THE UNIVERSITY OF MANITOBA

STUDIES ON A NITROGEN-FIXING SUBARCTIC SOIL SPECIES (LABORATORY NUMBER G-15)

A Thesis

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Master of Science

by

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ABSTRACT

A study of the general morphological, cultural and physiological characteristics of a subarctic isolate (designated G-15) able to fix atmospheric nitrogen is presented. Based upon this study and upon evidence for the presence of violacein in the organism it was tentatively classified in the genus Chromobacterium.

Although a lactate utilizing system could be demonstrated by good growth in a lactate medium, by respiration studies and by pyruvate production in a cell sonicate, spectrophotometric evidence for the lactate dehydrogenase enzyme was not obtained. Copious quantities of fat were found to accumulate in this organism and were implicated in a prolonged high rate of endogenous respiration.

The ability to fix atmospheric nitrogen was examined using a modification of recently developed radiotracer technique. The experimental apparatus and the means of producing the radioisotope of nitrogen, $^{13}N_2$, are described and photographs of the apparatus are presented. Components of the medium, judged best suited for support of this organism under nitrogen fixing conditions, were examined for their individual contribution to the growth response. Of a number of energy substrates studied, lactate, glucose, sucrose and mannitol were found to support growth under

nitrogen fixing conditions. Glucose is shown to be a potent inhibitor of nitrogen fixation in lactate grown cells. The presence of the enzyme hydrogenase could not be demonstrated and this is discussed in view of its postulated role in nitrogen fixation. Evidence of fixation in sonic extract of the cell suspension is presented.

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INTRODUCTION

INTRODUCTION

'Extending northward from approximately Latitude 55 degrees, the land mass of Canada, including the Arctic Archipelago, represents one of the last relatively untouched frontiers of the world'. Written fifteen years ago, this statement would have been largely true. With the exception of geological and geographic surveys and the limited penetration by the mining industry and the fur trade, little more information was available in 1950 than was known one hundred years prior to that time.

The last fifteen years, however, has seen an increasing interest develop in many phases of Arctic and Sub-Arctic research. Improved transportation has been the cornerstone of this interest and has provided the means for travel on or over the northern forest and tundra plains. Coupled with improvements in Arctic travel and communication the establishment of northern research centers such as those at Fort Churchill and Inuvik has done much to encourage an active interest in Arctic research.

Much of the work done in the past decade or so has been in the fields of geology, meteorology, climate studies, permafrost engineering and other aspects of the physical sciences. In the biological field, the outstanding contributions in Botany by Richie (43), Scoggan (45) and Porsild (42) should be mentioned along with the entomological pro-

grams of the Canada Department of Agriculture and the Defence Research Board. Ornithological studies by the Canadian Wildlife Services have given us much information on indigenous and migratory bird life.

A review of the literature of Arctic microbiology, however, reveals little to be complacent about in this respect. In a survey based on Vols. I to XI of the Arctic Bibliography, for example, it was noted that over eighty percent of the several hundred publications pertaining to Arctic microbiology were authored by Soviet investigators. Of the remaining twenty percent, American and Danish scientists contributed much the larger portion. Certainly, as far as North Central Canada is concerned the single report of James and Sutherland (27) constitutes the only Canadian contribution in soil microbiology.

In contrast, a number of interesting studies have been conducted in the Point Barrow region of Alaska (3,5,18) and in the delta of the MacKenzie River. For the most part, however, these studies have been of an ecological nature with only an indirect interest in the role Arctic micro-organisms play in those soil-building processes permissible under severe climatic conditions. Recently, personnel of the Biosciences Division of the National Research Council of Canada have examined Arctic marine microflora and sediments within the scope and aims of the Polar Continental

Shelf Project.

Since early in 1965, soils from the Fort Churchill region have been examined by Ram Dular and N.E.R. Campbell of the Department of Microbiology, the University of Manitoba and this program is continuing at this time. One aspect of this study deals with microbial influences in the nitrogen economy of the soils in question. In a region where soil temperatures seldom exceed 17 degrees centigrade during the brief summer interval, pediogenesis is slow and soils are, for the most part, immature.

Of particular interest in the program is the question of asymbiotic nitrogen fixation to determine its role, if any, in the soil nitrogen economy. It should be mentioned here that a recent preliminary survey of Irvine and Campbell (25) indicated that nodulated legumes and non-leguminous species were infrequently encountered. This may suggest a rather restricted contribution by the symbiotic nitrogen fixers whereas a systematic investigation of over 150 microbial isolates by Dular (21) showed that at least twenty different species, including several yeasts and one type of mold possessed the ability to grow well in a chemically defined nitrogen-free medium.

One of the bacterial isolates, laboratory number G-15, was selected as the organism of study, for the investigation presently being reported. An attempt has been made to

examine in some detail the morphological and cultural characteristics of this isolate, to identify its taxonomic position and to examine various parameters of its nitrogen fixing capacity.

HISTORICAL

HISTORICAL

In a review of the literature dealing with microflora of Arctic and Sub-Arctic soils of North America, the reader finds little published information pertaining to North Central Canada. A singular exception to this is the publication by James and Sutherland in 1942 (27) which describes a search for viable organisms in the permafrost. They were not able to show conclusively that the organisms encountered were, in fact, from the permafrost region; or represented, on the other hand, surface organisms carried down by ground water movement. In 1957 Brown-Beckel (6) reported on seasonal changes in the soil temperature gradient. Her data, based upon thermocouple probes placed at varying depths in different soils of the region, gave the first definitive picture of the influence of air temperature, wind velocities and relative humidity on soil temperature of the Hudson Bay lowland formation.

About the same time Boyd and associates, working from the Naval Research Lab at Point Barrow, Alaska, began a series of studies which demonstrated that bacteria concerned with soil transformation were active only in the brief summer interval. In 1962 Boyd and Boyd (4) reported the presence of Azotobacter species in Alaskan soils but felt that during the short active season little contribution to soil fertility could be expected by this group. They further reported in 1962 (3) on the basis of soil and water studies

in the vicinity of Point Barrow, the presence of thermophiles and coliforms but concluded their presence was due to recent contamination by human or animal feces. However, in 1964 (5) working on sample corings extending well into the permafrost, they reported thermophiles at relatively constant counts to the depth of the core (12 feet). Mesophiles were noted mainly in the active layer near the surface with molds restricted to the upper six inches. Psychrophilic bacteria were encountered to a depth of 12 inches.

Day and Rice in 1964 (18) and Ivarson in 1965 (26) reported on the microflora of the permafrost in the MacKenzie Valley, N.W.T., while McDonald et al in 1963 (31) working with marine sediments obtained from the Polar Continental Shelf group examined factors of cold tolerance of bacteria and studied the thermal lability of their enzymes in comparison to those of mesophilic organisms.

Clearly, of the few publications related to the microbiology of North American soils north of Latitude 55 degrees only two deal with North Central Canada; one, by James and Sutherland and the other, by Brown-Beckel. Of these, the temperature studies by Brown-Beckel formed the basis for the selection of soils for our study.

One of the major limitations to the breakdown of organic residues of plant and animal origin is the availability of nitrogen. In soils high in organic matter

available nitrogen tends to be low during periods of active decomposition. This situation seems also to be true for the Fort Churchill soils. The relatively large numbers of asymbiotic aerobic nitrogen fixers noted by Dular (21) suggested that these organisms may play a significant role in providing nitrogen to the soil from the otherwise unavailable resources in the atmosphere.

Until the early 1940's determination of nitrogen fixation from bacterial cultures was usually carried out by total nitrogen determinations based upon Kjeldahl method. These quantitative determinations of increase in cell mass under nitrogen-free conditions were supplemented by visual turbidometric observations. However, Burris et al in 1943 (12), reported using $^{15}N_2$ as an isotopic tracer of nitrogen fixation. This technique is far more sensitive than the Kjeldahl method and permits the use of smaller amounts of cell material. Essentially the method is based upon the use of $^{15}N_2$ enriched atmospheres and determination of above normal concentrations of $^{15}N_2$ in the protein hydrolysis products of the cell. From 1950 to the present time this assay technique has been modified and improved and presently constitutes the most widely used measure of nitrogen fixation. The major disadvantages are twofold. One, the cost of heavy nitrogen is high and two, the assay procedure requires a minimum of two days from start to finish.

