the fact that both parents were capable of producing detectable amounts. Rather than indicating a relationship to transformation, it is felt that the variation in $H_2S$ production was due mainly to chance since no degree of consistency of response by transformed isolates was evident.

Similar conclusions may be drawn in the evaluation of transformed isolate responses in the case of indole production and asparagine utilization. Transformed isolates behaved almost entirely as their acceptor parents. This form of response was also noted in the case of litmus milk and calcium glycerolphosphate agar. These differential media

elicit such variation in response within a species as to be valueless for a study of this kind.

No stimulation of growth by the various growth factor supplements was evident in results presented in Table 14. Moreover, it is evident on the basis of results presented in Table 15 that only a few of the transformed isolates in addition to the virulent parent A. rubi P60 showed dependence on certain amino acids. Similar responses to amino acids were reported by Sagen et al. (1934) for A. rhizogenes, a closely related organism.

It may be concluded that the acquisition of virulence does not appear to be related to a dependence on specific growth factors or amino acids.

## SUMMARY

The experimental evidence obtained confirms that transformations of virulence occur between species of the genus *Agrobacterium*, Conn. Competence for transformation is not always evident between members of the genus. The detection of acquired virulence was more readily determined by carrot assays than by seedling inoculations. It is recommended that assay methods based on this system be employed in future laboratory investigations of this nature.

The experimental evidence did not suggest any direct relationship between the nutritional requirements of the organisms concerned and the acquisition of virulence.

## PART II

## AN APPARENT

## TRANSFORMATION FOR NITRATE UTILIZATION

# AN APPARENT TRANSFORMATION FOR NITRATE UTILIZATION

## INTRODUCTION

Detailed information concerning pathways of nitrate assimilation by members of the genus Agrobacterium Conn is lacking. Nitrate assimilation by certain members of the closely related genus, Rhizobium, however, has been reported in some detail by Chenias and Evans (1956) and by Lilly and Leonian (1945). These investigators characterized the nitrate reductase involved in terms of its electron donors and flavine requirement. Similar information with respect to Agrobacterium spp. does not seem to be available.

An examination of the cultural characteristics of this group presented in Bergey's Manual of Determinative Bacteriology, 7th ed., indicates that nitrate reduction is considered only as a diagnostic tool. As indicated in the previous discussion, the validity of the nitrate reduction test may be questioned particularly in the case of a negative result. Such a result may arise either through the inability of the culture tested to reduce nitrate to nitrite or from subsequent nitrite utilization at a rate equivalent to its production. As well, the physiological significance of a positive test for nitrite is obscure unless, as suggested by Verhoeven (1956), more complete information respecting assimilatory pathways is known. The nitrate disappearance may be due to it's role as a non-essential hydrogen acceptor

or to it's reduction to provide ultimately nitrogenous material for cell protein. As far as *Agrobacterium* spp. are concerned, information with respect to these alternatives is not available.

Cultural studies dealing in part with growth by parent and transformed isolates in a medium with nitrate as sole nitrogen source presented in Tables 6, 7, 8 and 9 Part I indicated that *A. radiobacter*, strains 1012 and 590, grew well in the medium while *A. rubi* P60 displayed no growth despite repeated trials.

Accordingly, it seemed reasonable to attempt a transformation of the capacity to utilize nitrate from *A. radiobacter* to *A. rubi*, since competence between these species has been demonstrated in one direction at least by transformations of virulence. An examination of the transformed isolates and parent cultures by conventional manometric and enzyme methods could be used to extend present knowledge on the transformation phenomenon and on pathways of nitrate assimilation in the genus *Agrobacterium*.

## METHODS

Strains of the donor, A. radiobacter, were cultured in mannitol mineral salts medium with nitrate as the sole source of nitrogen at 25°C. for 24 hours and filtered twice in series through Pyrex U.F. sintered glass filters. Filtrates were checked for sterility by incubation at 25°C. for 96 hours.

The acceptor, A. rubi P60, was cultured in mannitol yeast extract medium at 25°C. for 24 hours, centrifuged and washed four times with sterile saline (0.85% NaCl) to remove traces of medium and excretory products; then respired for 18 hours in sterile saline. Two ml. of cells thus prepared were inoculated into the cell-free filtrate referred to above, and the temperature was lowered quickly to 10°C. and held for one hour. Following this cold shock treatment which was used to encourage synchronous division, incubation was at 25°C. for 24 hours.

## Manometry

A. radiobacter 1012 and the transformed A. rubi P60 (1012) were cultured at 25°C. for 48 hours in mannitol nitrate mineral salts medium (see Appendix). Since A. rubi P60 did not grow in this medium, mannitol yeast extract medium was used for its propagation.

Harvested cells were washed three times with sterile saline to remove traces of the medium and excretory products and then respired in a nitrogen-free mannitol medium for 18 hours.

Each of the four inorganic nitrogen sources, $NO_3$, $NO_2$, $NH_2OH$ and $NH_4$, was made up to yield a final concentration of $10^{-4}$ M in 2.7 ml. of the following reaction mixture.

1.0 ml. cells

1.0 ml. mannitol mineral salts N-free medium

0.2 ml. KOH (20%) in the center well

and 0.5 ml. of appropriate N source in the side arm. Respiration experiments were conducted at 25°C., and each inorganic nitrogen treatment and each Warburg trial was duplicated for each culture. After 30 minutes for stabilization, the contents of the side arm were tipped in and a zero reading noted. Readings were taken at 15-minute intervals for a period of six hours.

## ENZYME STUDIES

### Preparation of cells

Five gm. of cells (wet weight) were used for each extraction. After harvesting, cells were washed three times with sterile saline to remove residual medium and excretory growth products. Cells thus harvested were frozen at -15°C. and held at that temperature until required.

Nitrate reductase extraction

Frozen cells were ground vigorously in a cold mortar with twice their weight of levigated alumina for five minutes. Three volumes of cold buffer solution (0.2 M $K_2HPO_4$ with β mercaptoethanol, $10^{-4}$ M, final pH 8.5) were added slowly and grinding was continued for another five minutes.

The resultant suspension was centrifuged at 4°C. (10,000 r.p.m.) for 20 minutes yielding a supernatant or crude extract, which according to Nason and Evans (1955) usually contains 75% or more of the nitrate reductase activity.

Subsequent fractionations were carried out at 4°C. by precipitation with ammonium sulphate. The latter was prepared as the saturated solution at 25°C. and adjusted with sodium hydroxide to pH 7.0. Centrifugations were performed at 10,000 r.p.m. unless otherwise noted.

To 20 ml. crude extract 15 ml. saturated ammonium sulphate was added to yield 43% saturation. The mixture was allowed to stand at 4°C. for five minutes and then centrifuged for 10 minutes. The supernatant was discarded.

The precipitate was dissolved in 8.0 ml. cold phosphate buffer (0.1 M, pH 7.5). To this was added 2.5 ml. saturated ammonium sulphate and the mixture allowed to stand at 4°C. for 15 minutes. Centrifugation followed for 10 minutes, and the resultant precipitate discarded.

To the supernatant 4.3 ml. saturated ammonium sulphate was added and the mixture allowed to stand at 4°C. for 15 minutes. The precipitate was collected by centrifugation and dissolved in 3.2 ml. cold phosphate buffer (0.1 M, pH 7.5).

Nitrate reductase activity was determined using this fraction, no further purification of enzyme being carried out. Protein in the fraction was determined according to the method of Lowry (1951).

Nitrate reductase activity was determined with the following reaction mixture:

0.10 ml. $KNO_3$ (0.1 M)
0.05 ml. FMN ($10^{-4}$ M)
0.05 ml. DPNH or TPNH (7.0 $10^{-3}$ M)
0.45 ml. pyrophosphate buffer (0.1 M, pH 7.5)
0.05 ml. hydroxylamine HCl (0.1 M)
0.20 ml. EDTA ($10^{-3}$ M)
0.10 ml. enzyme preparation

The reaction mixture was incubated at 25°C. for 10 minutes, and 1.0 ml. of a 1% sulfonilamide solution was added to stop the reaction. Following this, 1.0 ml. of 0.02% N-napthyl ethylenediamine dihydrochloride was introduced and color allowed to develop at 25°C. for 10 minutes. The control consisted of a similar reaction mixture from which the coenzyme was omitted.

Abbreviations: FMN, flavine mononucleotide;
FAD, flavine dinucleotide;
DPNH, diphosphopyridine nucleotide, reduced;
TPNH, triphosphopyridine nucleotide, reduced;
EDTA, ethylene diamine tetraacetic acid, the disodium salt.

Nitrite formed was determined by optical density measurements at 540 mμ using a Hilger-Watts Uvispec spectrophotometer; by comparison of the optical density values obtained with a standard curve for nitrite previously prepared from known nitrite concentrations. Controls consisted of reaction mixtures without the coenzymes. In this case, volume in the cuvette was adjusted by increasing the buffer content by an appropriate amount.

<u>Nitrite and hydroxylamine reductase extractions</u>

The method followed was essentially that described by Spencer *et al*. (1957) with certain modifications.

Frozen cells were ground vigorously in a cold mortar with twice their weight of levigated alumina for five minutes. Three times their volume of cold tris buffer (0.05 M, pH 7.1) with β mercaptoethanol ($10^{-4}$ M) was added slowly and grinding was continued for another five minutes. The resultant suspension was centrifuged at 4°C. for 20 minutes, and the precipitate discarded.