Mortenson in 1961 (36) demonstrated a somewhat different approach to determining nitrogen fixation in the case of extracts of Clostridium pasteurianum. Using a micro diffusion system he showed that NH_3 liberated in extracts of the culture corresponded directly to a measure of the nitrogen fixed. That same year Nicholas et al (39) published details of an even more sensitive assay system based upon radioactive isotope $^{13}N_2$. Assay procedures based upon this radio tracer are probably more sensitive by a factor of ten than those available with $^{15}N_2$ system. The major difficulty with the radioisotope of nitrogen is its short half life of 10.05 minutes. This precludes the possibility of transporting and storing the gas which must be used immediately after production. As well, the short half life places restrictions on the design of experiments.

Studies of nitrogen fixation by whole cells remained as the basic approach until 1960 when Carnahan et al (17) published methods of preparing 'active' cell free extracts. Although several attempts had been made previously to demonstrate nitrogen fixation at the cell free level, results were inconsistent and seldom could be verified (14). Since 1960 the main emphasis has been placed on enzyme investigations in an attempt to elucidate the fixation mechanism. Partial purification of the nitrogenase involved has been carried out and several natural and artificial electron

donors capable of activating the reduction of N_2 to NH_3 have been discovered.

Dua and Burris in 1965 (20) reported a 121 fold purification of the Clostridium pasteurianum system which Mortenson et al (37) had fractionated into two components. Bulen (8) demonstrated a somewhat similar purification method for the Azotobacter system although achieving only a 7 fold increase in activity.

A hypothetical mechanistic scheme reported by Burris in 1966 (14) for the Clostridium pasteurianum system involves an initial phosphoroclastic split of pyruvate yielding reduced ferredoxin, acetyl phosphate and CO_2. The reduced ferredoxin can be oxidized in the presence of hydrogenase to release H_2 gas. This is a freely reversible reaction. The acetyl phosphate via a transphosphorylase can form ATP and acetate. The reduced ferredoxin and ATP thus formed can then interact with nitrogenase to reduce N_2 to NH_3 stepwise. No products are released from the enzyme until NH_3 is formed.

This scheme incorporates the known requirements for ferredoxin (Mortenson, (38)), for ATP (Dilworth et al, (19)) and for pyruvate. It also illustrates how H_2 can be coupled to ferredoxin via hydrogenase to supply necessary reducing power. However, it does not indicate a possible intermediate between ferredoxin and nitrogenase which is postulated by

Hardy et al (24). Their intermediate can be reduced either by ferredoxin or by dithionite and, when reduced, is activated by ATP to reduce N_2 to NH_3.

In studying nitrogen fixation it is important to bear in mind that while the basic N_2 conversion to NH_3 may be the same, the complete system, in fact, is likely to be very different in different organisms. The anaerobic system (Clostridium pasteurianum) has been separated into two components. The complete system is rapidly and irreversibly inactivated by O_2, and is cold labile although activity can be restored with reducing agents. It is soluble, has an absolute requirement for ferredoxin, and demands pyruvate as an energy source. On the other hand, the aerobic system (Azotobacter vinelandii) is inhibited by O_2 but not inactivated and is not cold labile. The 'complete' enzyme is particulate (sedimenting in 6 hours at 144,000 x g), will use ferredoxin or dithionite as an artificial electron donor but does not contain ferredoxin, and no definite source of reducing power has been established.

Both systems appear to have an ATP-dependent hydrogenase which parallels the nitrogen fixing system in purification and in conditions of optimal activity. It differs from the Burris concept of a 'normal' hydrogenase which, as has been pointed out by Thimann (49), is often implicated in nitrogenase activity by inference rather than by direct

observation. Moreover, it has been suggested (Burris (14)) that the ATP-dependent hydrogenase has a distinct site on the nitrogenase separate from the N_2 site.

This year, 1966 (16), Campbell, Dular, and Lees at the IX International Congress for Microbiology reported using $^{13}N_2$ to determine fixation in a number of subarctic isolates. They found approximately twenty species of bacteria, one species of Rhodotorula and an isolate identified as a member of the genus Pullularia all with nitrogen fixing capacity. One of these bacterial species, originally isolated from the soil of the Goose Creek area was designated laboratory number G-15 (Dular (21)).

Presently, studies with these organisms are continuing. From these studies, including the one herein reported, it is hoped to provide information concerning the role of asymbiotic nitrogen fixation in the soils of the region.

SECTION I

CULTURAL STUDIES OF THE ISOLATE

MATERIALS AND METHODS

ISOLATION AND PROPAGATION

On examination of the spread plates prepared from soils of the Goose Creek area, Dular noted the appearance of a number of purple pigmented colonies. His isolation procedure may be briefly described as follows: from 10 gm samples of soil suspended in 100 ml of sterile distilled water serial dilutions were prepared, shaken vigorously and allowed to settle. From the supernatant 0.1 ml aliquots were uniformly spread over the surface of McDonalds' (31) medium B using a sterile bent glass rod. Plates prepared in this way were incubated at 10 degrees centigrade for one week following which total colonies were counted with the aid of a Darkfield Quebec Colony Counter.

McDonalds' medium B (31) contained in one liter of distilled water K_2HPO_4, 1.0 gm; $MgSO_4.7H_2O$, 0.2 gm; $FeSO_4.7H_2O$, 0.05 gm; $CaCl_2$, 0.02 gm; $MnCl_2.4H_2O$, 0.002 gm; Na_2MoO_4, 0.001 g; NH_4Cl, 1.0 gm; yeast extract, 1.0 gm; glucose, 0.1 gm, and agar (Noble) at a concentration of 1.5%. Final pH adjustment was made to 7.2 with NaOH.

The initial cultural survey of these purple pigmented organisms demonstrated their ability to grow well in a nitrogen free medium under an ammonium free atmosphere and,

accordingly, presented evidence for their ability to fix N_2. Therefore, this culture and others isolated at the same time which possessed the N_2 fixing potential, were selected for study of their contribution to soil fertility.

The culture designated G-15, was hereafter routinely propagated on nitrogen-free medium*. For general cultural and taxonomic investigations a variety of media were employed. Unless otherwise stated, the media, stains and reagents for biochemical tests were prepared according to "The Laboratory Manual for General Bacteriology" (5th edition, 1961) by G.L. Peltier, G.E. Georgi, and L.F. Lindgren (41).

Customarily, for growing large masses of cells for experimentation, colony material was transferred from an N_2-free slant culture either with a sterile loop or by adding sterile medium, emulsifying the colonies and transferring the cell suspension entirely. An initial transfer to a 7.5 ml volume of nitrogen-free broth was propagated, for 4-5 days at room temperature, then aseptically added to a 100 ml volume of the same medium and incubated for a further 3-7 days. The resultant cell population was used as an inoculum for 1.5 liter flask culture of the same medium.

It should be pointed out that soil samples from

*See Section II for a description of this medium.

which this organism was isolated ranged in pH from 5.7 to 6.4.

GROWTH TEMPERATURE STUDIES

Growth temperature responses were examined in unshaken liquid cultures at 11°C, 22°C, 28°C and 37°C in nitrogen-free medium and in medium containing a NO_3^- nitrogen source. Stationary (unshaken) cultures were used because it was felt that precise replication of aeration conditions in each flask would not be possible and since it was observed that aeration stimulated growth, lack of uniformity in aeration would lead to erroneous results. The medium, identical except for nitrogen content, in one liter contained sodium lactate, 6.0 gm; K_2HPO_4, 0.1 gm; NaCl, 0.1 gm; Na_2MoO_4, 0.001 gm; $MgSO_4 \cdot 7H_2O$, 0.3 gm; and Larsen's * (29) trace element solution. In the nitrogen containing medium 0.5 gm of KNO_3 was added. The final pH was 6.9-7.1 and no further pH adjustment was required.

PIGMENT STUDIES

In the beginning it was noted that aside from the rather creamy color on nutrient agar this organism also produced two distinct pigments; one purple and one red. The purple pigment was initially examined for solubility

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*See page 39.

in dilute NaOH and in dilute HCl. Subsequently, the solubility in water, ethanol, acetone, methanol and ethyl acetate were also considered. Pigment extraction and partial purification proceeded as follows:

Approximately equal volumes of ethyl acetate were added directly to the broth culture and the mixture shaken for two hours at room temperature. Then, the resultant purple-blue layer separated by separatory funnel. The pigment solution was evaporated to dryness under vacuum, washed several times with H_2O, again dried, and then dissolved in acetone. The partial purification from acetone was achieved by adding H_2O until a faint blue haze began to appear. The water-acetone mixture was stored at $4^{\circ}C$ for 12 hours. Following this, the particulate (haze) fraction was collected by centrifugation at 10,000 x g, washed with water, air dried and again dissolved in acetone. The crystallization procedure was carried out three times. A spectrum scan of the pigment in ethanolic solution was carried out using a Unicam SP700 spectrophotometer and is given in Figure 3.

RESPONSE TO VARIOUS NITROGEN SOURCES

Although the organism was recognized as an asymbiotic nitrogen fixer it was decided as part of a general cultural study to examine growth performance in a mineral salts medium

with various sources of nitrogen. These different forms of inorganic nitrogen; KNO_3, $NaNO_2$ and $(NH_4)_2SO_4$, were added at levels of 0.5 gm/liter.

ENERGY SUBSTRATES

Again, as a part of the general cultural studies a number of different energy substrates were examined for their ability to support growth of the culture. The initial isolation had been made, as previously noted, using glucose as the energy source in the medium. In addition to glucose, sucrose, mannitol, and lactate were added to separate flasks of the basal salts medium at a concentration of 5 gm/l.

To assess the ability of the culture to produce acid and gas from the more commonly used energy sources fermentation vial tubes were prepared with a purple broth (Difco) base to which had been added glucose, lactose, sucrose, maltose, mannitol, glycerol, and sodium lactate at a concentration of 5 gm/l.

GENERAL CULTURAL PROPERTIES

The behavior of the culture in other routine diagnostic media was also considered. The growth response in citrate broth, tryptic soy broth, urea medium, nitrate broth and gelatin stab cultures was noted. As well the culture

was inoculated to litmus milk, high salt (NaCl) broth, nutrient broth pH 9.7, and brain-heart-infusion broth; streaked on plates of eosin methylene blue medium, McConkey's medium, tellurite medium, nutrient agar, tryptic agar, glucose agar and blood agar. Methyl red, Voges-Proskauer and catalase tests were also carried out.

STAINING CHARACTERISTICS

Staining procedures included Gram's stain, spore stain, simple stain to determine cell grouping, iodine stain for starch and Sudan Black B stain for evidence of fat production.

PHYSIOLOGICAL EXPERIMENTATION

ENDOGENOUS RESPIRATION IN THE CULTURE

Of the energy sources examined in a qualitative sense, two, namely glucose and lactate were chosen for further quantitative studies. Preliminary Warburg experiments had shown an apparently high rate of endogenous respiration suggestive of the fact that energy products may have been stored in the cell during active growth. To evaluate this aspect of the problem a series of Warburg studies were conducted using lactate in various concentrations over periods of time, including a starvation interval

in each case of 60 hours. A typical curve series is presented in Figure 4.

LACTIC DEHYDROGENASE

Since the organism readily utilized lactate as a carbon energy source it was felt pertinent to briefly study the lactic dehydrogenase system likely present in the conversion of lactate to pyruvate. The enzyme was assayed by a number of different procedures or techniques. In addition to whole cell respiration (as previously noted) they are as follows: determination of pyruvate production and spectrophotometric determination of (i) appearance or disappearance of NADH in the reaction and (ii) reduction of ferricyanide.

Pyruvate production was determined using a sonic extract of the culture with added lactate. Essentially, this experimental design was similar to that used in the Warburg study of whole cells and O_2 uptake was accepted as indicating the reaction was proceeding. At the end of a 60 minute interval a 20 ml sample was removed from the Warburg flask and treated with 5 volumes of trichloroacetic acid. Pyruvate was determined by the hydrazine colorimetric method as reported by Friedemann (23).

Aliquots of the sonicate-trichloroacetic acid mixture,

in the amount of 3.0 ml each, were dispensed to clean test tubes and were then incubated at 25°C for 10 minutes in a water bath. At precise intervals of 30 seconds 1.0 ml volumes of a 0.1% hydrazine solution were added in series and incubations were continued for a further 5 minute interval in each case. At the end of the 5 minute incubation interval to each tube 3.0 ml of benzene were added, thoroughly mixed, and the aqueous phase in each case removed by pasteur pipette. To each sample so obtained 6.0 ml of 10% Na_2CO_3 solution was added and thoroughly mixed. Five ml portions from the carbonate layer thus formed were transferred to colorimeter tubes and stored in a water bath at 25°C. At 30 second intervals each tube, in turn, received 5.0 ml of a 1.5 N NaOH solution. Allowing 5 minutes in each case for development of color, readings were taken at 420 mμ (blue filter) in a Klett-Summerson colorimeter and were compared to a standard curve for pyruvate. The results are presented in Table II.

Spectrophotometric assays for lactic dehydrogenase were carried out by measuring the appearance or disappearance of the reduced form of the pyridine nucleotide (NAD) at 340 mμ with both a Gilford Model 2000 recording spectrophotometer and a Unicam SP700 spectrophotometer.

The assay system for the forward reaction (lactate to pyruvate) contained in a volume of 3.0 ml, 10.0 umoles lactate, 1.0 μmoles NAD, various levels of enzyme prepara-

tion, and 0.1 M potassium phosphate buffer pH 7.2.

A measure of the back reaction (pyruvate to lactate) was carried out by measuring the disappearance of NADH as follows: in a final volume of 3.0 ml, 0.2 μmoles NADH, 1.0 μmoles sodium pyruvate, varying amounts of enzyme preparation and 0.1 M potassium phosphate buffer pH 6.0. It was considered necessary in both these assays to include a control to account for the possible presence of NADH oxidase. For the back reaction the control was similar to the test assay system but did not include pyruvate. For both the forward and reverse reactions a control, using the same systems, was incubated in anaerobic cuvettes during measurement.

The reduction of ferricyanide was assayed using a system containing 3.0 μmoles $K_3Fe(CN)_6$, 10.0 μmoles lactate, enzyme preparation and 0.1 M potassium phosphate buffer pH 7.5 in a final volume of 3.0 ml. The reaction was measured both anaerobically and aerobically, in a spectrophotometer at 420 mμ.

RESULTS AND DISCUSSION

GENERAL DESCRIPTION OF THE ORGANISM

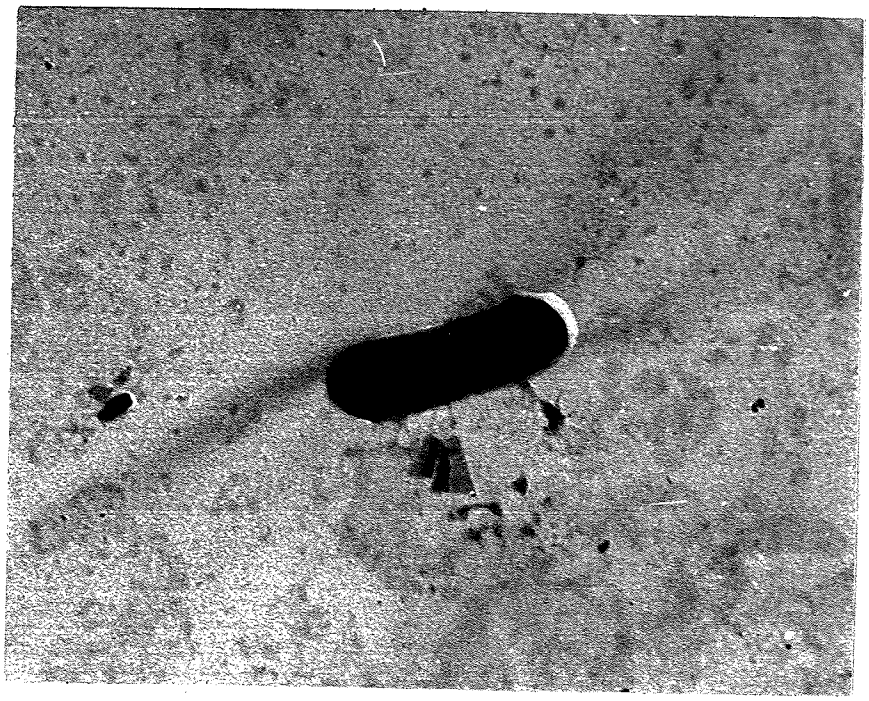
TABLE Ia. Morphology, Staining and Colony Appearance.