The cell-free supernatant was treated with one-half its volume of alumina C γ gel (14 mg. dry weight/ml.) for 20 minutes with intermittent stirring. The gel was collected at 5,000 r.p.m. after five minutes, washed twice with five times its volume of tris buffer (0.05 M, pH 7.1), and eluted twice with potassium phosphate buffer (0.1 M, pH 7.5) containing β mercaptoethanol ($10^{-4}$ M). For each elution

carried out for 20 minutes with occasional stirring, the buffer volume was one-quarter of the original volume of the cell-free extract. The two eluates were combined and this fraction used for the activity determinations.

Nitrite reductase activity was determined with the following reaction mixture:

0.15 ml. $KNO_2$ ($10^{-4}$ M)

0.05 ml. FAD ($10^{-4}$ M)

0.05 ml. TPNH or DPNH ($7.0 \times 10^{-3}$ M)

0.50 ml. tris buffer (0.1 M, pH 7.5)

0.15 ml. $H_2O$

0.10 ml. enzyme preparation

The treatments of the reaction mixture for the development of color and the subsequent spectrophotometric evaluation of color intensities were the same as those for nitrate reductase estimation.

Two sets of controls were used. One consisted of the reaction mixture from which the coenzyme was omitted. The second contained all reaction mixture components except the nitrite source.

Hydroxylamine reductase activity was determined on the following reaction mixture dispensed directly into a silica cuvette.

2.60 ml. phosphate buffer (0.1 M, pH 7.5)
0.05 ml. FAD ($10^{-4}$ M)
0.05 ml. TPNH ($7.0 \times 10^{-3}$ M)
0.10 ml. distilled water
0.10 ml. enzyme preparation

Prior to the introduction of the enzyme preparation, the cuvette was placed in position in the spectrophotometer. As soon as the enzyme aliquot was added to the cuvette and gently stirred to ensure uniform distribution, the optical density scale was adjusted to an arbitrary value of 0.10 (zero time).

Optical density readings were taken at 30 second intervals for two minutes at 340 mμ to establish the endogenous rate of TPNH oxidation. Following this, 0.10 ml. of hydroxylamine hydrochloride (0.4 M) was added. Optical density readings were continued at 30 second intervals for two to two and one-half minutes to establish the rate of TPNH oxidation in the presence of the substrate.

## RESULTS

Growth was evident in the *A. radiobacter* 1012 filtrate within 24 hours after the addition of the *A. rubi* F60 cell suspension. Subsequent transfers to fresh mannitol nitrate medium gave good growth although at a somewhat slower rate than that displayed by either parent in their respective media. The heavy slime common to *A. radiobacter* 1012 cultured in mannitol nitrate medium was not evident in the transformed culture.

### Manometry

The development of extensive slime by *A. radiobacter* 1012 in the mannitol nitrate medium led to difficulty in estimation of cell concentrations for manometric purposes. It was inevitable that variations in cell suspension densities would occur between replicate trials. In order to present a more logical comparison of the different cultural responses, values reported in Figs. 2, 3 and 4 have been adjusted to an equivalent cell-nitrogen basis.

A most noticeable inhibition response by the parent *A. radiobacter* 1012 in the presence of nitrite is indicated in Fig. 2. A positive gas pressure in the manometers was evident the first 45 minutes following the initiation of the experiment. By the end of the first hour, however, this pressure was reduced and oxygen uptake became evident.

The transformed A. rubi F60 (1012) gave a strong respiratory response in the presence of nitrate as indicated in Fig. 3. After an initial strong response for two hours, the rate of oxygen uptake in the presence of ammonia decreased sharply and remained at a sub-endogenous level for the remaining four hours.

The two most noticeable features of the manometric response by the parent A. rubi F60, Fig. 4, were first, the strong oxygen uptake in the presence of nitrite and second, the endogenous-like rate of oxygen uptake in the presence of nitrate. Oxygen uptake decreased noticeably at the end of the fourth hour for all substrates except nitrite.

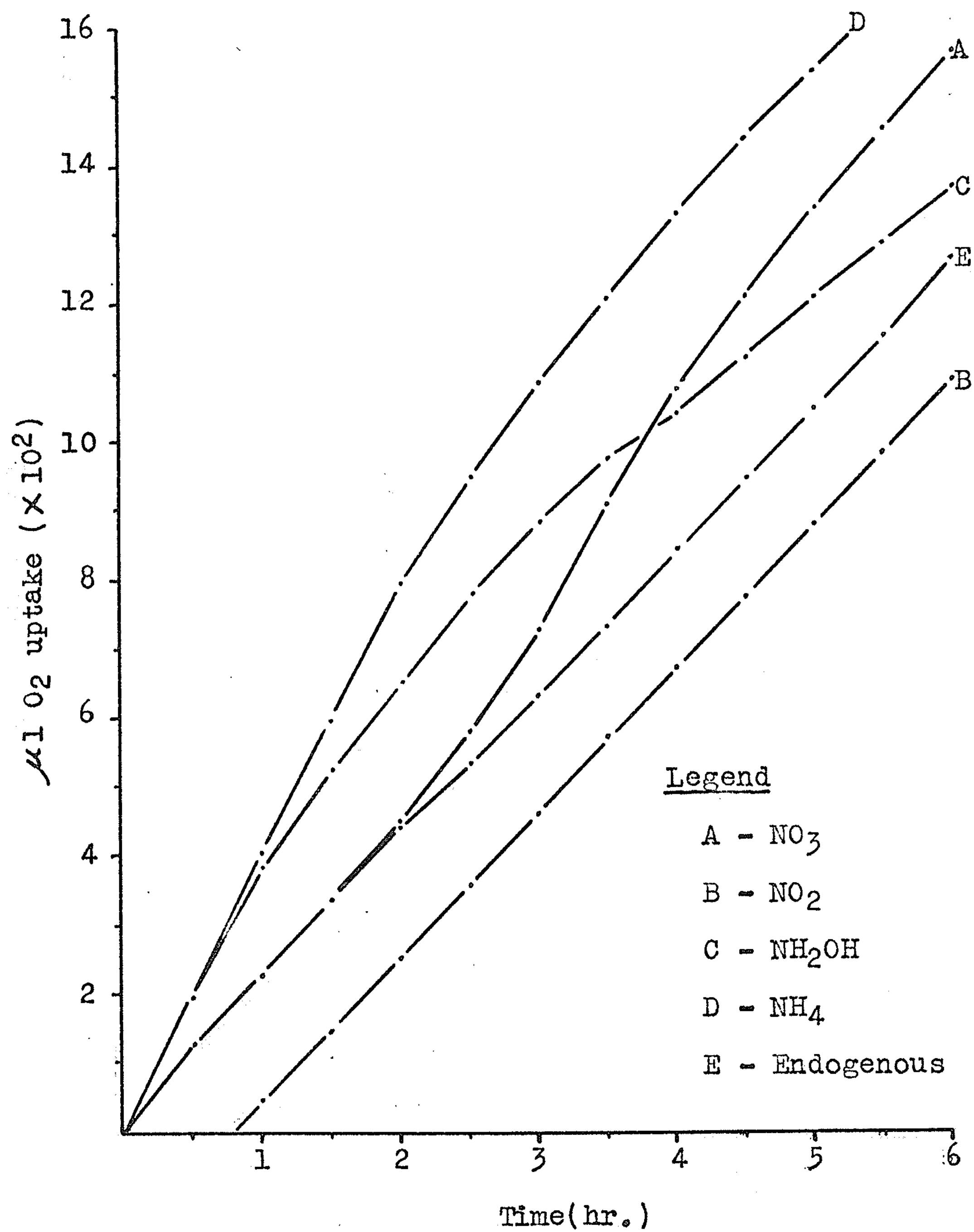


Fig. 2. Utilization of various nitrogen sources by the parent A. radiobacter 1012 as measured by oxygen uptake.