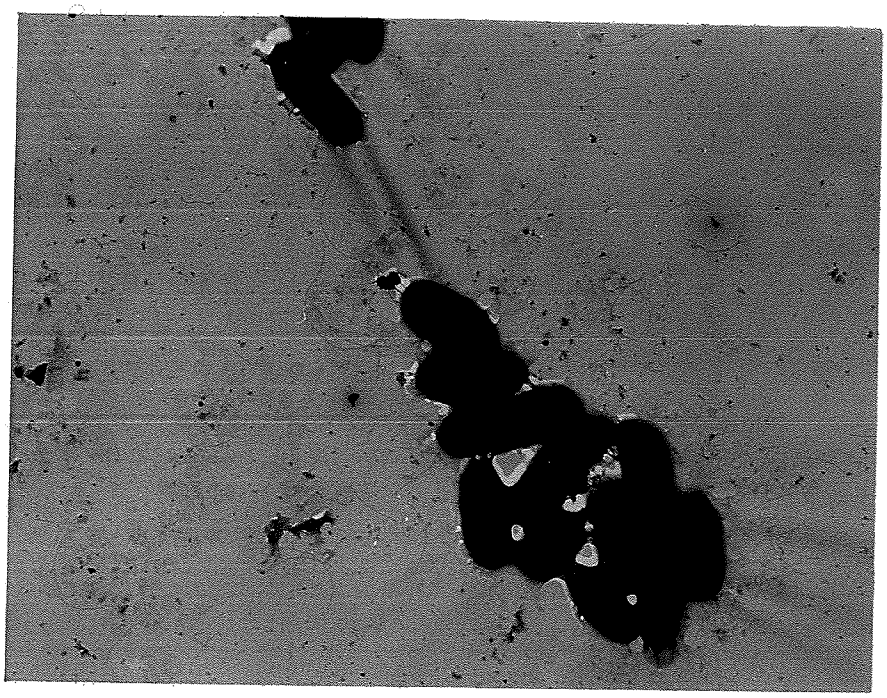
The culture designated G-15 is comprised of small rods with rounded ends, showing some variation in size: average length of 1.5 mu and average width of 0.75 mu. Gram negative, no endospores, no starch inclusions by iodine stain, fat distribution throughout the cytoplasm as demonstrated by Burdon's stain.
Electron micrographs show typical morphology of the isolate as illustrated in Figure 1.

In liquid media the cells tend to clump but, on staining, no regular aggregation is apparent. In an unshaken medium cell clumps settle out rapidly. In a culture medium containing a high carbohydrate concentration (up to 20 gm/l of fermentable carbohydrate) large quantities of slime with a very gummy consistancy are produced. Even at low carbohydrate concentrations some slime production is apparent.

On nutrient agar streak plates the colonies developed to a diameter of 3-5 mm within 36 hours. They were raised, with a glistening smooth surface and entire margins; were creamy yellow in color and developed a slightly sour, musty odor. On nitrogen-free agar medium the colonies were raised with a glistening smooth surface and entire margin; were translucent and very strongly adherent to the agar surface. Moreover, it required 3-5 days to produce colonies

FIGURE 1. Electronphotomicrographs of 48 hour old cells of the isolate G-15.





similar in size to those produced on nutrient agar in 36 hours and no odor was detectable.

TENTATIVE IDENTIFICATION OF G-15

From a consideration of morphology, pigmentation and biochemical and cultural tests, as noted in Table I, it is apparent that G-15, if not a member of the genus Chromobacterium, is closely related to it. A consideration of the classification schemes proposed by Bergey (1) and by Skerman (46) suggests the organism belongs in the genus Chromobacterium but no precise species identification could be made. The principal variance with recognized species of Chromobacterium lies in the matter of growth temperature. Unlike the recognized species of Chromobacterium which show distinct temperature preferences this culture grows better at 11°C than at 37°C as indicated in Figure 2. The listed species of Chromobacterium on the other hand, are roughly divided on the basis of temperature into two groups; those which grow well at 4°C but not at 37°C, and those which grow well at 37°C but not at 4°C. Considering the natural habitat of this culture it may well be a member of the genus Chromobacterium which has become adapted to growth at the lower temperature ranges. The culture G-15 does not bear a close resemblance to any of the known asymbiotic nitrogen fixers with the

TABLE Ib. General Cultural Responses of G-15

Culture Medium	Response	Culture Medium	Response
Glucose in purple broth	-	Nitrate broth	+
Lactose in purple broth	-	-production of NO_2^-	+
Sucrose in purple broth	-	Gelatin	liqui-fication
Maltose in purple broth	-	Eosin methylene blue	Growth
Mannitol in purple broth	-	McConkey's	-
Glycerol in purple broth	-	Tellurite	-
Lactate in purple broth	-	Brain-heart-infusion	-
Litmus milk	Coagulation	Nutrient agar	+
Methyl red	-	Tryptose agar	+
Vogues-Proskouer	+	Glucose agar	+
Citrate	Weakly +	Blood agar	-
Tryptic soy broth	+	NaCl broth 9%	-
Urea	-	Hugh Leifson's (glucose)	-
Catalase	+	Nutrient broth pH 9.7	weakly +

possible exception of Beijerinkia. However, on the basis of these studies the culture seems more closely related to Chromobacterium.

OPTIMAL GROWTH TEMPERATURE

As previously noted the organism grows better at 11°C than at 37°C, however, a growth temperature of approximately 22°C seems to provide optimal conditions for growth. As noted in Figure 2, growth at 11°C approximated 60% of that at 22°C for the same intervals of time. This suggests that in its natural temperature range the organism performs quite well in terms of growth and potentially in terms of nitrogen fixation.

PIGMENT STUDIES

The production of the violet pigment, violacein, is characteristic of members of the genus Chromobacterium. Sneath in 1956 (47) reported that violacein has an absorption maxima of 565 mμ and that in the presence of alkali the pigment turns green then, in a few seconds, to a red-brown color. He used this criterion to demonstrate similarity of pigment in 38 strains of Chromobacterium. The G-15 pigment also has an absorption maxima of 565 mμ (Fig.3), and turns green then red-brown in the presence of alkali.

FIGURE 2. Temperature growth response by the isolate G-15.

Cells were incubated at the indicated temperatures in the medium listed in the Materials and Methods section. The curves above were obtained in a medium contain 0.5 gm KNO_3/liter, under stationary conditions.

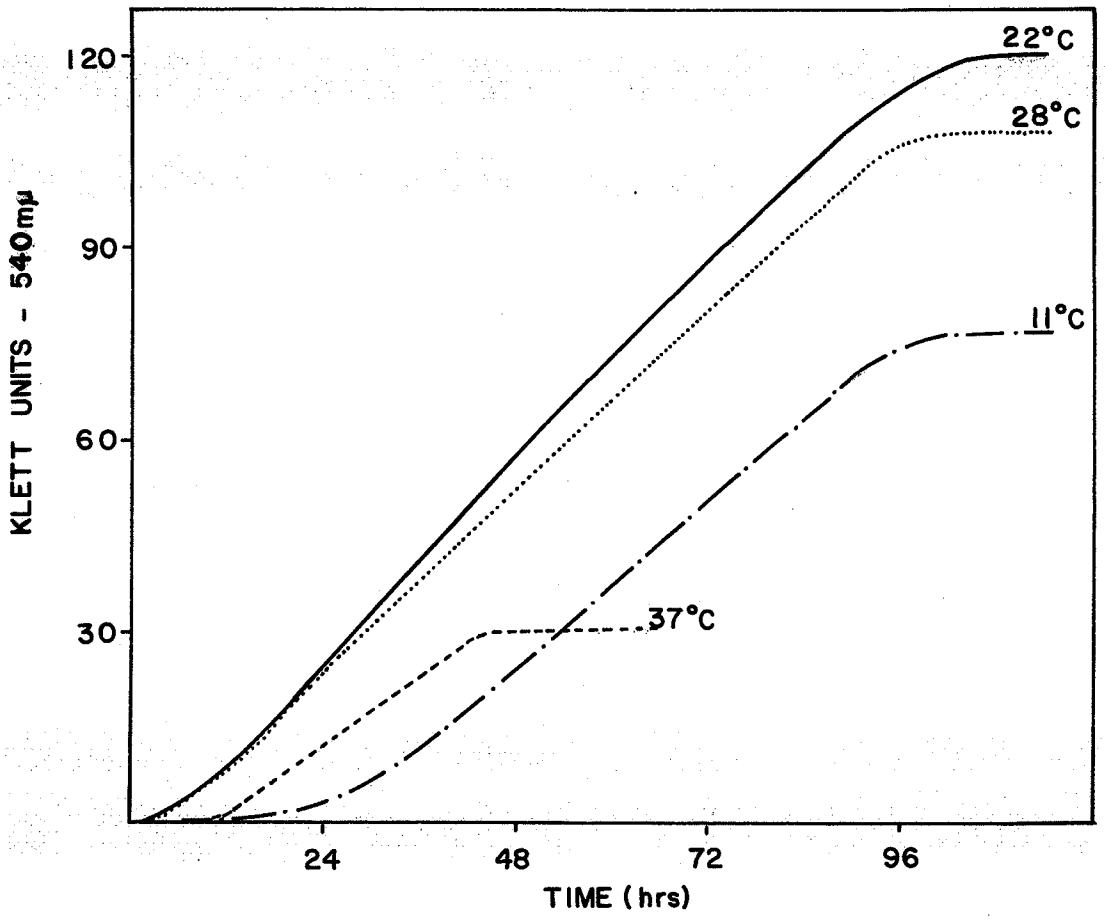
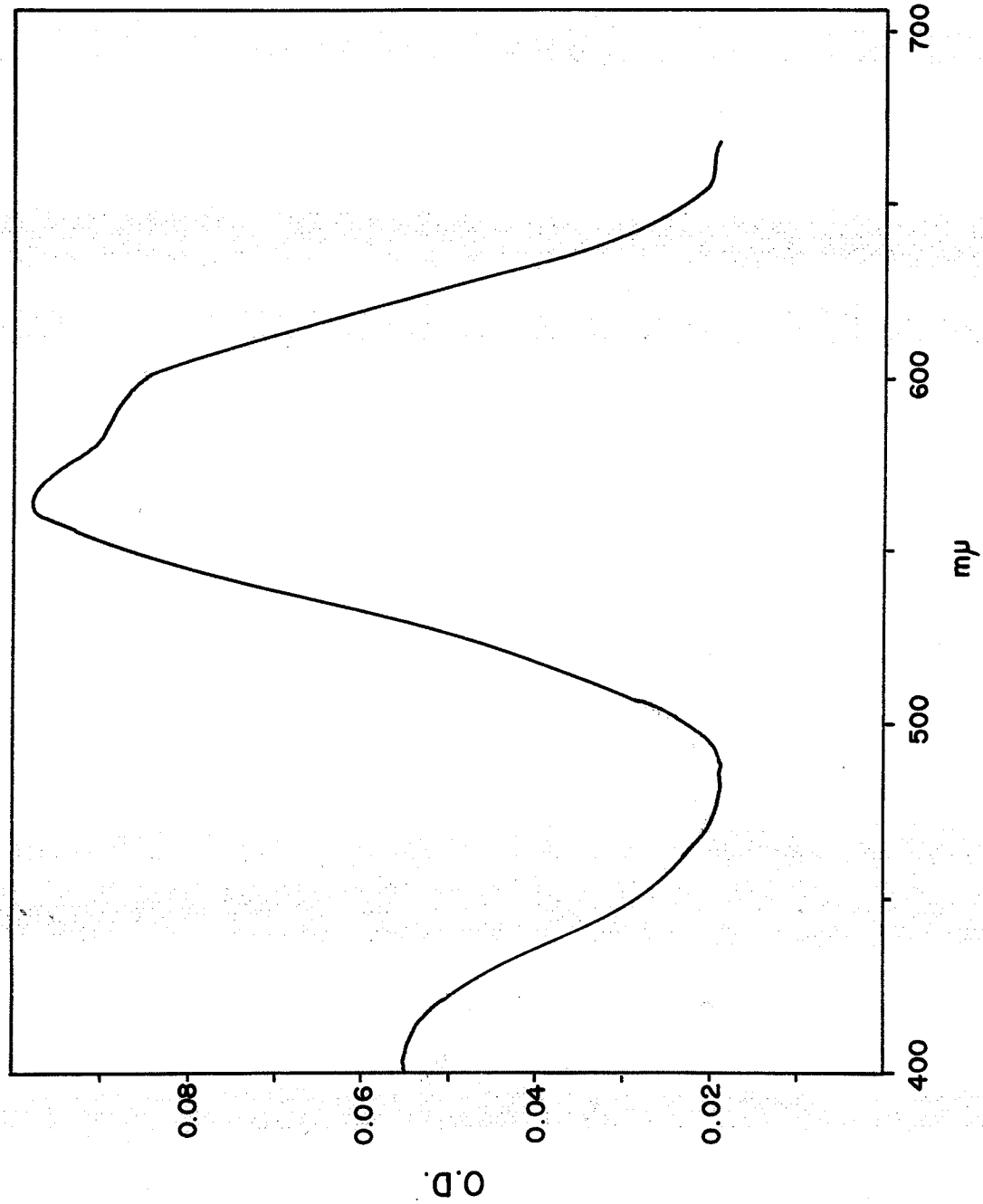

KLETT UNITS - 540mμ
120
90
60
30
22°C
28°C
11°C
37°C
24
48
72
96
TIME (hrs)

FIGURE 3. Absorption spectrum of an ethanolic solution of the purple pigment.


0.08
0.06
0.04
0.02
O.D.
400
500
600
700
mμ

It must be pointed out that similarity in absorption maxima and in alkali reaction does not definitely establish the pigment as violacein. It does, however, lend definite credance to the proposed taxonomic position of the culture; which, in turn, lends credance to the identification of the pigment as violacein.

NITROGEN SOURCES

Measured in terms of growth response within a given interval of time and with constant temperature, the preferred nitrogen source seems to be nitrate. Ammonia, as ammonium sulfate, is also readily acceptable, while peptone supports only a weak growth. Under nitrogen fixing conditions growth was, naturally, considerably less in extent for a given period of incubation. Under ideal cultural conditions the best growth response supported by nitrogen fixation was something less than 50% of that demonstrable in the case where nitrate was supplied to the medium. Nitrite, on the other hand, did not support growth at all, although, it should be pointed out that small amounts of nitrite were detectable in the medium during growth when the culture was initially supplied with nitrate. This finding is not entirely unexpected since, obviously, the culture reduces nitrate, possibly to ammonia, before incorporation into the cell synthesis program.