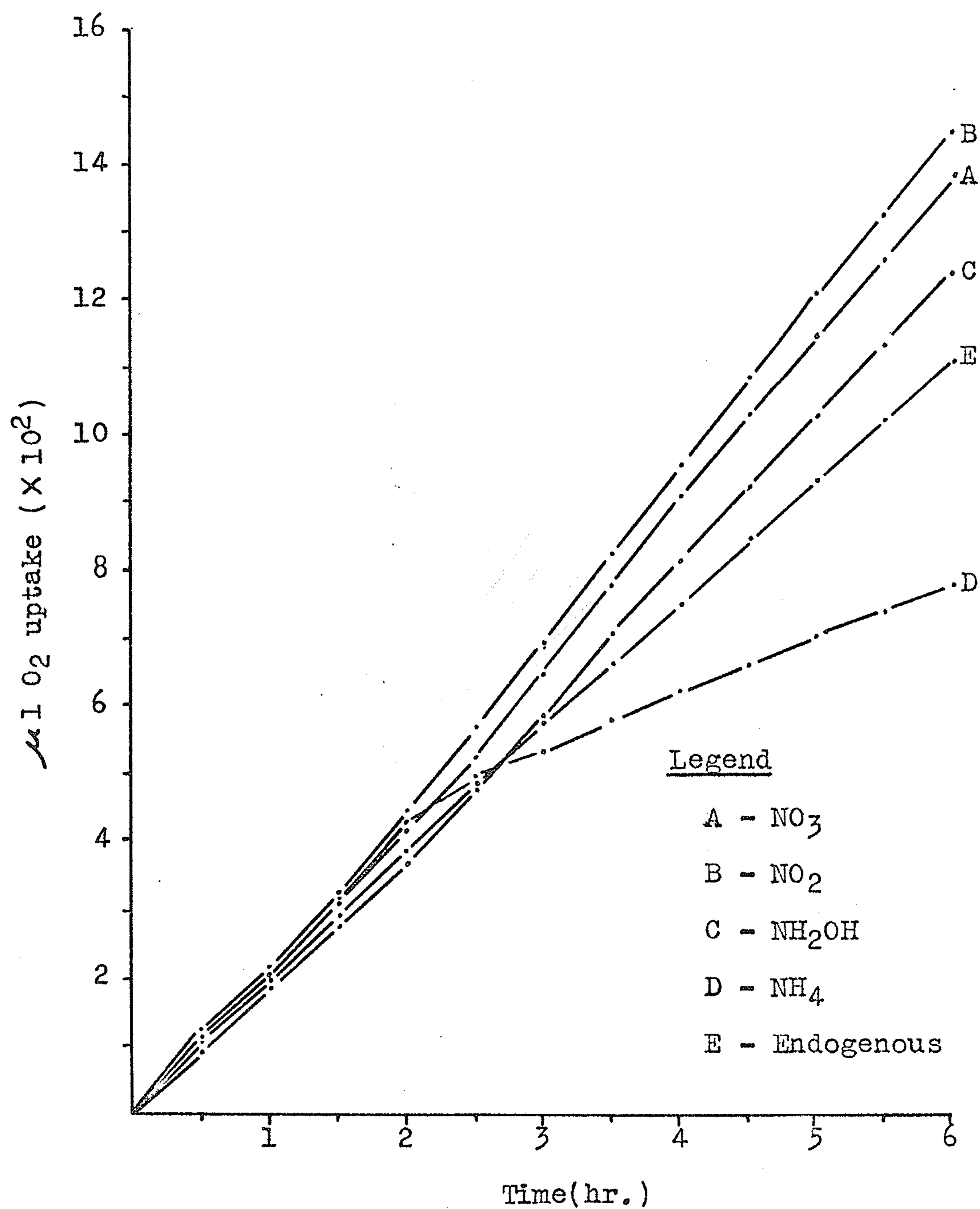


Fig. 3. Utilization of various nitrogen sources by the transformed A. rubi P60(1012) as measured by oxygen uptake.

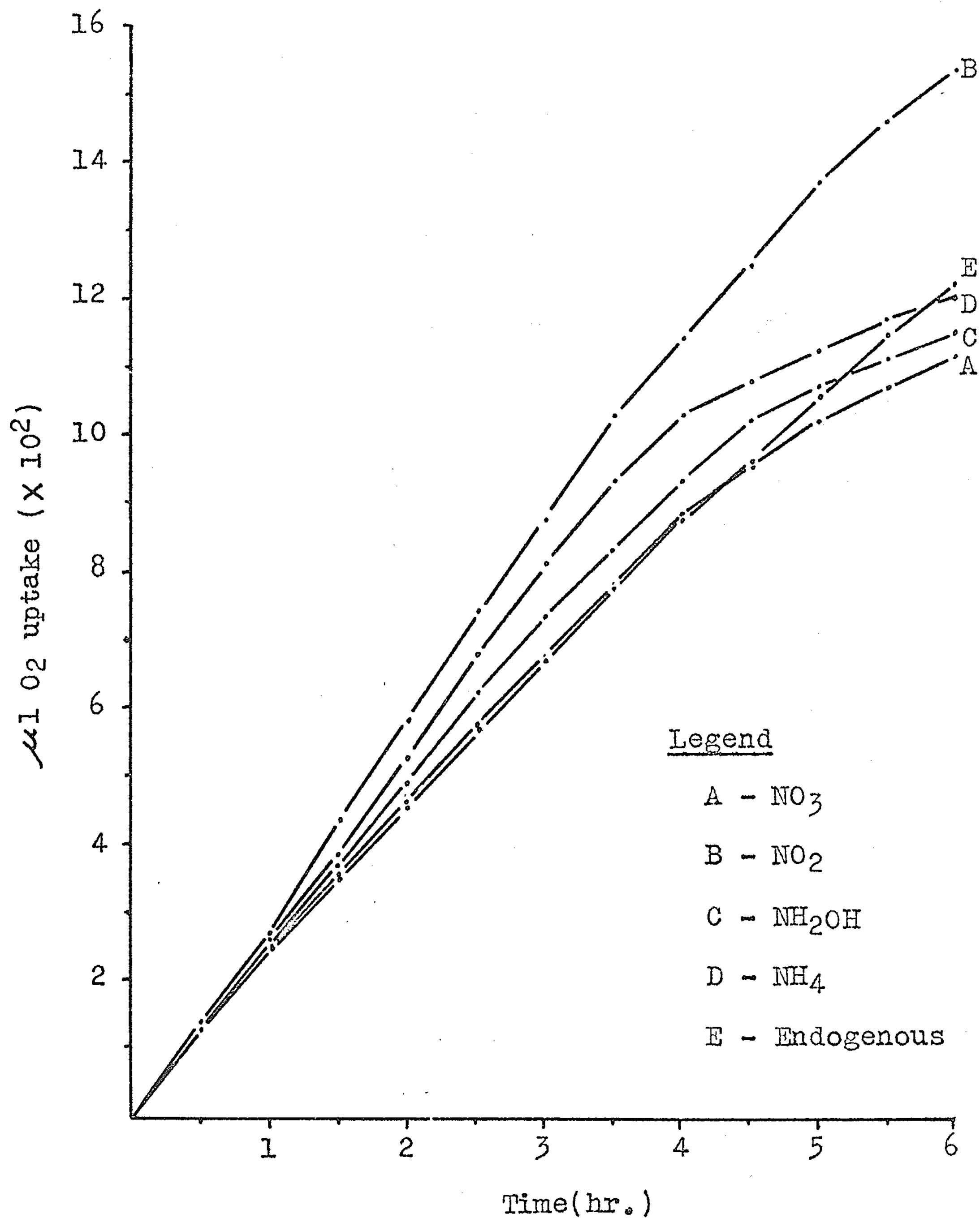


Fig. 4. Utilization of various nitrogen sources by the parent A. rubi P60 as measured by oxygen uptake.

## Enzyme Studies

The greater amounts of nitrite formed by extracts from parent and transformed isolates were evident when DPNH served as the coenzyme. No appreciable amount of nitrite could be detected in the reaction mixture with A. rubi P60 extract present. These results are presented in Table 16.

Table 16. Reduction of $NO_3$ by parent and transformed cultures

| | mg. $NO_2$ formed/mg. protein in 10 min. at pH 7.5[i] | |
|---|---|---|
| Culture | DPNH | TPNH |
| 1012 | 0.47 | 0.20 |
| P60 (1012) | 0.23 | 0.13 |
| P60 | 0.02[ii] | 0.00 |

i The values reported represent the means of four determinations conducted on each culture extract.

ii The mean value reported here represents activity at pH 8.0.

The optimum pH of the nitrate reductase activity in A. radiobacter 1012 extracts was at pH 7.5 for both coenzymes. In the case of the transformed A. rubi P60 (1012), the optimum pH for activity was at pH 7.5 with DPNH and at pH 8.0 with TPNH. Slight activity from A. rubi P60 extracts was evident at pH 7.0 and at pH 8.0 with DPNH as the coenzyme. These results are presented in Figs. 5, 6 and 7.

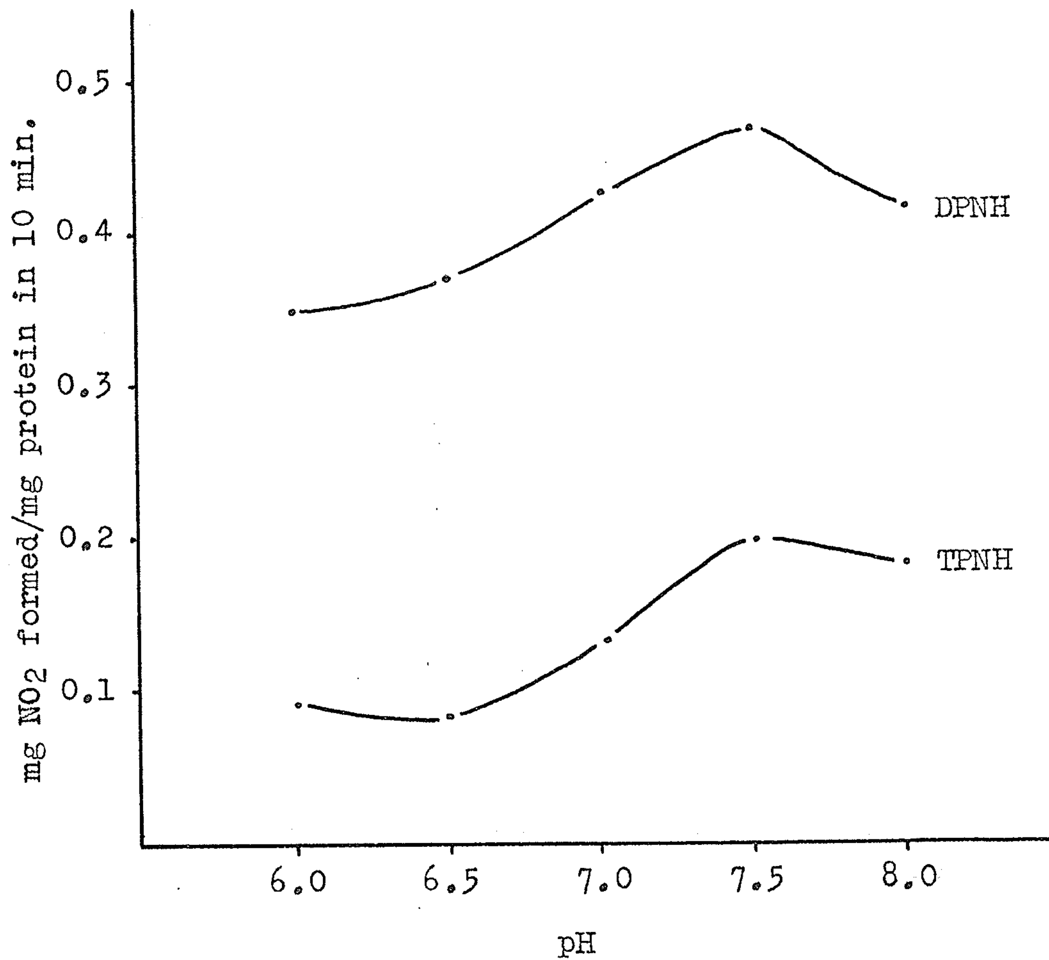


Fig. 5. Nitrate reductase activity in extracts from the parent A. radiobacter 1012.