ENERGY SUBSTRATES

In the mineral salts medium, glucose, sucrose, and lactate as energy sources supported growth quite well, whereas mannitol when added supported growth only to a limited extent. When these and other energy sources were added to Purple Broth (Difco) and inoculated in no instance did the culture show even slight growth. On the assumption that some component of the Purple Broth, possibly the Brom Cresol Purple indicator, might be interfering with the growth of the culture an inoculation series was carried out in a medium comprised of a mixture of mineral salts and Purple Broth with the addition of the various mentioned energy sources. Again no growth was evident even after prolonged incubation. No attempt was made to further explore this inhibition phenomenon. It should be pointed out, however, that the routine assessment of acid and gas production from various carbohydrates could not, under the circumstances, be evaluated.

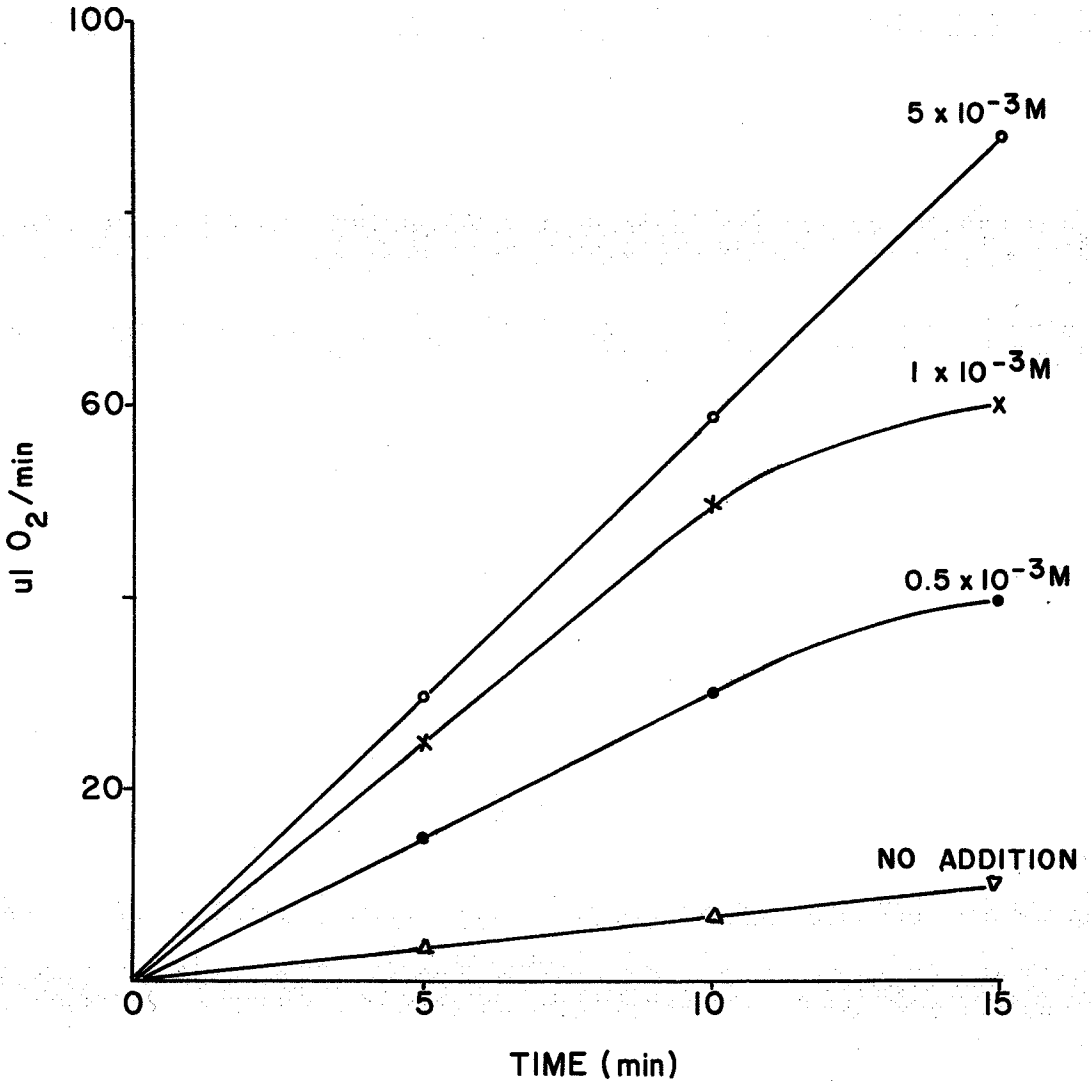
PHYSIOLOGICAL STUDIES

ENDOGENOUS RESPIRATION

As can be seen in Figure 4 endogenous respiration remained rather high even after the culture had been respired in the absence of added substrate for 60 hours on

FIGURE 4. Oxygen uptake by starved cells in the presence of added lactate.

Lactate, at the indicated concentrations, was added to the Warburg flask suspension of cells previously starved for 60 hours.


100
60
20
ul O_2/min
5×10^{-3}M
1×10^{-3}M
0.5×10^{-3}M
NO ADDITION
0
5
10
15
TIME (min)

a rotary shaker at 28 degrees centigrade. Fat staining by the Sudan Black B procedure (9) showed rather large amounts of fat in the cells at the beginning of the respiration interval. As respiration proceeded fat content as measured by the fat stain procedure decreased until by 60 hours fat was scarcely detectable by this method. The appearance of the fat soluble stain distributed more or less throughout the organism is somewhat unusual. This may be considered as partial evidence that fat was intimately bound to some surface component. However, this finding is at variance with that noted in Figure 5 which represents electron micrographs of thin sections of cells (imbedded) treated with osmium tetroxide. Here, as pointed by arrow, uniformly stained areas suggest extensive fat accumulations in the cytoplasm. It should be pointed out that the intensity of the osmium tetroxide staining of fat is directly proportional to the degree of unsaturation of the fat in question.

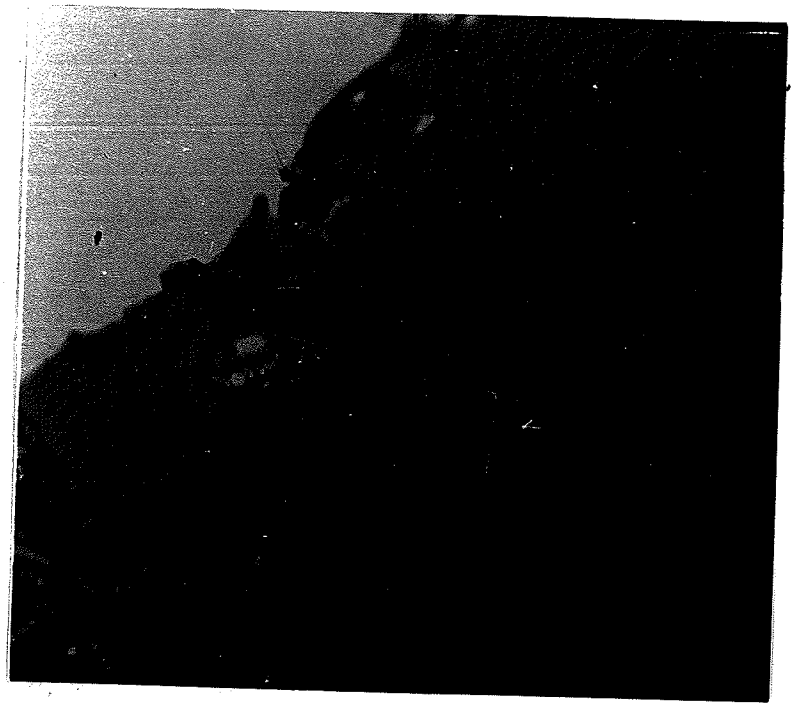
No detectable starch inclusions could be determined by staining with Lugols' iodine.

LACTIC DEHYDROGENASE

In view of the respiration by the culture in the medium with lactate as the energy source, Figure 4, and in the case of the appearance of pyruvate in the presence

FIGURE 5. Thin sections of methacrylate embedded cells of the isolate G-15 stained with osmium tetroxide.