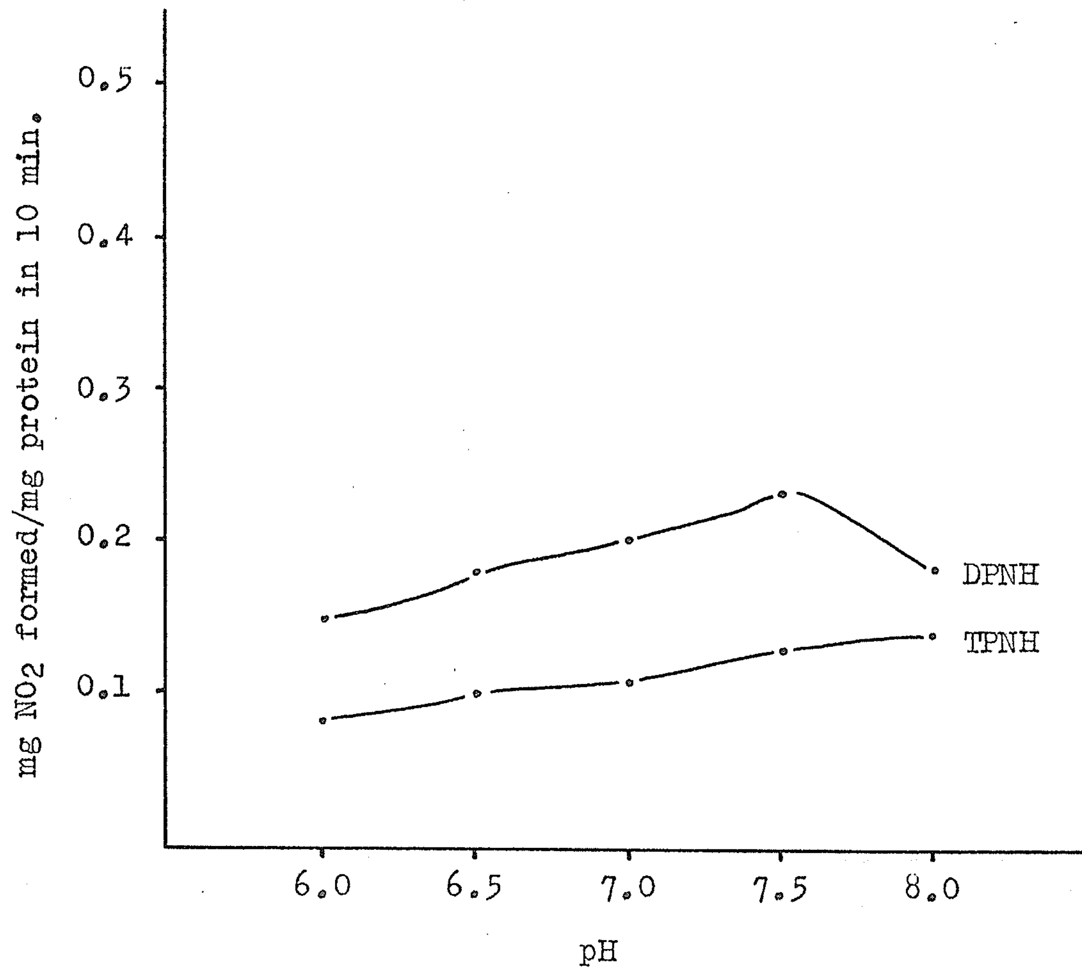


Fig. 6. Nitrate reductase activity in extracts from the transformed A. rubi culture P60(1012).

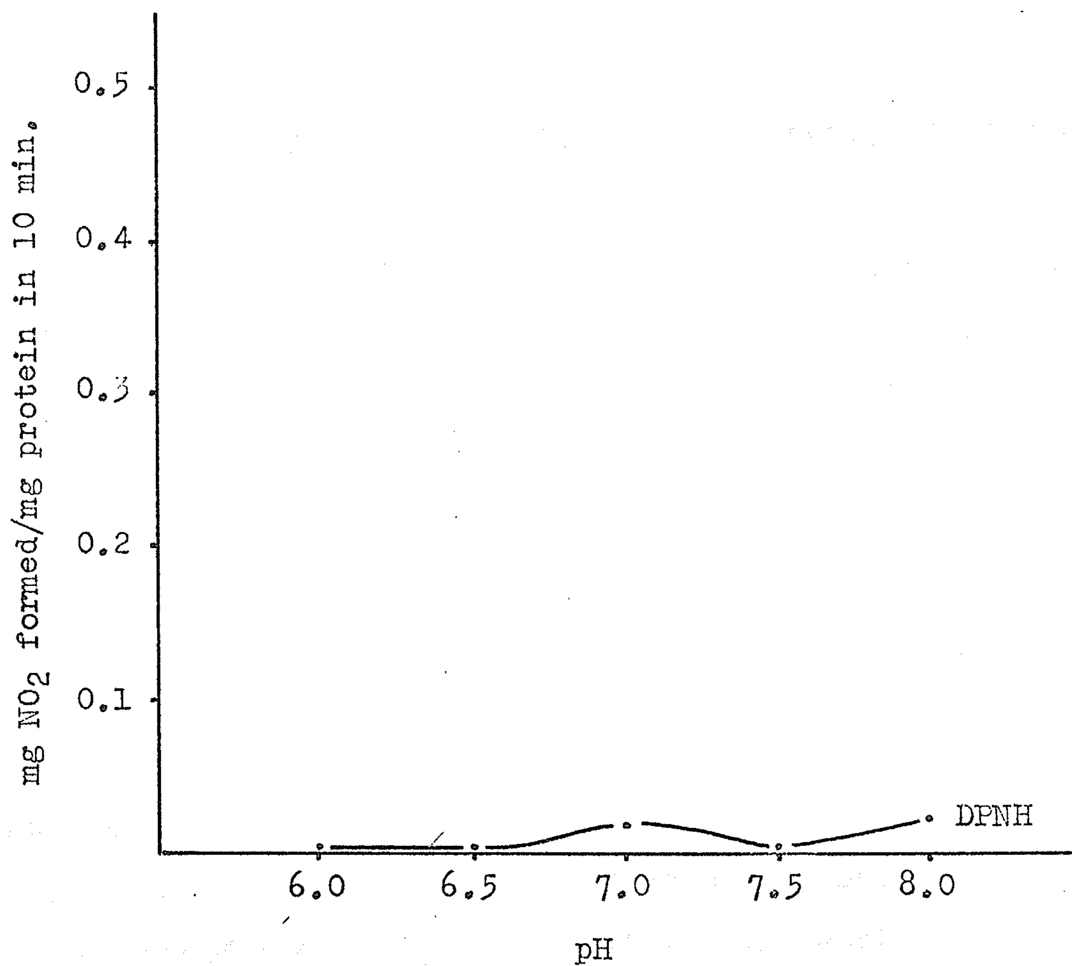


Fig. 7. Nitrate reductase activity in extracts from the parent _A. rubi_ P60

Conversion of nitrite by extracts from parent and transformed isolates was greater with TPNH than when DPNH served as the coenzyme. These results are presented in Table 17.

Table 17. Reduction of $NO_2$ by parent and transformed cultures.

| Culture | µg. $NO_2$ converted/mg. protein in 10 min. at pH 7.5[1] DPNH | TPNH |
|---|---|---|
| 1012 | 22.2 | 48.1 |
| 1012 (F60) | 59.3 | 72.0 |
| F60 | 203.0 | 296.5 |

1 The values reported represent the means of four determinations conducted on each culture extract.

The optimum pH for nitrite reductase activity in A. radiobacter 1012 extracts was at pH 8.0 for both coenzymes. In the case of the transformed A. rubi F60 (1012), the optimum pH nitrite reductase activity was at pH 7.5 for both coenzymes. Strong activity in A. rubi F60 extracts was evident at pH 7.5 with TPNH and at pH 8.0 with DPNH. These results are presented in Figs. 8, 9 and 10.

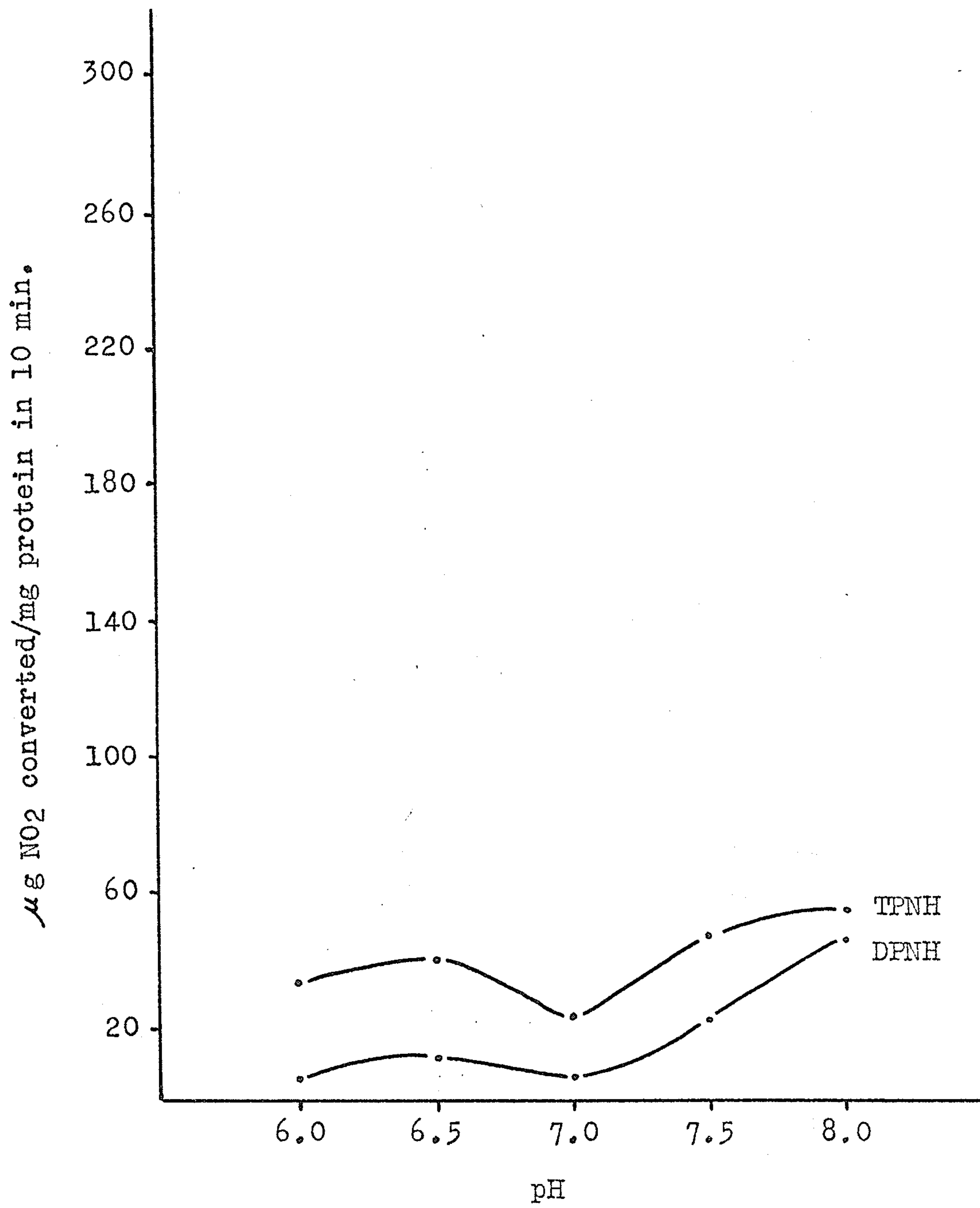


Fig. 8. Nitrite reductase activity in extracts from the parent A. radiobacter 1012.

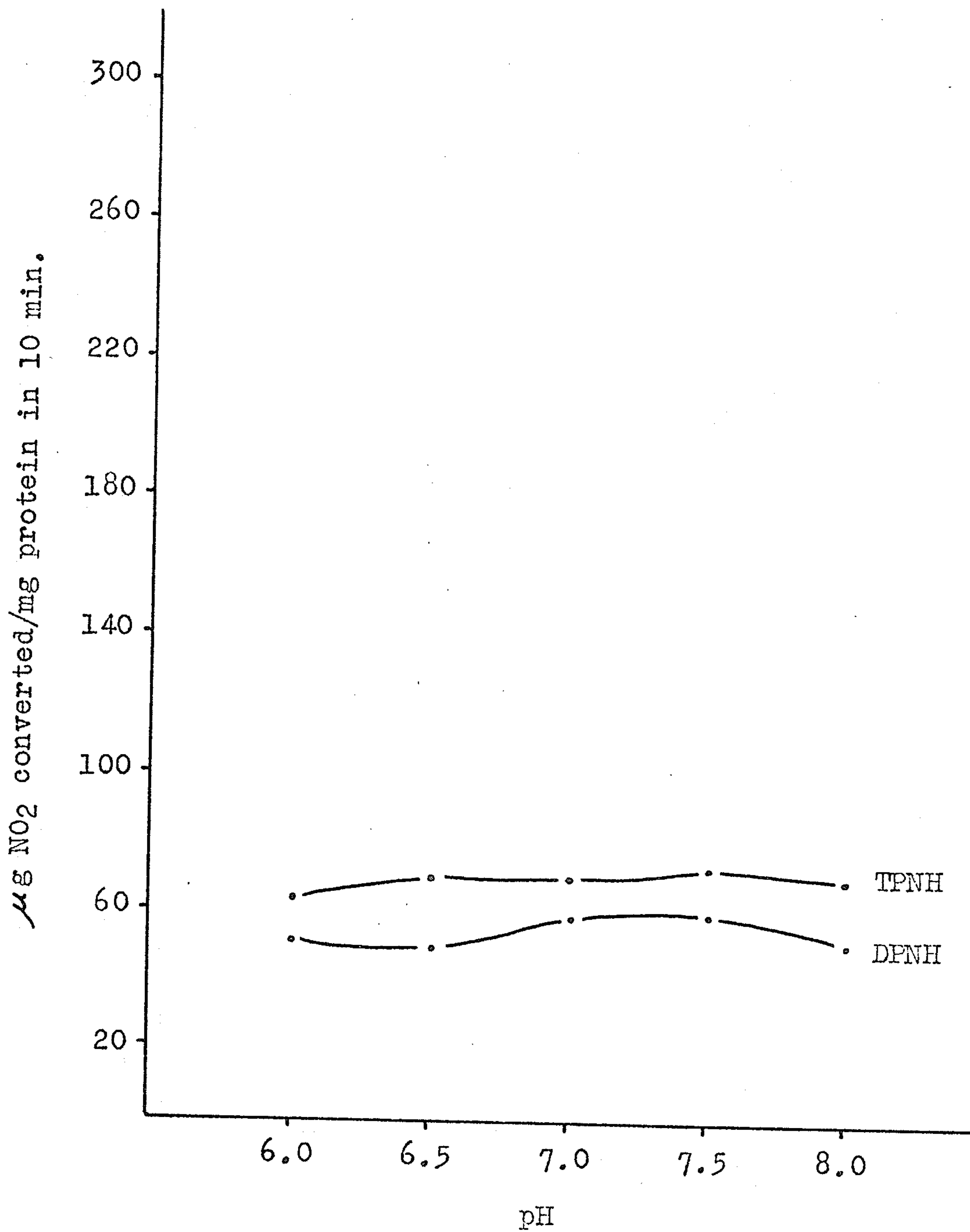


Fig. 9. Nitrite reductase activity in extracts from the transformed A. rubi culture P60(1012).