- arrows indicate areas of fat inclusion.



of a cell sonicate and added lactate, (Table II) it is reasonable to expect an easily demonstrable lactic dehydrogenase activity. However, when spectrophotometric assays were conducted they consistently failed to show a specific lactic dehydrogenase, either, on the basis of extracts of the culture prepared by sonication or, by assay of acetone cell powders. On the assumption that strong NADH oxidase activity might be responsible for failure to demonstrate a specific lactic dehydrogenase, controls were designed to take this into consideration. Again, it was not possible to demonstrate the presence of the enzyme. Although it was possible to couple the system to show ferricyanide reduction, controls, in this instance, indicated that ferricyanide could be reduced in the absence of added energy substrate.

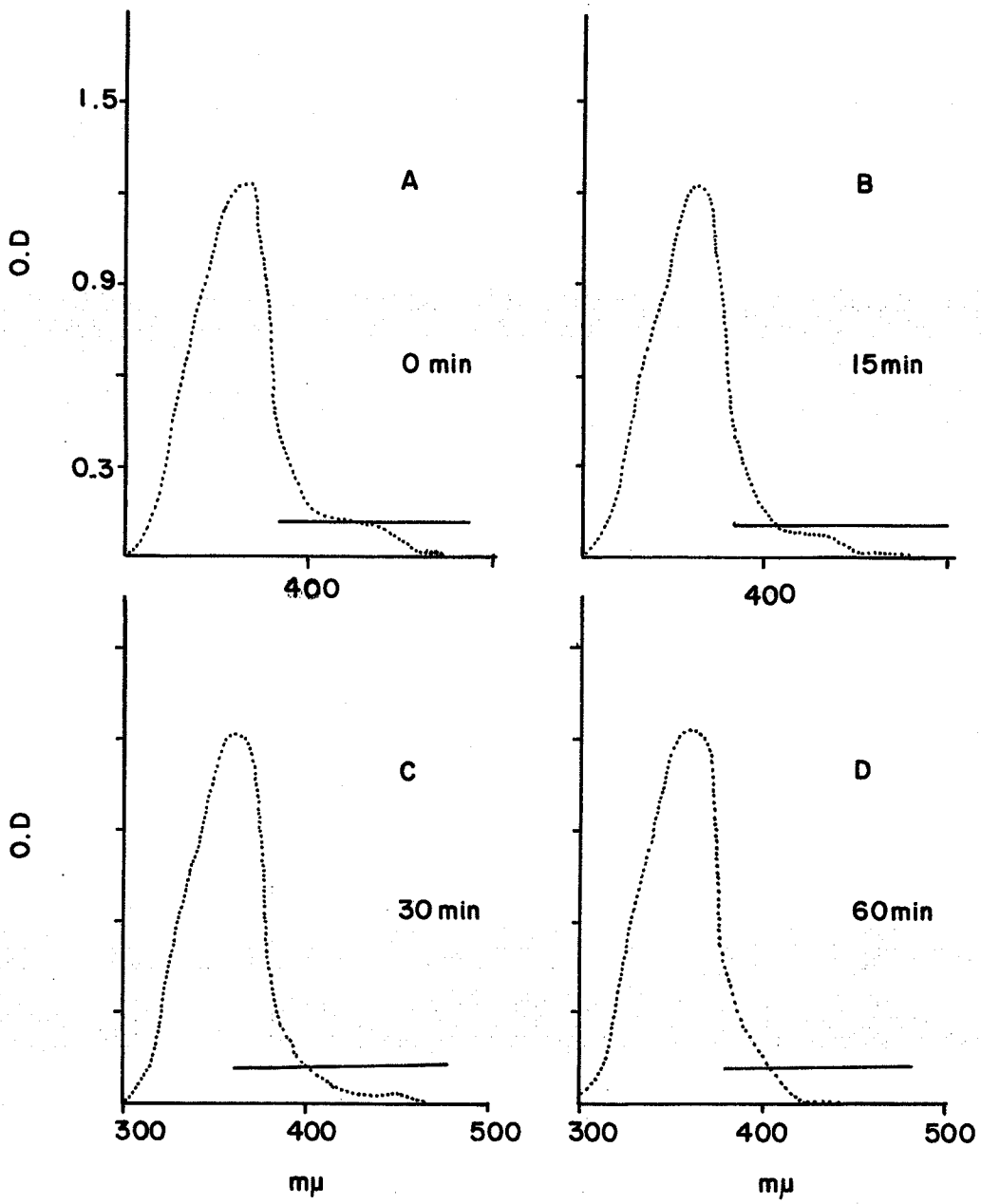
TABLE II. Production of pyruvate from lactate by cell-free fractions of the isolate, G-15 at 30°C.

	Lactate control	TCA Blank	CFE only	CFE + lactate
µmoles pyruvate formed in 60 min.	4.5	0.0	12.6	33.0

Time lapse spectra, (Fig.6a,b,c,d) indicate the disappearance of a peak originally noted at 420 mµ in the cell

FIGURE 6. The effect of ferricyanide on the absorption peak at 420 mμ.

The assay mixture contained in 3.0 ml: 3.0 μmoles $K_3Fe(CN)_6$, an acetone powder extract and 0.1 M potassium phosphate buffer, pH 7.5.


1.5
0.9
0.3
O.D
A
0 min
400
B
15min
400
C
30 min
D
60min
O.D
300
400
500
mμ
300
400
500
mμ

extract. This peak, suggesting a cytochrome involvement, gradually disappeared in an assay system containing cell extract and ferricyanide.

A distinctive peak at 360 mμ (Fig. 6) which appeared consistently in the assay spectra might be interpreted as indicative of the presence of vitamin B_{12} (Weissbach et al (51)). However, when a replicative assay system was treated with β-mercaptoethanol following the method of Weissbach et al no reduction of the 360 mμ peak concomitant with the development of a peak at 311 mμ could be demonstrated. The possibility exists, although relatively slight, that the 360 mμ peak might represent a flavin component. However, in view of the much broader peaks typical of the presence of flavin this supposition is unlikely.

Despite the inability to demonstrate spectrophotometrically the presence of lactic dehydrogenase in the system the evidence presented, i) by the high respiration rate and growth in the presence of lactate and ii) the formation of pyruvate in quite significant amounts in the assay system can lead only to the supposition that an active lactic dehydrogenase is operative. The failure to demonstrate the enzyme spectrophotometrically may then be assumed to arise from interferences or inhibitions by some unrecognized component in the cell system. Aware of the rather considerable difficulties involved in identification of the

the inhibitory factor or factors and in view of the time limitations placed upon this investigation it was not feasible to pursue this question further.

SECTION II

NITROGEN FIXATION

INTRODUCTION

As reported in Section I the culture G-15 tentatively identified as a member of the genus Chromobacterium displayed an ability to grow well in a medium in which the only available source of nitrogen was the atmosphere. Since the culture is not recognizable as belonging to any of the known nitrogen fixing groups it was considered appropriate to examine some of the basic relationships pertinent to bacterial nitrogen fixation. In this sense a consideration of what might be deemed a good supporting medium was undertaken. It should be realized that a medium well able to support growth under nitrogen fixing conditions is not necessarily the same medium which may be used for routine cultural studies and observations. The rather unique demands placed upon the nutritional environment during fixation, in terms for example, of trace element requirements, of energy sources, of vitamin requirements, and the like must be evaluated to insure a growth response directly interpretable in terms of the amount of nitrogen fixed. Although G-15 is aerobic and may accordingly be presumed to react differently in a fixation sense as compared to the anaerobic Clostridium species it was considered worthwhile to examine the culture medium after growth for nitrogenous

excretory products, specifically, amino acids and ammonia.

The highly sensitive nitrogen fixation assay procedure based upon incorporation of the radioisotope of nitrogen $^{13}N_2$ was undertaken to provide further and perhaps more definitive evidence of the culture's ability to fix nitrogen. Details of the $^{13}N_2$ procedure are available in (15) to be published in a later issue of the Canadian Journal of Microbiology. In the Materials and Methods Section which follows is a brief description of the radioisotope experimental design as it applied to the organism in question.