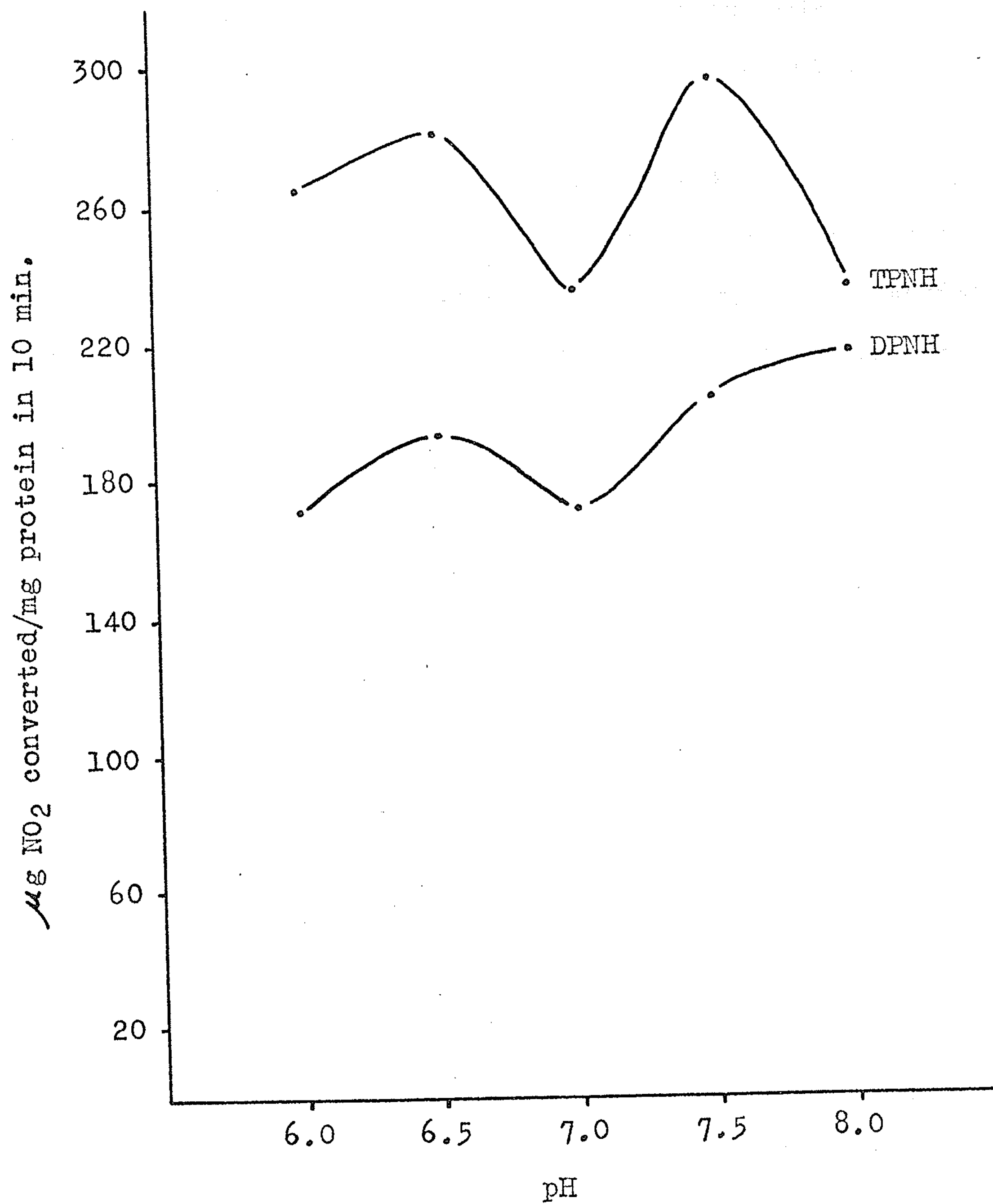


Fig. 10. Nitrite reductase activity in extracts from the parent *A. rubi* P60.

Hydroxylamine reductase activities in extracts from the parent and transformed cultures are presented in Figs. 11, 12 and 13. The plotted values represent the means of two sets of optical density readings taken at pH 7.5 with TPNH as the coenzyme.

The extraction procedures used in this case yielded enzyme preparations which displayed a high endogenous oxidation of DPNH. Several attempts to remove this activity by dialysis against a number of different buffer solutions were unsuccessful.

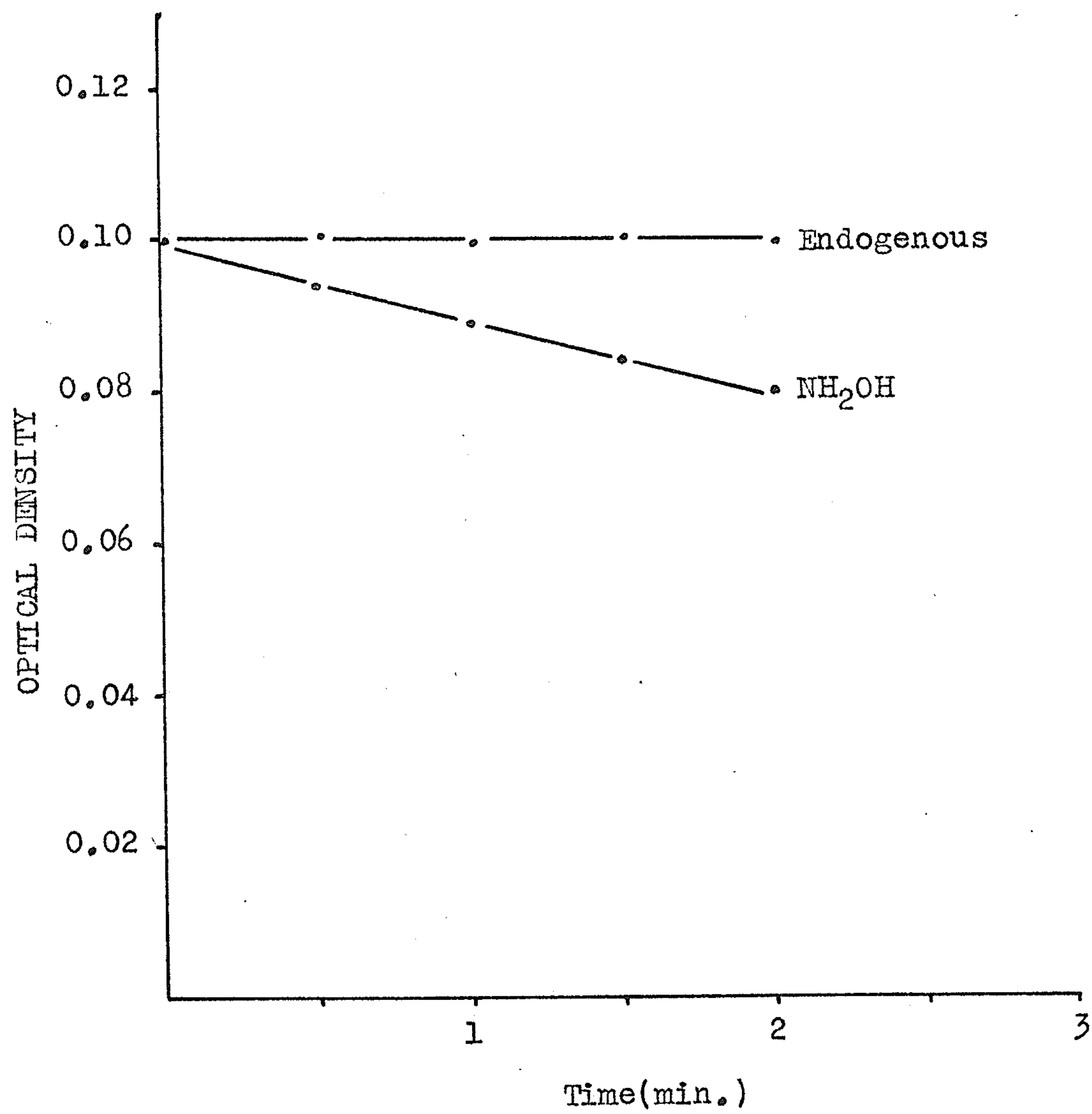


Fig. 11. Hydroxylamine reductase activity in extracts from the parent A. radiobacter 1012.

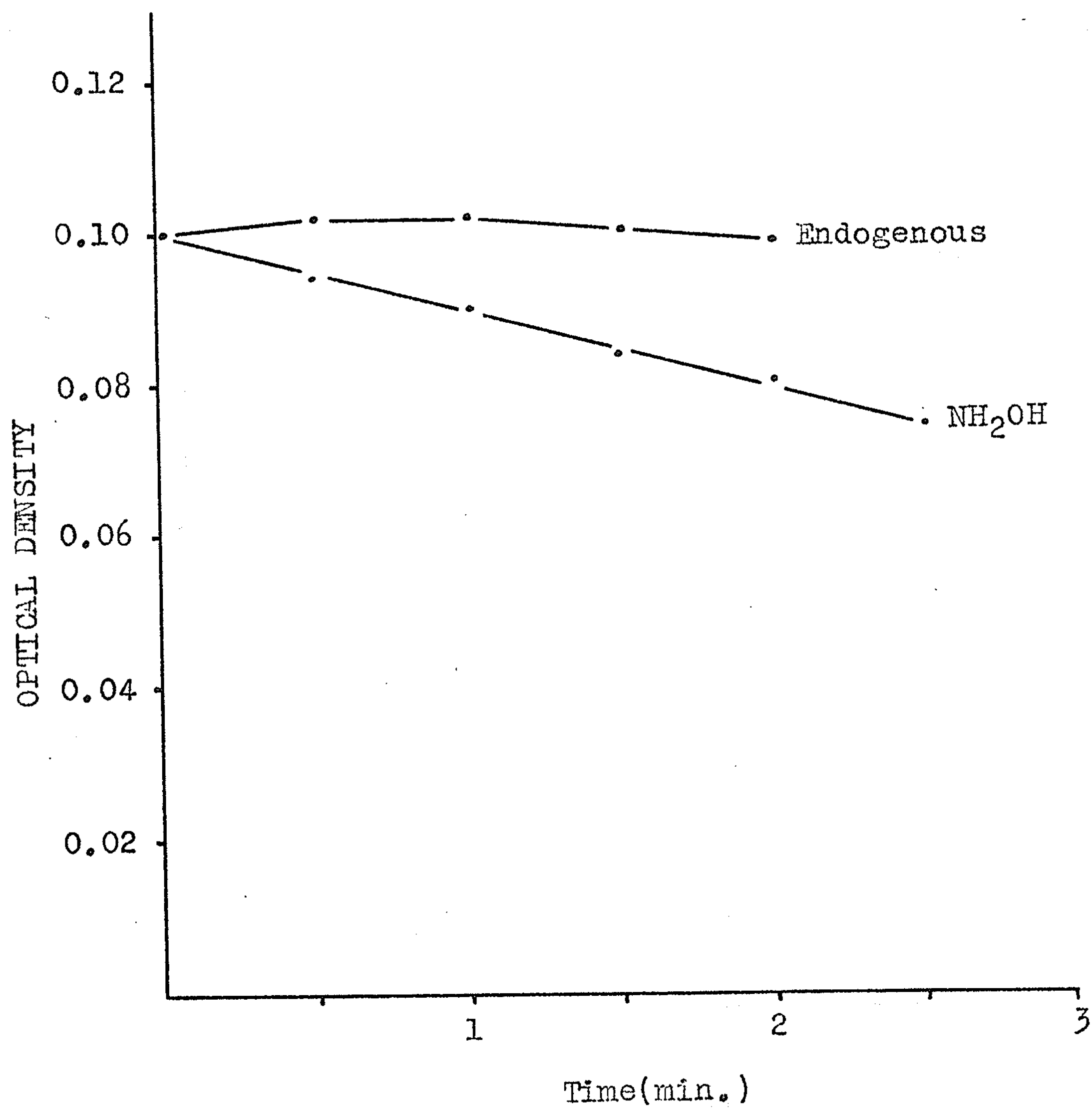


Fig. 12. Hydroxylamine reductase activity in extracts from the transformed <u>A. rubi</u> culture P60(1012).

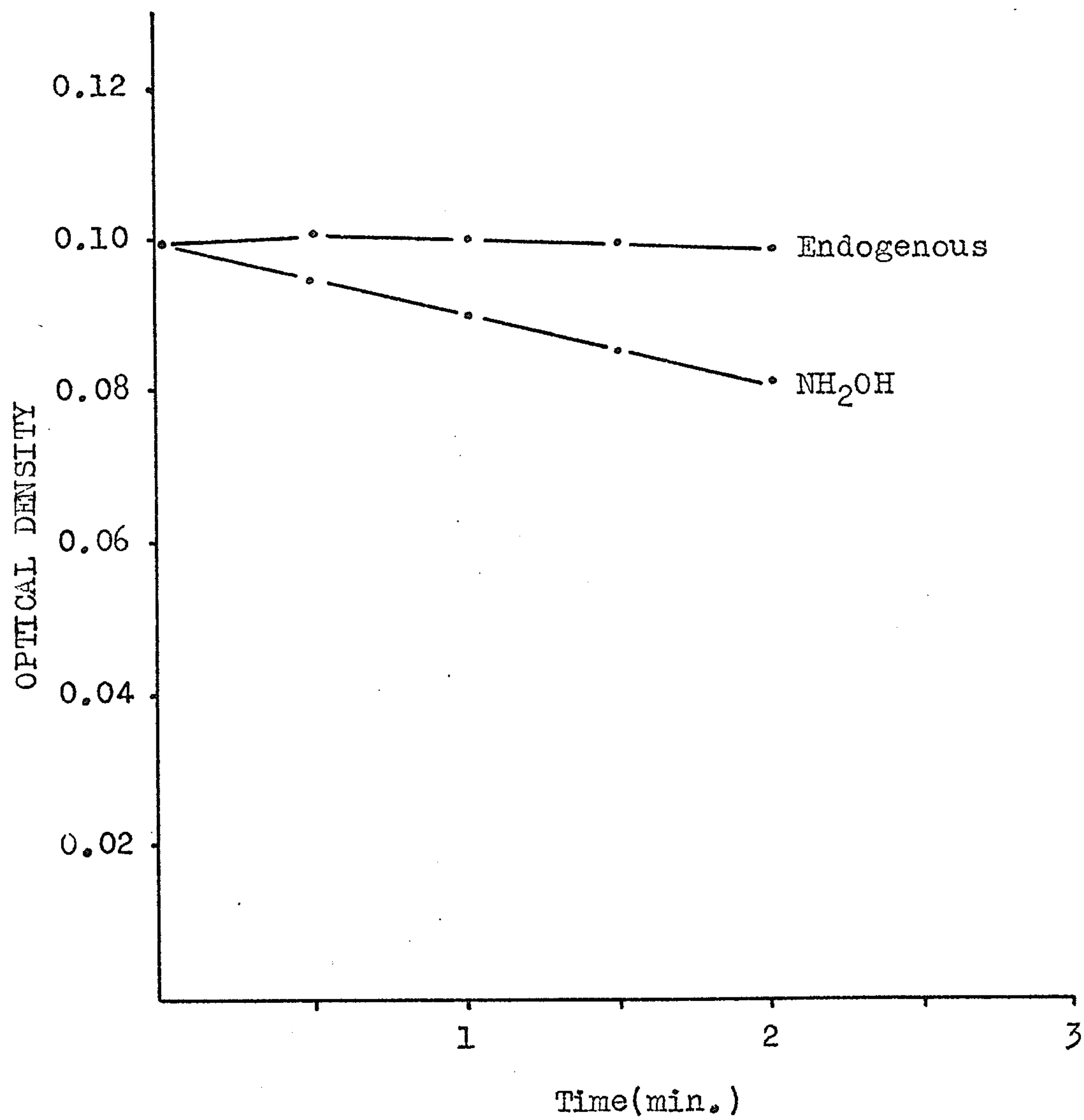


Fig. 13. Hydroxylamine reductase activity in extracts from the parent A. rubi P60.

# DISCUSSION

## DISCUSSION

Failure of growth to develop during incubation of the *A. radiobacter* 1012 filtrate at 25°C. for 96 hours was accepted to be evidence of the reliability of the filtration series.

Several possibilities should be considered in an evaluation of the growth response observed when *A. rubi* P60 cells were inoculated into the *A. radiobacter* 1012 filtrate. In the first instance, *A. radiobacter* 1012 cells may have excreted nitrogenous materials capable of sustaining growth of the *A. rubi* culture. Subsequent transfers by loop to fresh mannitol nitrate medium, however, would not likely carry significant amounts of such material. The growth response noted in repeated transfers could scarcely be attributed to substrate carry-over from the filtrate. Second, the possibility of adaptation by *A. rubi* P60 to the mannitol nitrate medium should be considered. Previous inoculations of *A. rubi* P60 culture to this medium gave no response suggestive of an adaptive process although incubations at optimum temperature were continued for several days. On the other hand, a good growth response by *A. rubi* P60 in the *A. radiobacter* filtrate occurred within 24 hours. As a third possibility, the growth response by *A. rubi* P60 in the filtrate represented the acquisition by transformation of the ability to use nitrate as the nitrogen source.

Lack of detailed information concerning nitrate assimilation pathways for these organisms makes it difficult to assess the failure of the _A. rubi_ P60 culture to grow with nitrate as the nitrogen source. If members of the genus _Agrobacterium_ have a nitrate assimilation pathway similar to that reported for _Rhizobium_ spp. by Chenese and Evans (1956) then failure to grow, or to respire significantly in the presence of nitrate, Fig. 4, might imply a lack of nitrate reductase activity. This implication was supported subsequently by results obtained from enzyme studies conducted on the parent _A. rubi_ P60 culture. These results presented in Table 16 indicate an insignificantly small nitrate reductase activity. Indeed, the only value reported could be attributed to experimental error inherent in the method of measurement. On the other hand, nitrite and hydroxylamine reductase activities by the culture were clearly indicated both in manometric studies, Fig. 4, and from results derived from enzyme experiments, Table 17 and Fig. 13.

The behaviour of the parent _A. radiobacter_ 1012 culture in manometric studies, Fig. 2, presented quite different results from those obtained with _A. rubi_ P60. A strong nitrate reductase activity suggested by manometric observations was later confirmed by enzyme studies, Table 16.

Again, departure from A. rubi P60 performance was noted in the case of respiration inhibition in the presence of nitrite by A. radiobacter 1012. Two possibilities may be considered in an attempt to account for this observation. One, nitrite was not an intermediate in the assimilation of nitrate by A. radiobacter 1012 and, moreover, was toxic to the cell. Second, nitrite was an intermediate and not toxic except at concentrations in excess of those produced by the cell under 'normal conditions'. Of these two possibilities, the latter is the more reasonable one on the basis of results reported in Table 17. Although A. radiobacter 1012 gave the lowest nitrite reductase activity of the three cultures examined, the enzyme's presence was clearly indicated. Such activity would scarcely be expected if nitrite was not an intermediate in the pathway. The nitrite concentration employed for manometric purposes, however, may have been at a level in excess of that which could be handled by the cell's nitrite reductase system. This might result in toxic accumulation of nitrite in the cell and ultimately in inhibition of respiration.

As in the case of A. rubi P60, a significant respiration activity was displayed by the parent A. radiobacter 1012 in the presence of hydroxylamine, Fig. 2. As well, hydroxylamine reductase activity was clearly demonstrated in the results presented in Fig. 11.

The culture designated as A. *rubi* F60 (1012) gave a strongly significant respiration response in the presence of nitrate as shown in Fig. 3. As well, moderately strong nitrate reductase activity was evident according to results presented in Table 16. With respect to respiration in the presence of nitrate, this culture behaved in the same way as the parent A. *radiobacter* 1012, from which, presumably, it acquired, by transformation, the ability to utilize nitrate.

Final confirmation of the reliability of the filtration method seems assured by an examination of A. *rubi* F60 (1012) respiration behaviour in the presence of nitrite. This respiration response reported in Fig. 3 closely resembles that of the parent A. *rubi* F60. If the post-inoculation growth response observed in A. *radiobacter* filtrate was due, in fact, to failure of the filtration to remove all A. *radiobacter* 1012 cells, then respiration in the presence of nitrite should be the same as for A. *radiobacter* 1012. A similar relationship would be expected in the case of ammonia as the nitrogen source.

In the case of nitrate and nitrite reductase activities reported respectively in Tables 16 and 17, activity values were intermediate between those reported for the parents, A. *radiobacter* 1012 and A. *rubi* F60.

Nitrite reductase activity values reported in Table 17 for _A. rubi_ P60 (1012) were probably under-estimations for the following reason. Activity measurements were made on cell extracts having some variation in protein content. In the case of the nitrite reductase measurements for _A. rubi_ P60 (1012) the cell extracts were considerably more dense than usual although extraction procedures were unchanged. A specific activity measured in such a preparation would tend to give a lower value relatively when compared to activities measured on purer preparations. This disparity would become even more pronounced when activity values are calculated on a one-milligram protein basis.

The respiration response by _A. rubi_ P60 (1012) in the presence of ammonia, Fig. 3, was unexpected. All replicate trials for this treatment gave similar results, i.e. an abrupt drop in oxygen uptake beginning near the end of the second hour. Although the parent _A. rubi_ P60 culture, Fig. 4, displayed a slow decline in respiration with ammonia as the nitrogen source, the decline began after the fourth hour and was gradual.

## SUMMARY

Competence for transformations between species in this genus does not seem to be unilateral. This is indicated by the apparent ability of an avirulent species to transfer a specific enzyme activity to a virulent member of the genus. On the other hand, pathogenicity may also be transferred from the virulent to the avirulent strain.

The results obtained have indicated that the nitrogen metabolism of the related species under consideration differ but may be altered in part by the phenomenon of transformation.

# GENERAL DISCUSSION

## GENERAL DISCUSSION

As stated in the Introduction to Part I, the purpose of this study was to determine whether any marked alterations in cultural or nutritional behaviour were associated with transformation of virulence between certain members of the genus *Agrobacterium*, Conn.

The assumption that alterations might reasonably be expected was based on the premise that the acquisition of virulence might place extra nutritional demands on the cell. These demands would be indicated, presumably, by changes in cultural response or in nutritional behaviour in a defined medium.

From the results obtained in the cultural and nutritional survey, it was evident that in a majority of the tests; such as growth in various carbohydrate broths, indole production, asparagine utilization, growth in litmus milk and on calcium glycerolphosphate agar; the virulent transformed isolates behaved essentially in the same manner as their avirulent acceptor parents. It may be assumed, therefore, that these tests did not indicate any changes in behaviour that were consequent to the acquisition of virulence.

Some transformed isolates, however, displayed cultural characteristics of the virulent donor parents. These were evident in growth response in mannitol nitrate medium, citrate utilization, $H_2S$ production and in nitrate reduction.

The responses by all transformed isolates, however, were not consistent in these differential tests. This suggests that the physiological activities upon which these tests are based were not involved in the acquisition of virulence.

Additional nutritional demands, as a consequence of the acquisition of virulence, were not indicated on the basis of the evidence presented in Table 15. Certain amino acid supplements were necessary for the growth of a few transformed isolates of the 1012 (P60) group. This dependence was not general for the group as a whole.

A recapitulation of results obtained on the basis of the cultural and nutritional tests employed leads to the conclusion that the acquisition of virulence through transformation was not accompanied by additional nutritional demands.

Consideration of the differential tests used in this study led to the conclusion that some were of doubtful value. As mentioned previously, difficulty was encountered in interpreting results of the nitrate reduction test. A negative result, except in the absence of growth, was meaningless, while a positive result merely indicated the presence of nitrite without indicating its physiological significance.

The fundamental importance of nitrate utilization and particularly the lack of detailed information in this respect concerning members of the genus <u>Agrobacterium</u> led to the investigations forming Part II of this study.

The results of manometric and enzyme studies suggested that the parent A. radiobacter and A. rubi strains possessed quite different assimilatory pathways for inorganic nitrogen sources. The demonstration of reductase activities in the presence of nitrate, nitrite and hydroxylamine led to the conclusion that the parent A. radiobacter 1012 possessed a nitrate assimilation pathway similar to that reported for Neurospora crassa by Evans and Nason (1953). In the case of the nitrate reductase activity by A. radiobacter 1012, however, a higher specificity for DPNH than for TPNH was indicated. Cheniae and Evans (1956) reported a similar DPNH specificity for a nitrate reductase isolated from a Rhizobium species.

A possible pathway for nitrate utilization by A. radiobacter is postulated as nitrate ⟶ nitrite ⟶ ? ⟶ hydroxylamine ⟶ ammonia. An unidentified intermediate between nitrite and hydroxylamine is indicated by the nature of the electron transfer system involved. As stated by Silver and McElroy (1954), "it is assumed that there are at least two enzymatic steps leading from nitrite to hydroxylamine, since most reductions of this type involve the transfer of two electrons". The nature of the unidentified intermediate has been investigated by Virtanen et al. (1948) and by Silver and McElroy (1954) among others. These investigators reported the presence of various oximino compounds as possible intermediates between nitrite and hydroxylamine. The participation of some organo-nitrogen complex in the case of A. radiobacter 1012 should be investigated.

As reported previously, _A. rubi_ P60 differed from _A. radiobacter_ 1012 in two major respects as far as the assimilation of nitrogen sources was concerned. The lack of nitrate reductase activity combined with weak responses in the presence of ammonia suggested a quite different assimilatory pathway. This contention was supported by the evidence of strong nitrite and hydroxylamine reductase activities displayed by the virulent parent. The nature of such a pathway remains open to conjecture. It seems reasonable, however, that in view of the strong nitrate reductase activity some steps involving organo-nitrogen compounds would follow, since growth by this species with ammonia as the sole nitrogen source was very weak. Whether such organo-nitrogen compounds represent the main entry for inorganic nitrogen in this case is still unknown.

The development of nitrate, nitrite and hydroxylamine reductases is considered by some investigators to be an adaptive response, according to Glass (1956). Although this contention may be a valid one for the reductases in _A. radiobacter_ 1012, the fact remains that _A. rubi_ P60 was cultured in a mannitol yeast extract medium containing, presumably, only trace amounts of nitrate or nitrite. Accordingly, it seems unreasonable to attribute the presence of strong nitrite and hydroxylamine reductase activities of _A. rubi_ P60 to an adaptive process.

On the bases of results obtained, the transformation of nitrate utilization capacity from A. radiobacter 1012 to A. rubi P60 seems clearly indicated. Moreover, the transformation was concerned only with the production of nitrate reductase; the remainder of the assimilatory pathway in A. rubi P60 was essentially unchanged.

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# APPENDIX

## Carbohydrate Media

Carbohydrate media were prepared by the addition of the various carbohydrate sources to Purple Broth Base, Difco, reconstituted with distilled water. Each carbohydrate source was added to give a concentration of 0.5% in the medium.

## Miscellaneous Differential Media

Nitrate Broth, Difco

The medium was reconstituted with distilled water and dispensed at the rate of 7.0 ml. per tube.

Tryptophane Broth

Nutrient Broth, Difco was used as the base in distilled water, Bacto Tryptophane was added to give a final concentration of 0.2% in the reconstituted medium.

Asparagine Broth

| | | |
|---|---|---|
| $MgSO_4 \cdot 7H_2O$ | .......... | 0.2 gm. |
| $K_2HPO_4$ | .......... | 0.2 gm. |
| NaCl | .......... | 0.2 gm. |
| $CaCl_2 \cdot 6H_2O$ | .......... | 0.1 gm. |
| Asparagine | .......... | 5.0 gm. |
| Tap water | .......... | 1000.0 ml. |

Adjusted to pH 7.0

Ferric Ammonium Citrate Broth

| | | |
|---|---|---|
| $(NH_4)_2SO_4$ | .......... | 0.5 gm. |
| $K_2HPO_4$ | .......... | 0.5 gm. |
| $MgSO_4 \cdot 7H_2O$ | .......... | 0.5 gm. |
| $CaCl_2 \cdot 6H_2O$ | .......... | 0.1 gm. |
| Distilled $H_2O$ | .......... | 1000.0 ml. |

Adjusted to pH 7.0

Litmus Milk

Litmus milk, Difco was reconstituted with tap water and dispensed at the rate of 5.0 ml. per tube. Sterilization was by autoclave at 120°C. for 10 minutes.

Lead Acetate Agar, Difco

The medium was reconstituted with distilled water.

Mannitol Calcium Glycerolphosphate Agar

| | | |
|---|---|---|
| Mannitol | .......... | 10.0 gm. |
| $KNO_3$ | .......... | 0.5 gm. |
| $MgSO_4 \cdot 7H_2O$ | .......... | 0.3 gm. |
| NaCl | .......... | 0.2 gm. |
| Calcium glycerolphosphate | ...... | 1.0 gm. |
| Agar | .......... | 15.0 gm. |
| Distilled $H_2O$ | .......... | 1000.0 ml. |

Adjusted to pH 7.2

Inorganic Basal Salts Broth, with Mannitol

| | | |
|---|---|---|
| Mannitol | .......... | 2.0 gm. |
| $KNO_3$ | .......... | 0.2 gm. |
| Salts A | .......... | 6.0 ml. |

Salts A

| | | |
|---|---|---|
| $MgSO_4 \cdot 7H_2O$ | .......... | 1.00 gm. |
| NaCl | .......... | 0.05 gm. |
| $FeSO_4 \cdot 7H_2O$ | .......... | 0.05 gm. |
| $MnSO_4 \cdot H_2O$ | .......... | 0.05 gm. |
| HCl (conc.) | .......... | 0.10 ml. |
| Distilled $H_2O$ to make | .......... | 25.0 ml. |

Salts B .... 6.0 ml.

Salts B

| | | |
|---|---|---|
| $K_2HPO_4$ | .......... | 2.50 gm. |
| $KH_2PO_4$ | .......... | 2.50 gm. |
| Distilled $H_2O$ to make | .......... | 25.0 ml. |

Distilled $H_2O$ .... 1000.0 ml.

The medium was adjusted to give a final pH of 7.2 after sterilization.