The uptake of the radioisotope was assessed in the presence of certain energy substrates which, on the basis of results in part reported in Section I, were found to be most readily accepted by the culture. Preliminary results of fixation by sonically disrupted cells are also presented along with a consideration of the possible participation by hydrogenase in the fixation process.

MATERIALS AND METHODS

THE MEDIUM

The original medium used by Dular in screening isolates for nitrogen fixing capacity contained in one liter sucrose, 20.0 gm; K_2HPO_4, 0.16 gm; $MgSO_4.7H_2O$, 0.2 gm; NaCl, 0.2 gm; $CaSO_4.2H_2O$, 0.05 gm; Na_2MoO_4, 0.001 gm;

$FeSO_4$, 0.003 gm and agar in the amount of 1.5%. Streak plates of the medium were aseptically inoculated and enclosed in an airtight plastic box through which ammonia-free air was forced at a relatively high rate.

From the various colony types which developed on the medium under these conditions, the one designated G-15 was selected and propagated in a modified Burk's (44) nitrogen-free liquid medium. This basal medium, differing in some respects from the initial screening medium, contained in one liter of distilled water glucose, 10.0 gm; K_2HPO_4, 0.1 gm; NaCl, 0.1 gm; $MgSO_4 \cdot 7H_2O$, 0.4 gm; Na_2MoO_4, 0.001 gm; Ca acetate, 0.2 gm; Na citrate, 0.2 gm; biotin, 0.002 gm and Larsen's (29) trace elements solution.

To assess the contributions of biotin, acetate and citrate modifications of the basal medium were prepared in which these were individually or collectively omitted. The addition of Larsen's (29) trace element solution was made on the assumption that specific trace elements, as reported by other workers, might be necessary for fixation activity. Final trace element concentrations in the medium on the basis of one liter were as follows: B, 100 µgm as H_3BO_4; Zn, 100 µgm as $ZnSO_4 \cdot 7H_2O$; Co, 50 µgm as $Co(NO_3)_2 \cdot 6H_2O$ or as $CoCl_2 \cdot 6H_2O$; Cu, 5 µgm as $CuSO_4 \cdot 5H_2O$; Mn, 5 µgm as $MnCl_2 \cdot 4H_2O$; and Fe, 100 µgm as $FeCl_3 \cdot 6H_2O$.

Evidence that the trace element solution stimulated

growth is presented in Fig. 8. Prompted by this result, an experiment to determine the effect of the individual components of the trace element solution was carried out. Aliquots of the nitrogen-free growth medium minus the trace element solution were dispensed into eight growth flasks. Components of the trace element solution were individually added to each of six flasks at concentrations equivalent to those provided by the complete trace element solution. To the seventh flask was added the complete trace element solution at the recommended level while flask number eight served as a control without any trace element addition. All flasks were uniformly inoculated and the growth response at room temperature for seven days is presented in Table III.

Since the cobaltic component of the trace element solution is the NO_3^- form, an assay was carried out according to the method of Montgomery and Dymock (34) to determine the final concentration of NO_3^--nitrogen in the whole medium. The assay determination was briefly as follows: 0.1 ml of sample and 0.1 ml of the 2,6-dimethyl phenol reagent (0.122 gm of 2,6-dimethyl phenol in 50 ml of glacial acetic acid added to 50 ml of 24% $(NH_4)_2SO_4$) were added to 1.6 ml of 75% H_2SO_4 precooled to 0°C. Three milliliters of water were added and the reaction incubated at room temperature for 15 minutes.

ENERGY SOURCES

The nitrogen-free agar medium was prepared with glucose, sucrose and mannitol as the energy source. Growth of the culture under each of these conditions was compared by measuring the amount of growth in terms of time required for colonial development.

A similar comparison was made in the case of aliquots of nitrogen-free liquid medium containing glucose, sucrose and lactate respectively. A bar graph (Fig. 9) illustrates the observed results.

AMMONIUM AS A CATALYST FOR NITROGEN FIXATION

As reported by Jensen (28) and others, low concentrations of ammonium ion seem to act catalytically for the N_2 fixation reaction. To examine the effect of low ammonium concentration on the growth of this culture 8 parts per million (p.p.m.) as NH_4^+ were incorporated into the medium and growth response of the culture at 15°C and at 37°C was determined. The results were compared with the growth response at the same temperature in the absence of ammonium. It should be noted that concentrations of NH_4^+ equivalent to 8 p.p.m. cannot be considered as even remotely approaching substrate levels.

CULTURAL EXCRETIONS

A nitrogen fixing organism can be assumed to contribute to the nitrogen economy of the soil in two ways. It may excrete nitrogenous compounds during growth and fixation, as in the case of NH_4^+ liberation by Clostridium (49) or its contribution may come only on the death and autolysis of the cell. The prospect that G-15 contributed nitrogenous excretory products, ammonium and possibly amino acids, to the culture medium during growth and fixation was examined.

Amino acids in the culture medium were assayed for by the copper salt method of Spies reported in 1952 (48). Five milliliter aliquots of culture medium from which cells had been centrifuged after growth were dispensed to a series of 12 ml stoppered centrifuge tubes each containing 5.0 ml sodium borate buffer pH 9.1. To this mixture was added 0.1 ml of 0.05 M $CuCl_2$; the reaction was allowed to proceed at room temperature for 10 minutes. The complete mixture was then centrifuged at 4000 x g and the supernatant was carefully transferred by pasteur pipette to cuvettes where optical density at 230 mμ was determined in a Beckman DB spectrophotometer. Values so obtained were interpreted on the basis of a standard curve prepared for alanine.

Excreted ammonium in the medium was assayed for by the p-nitrobenzene diazonium chloride test reported by

Welcher (52). It should be pointed out that this test is qualitative with a lower sensitivity level equivalent to 0.7 μgm of NH_4^+.

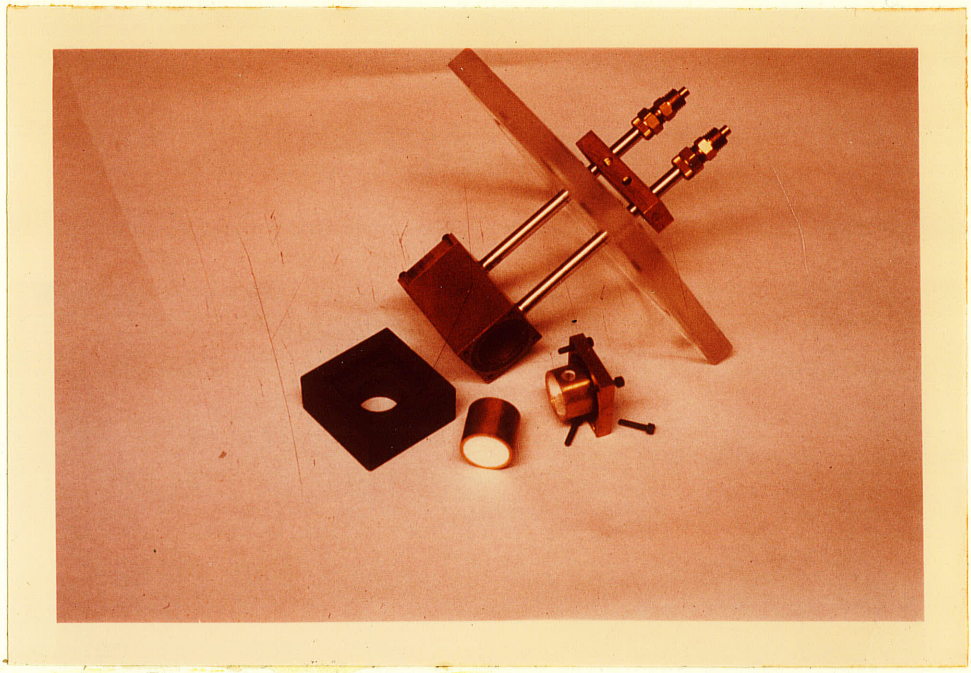
$^{13}N_2$ STUDIES

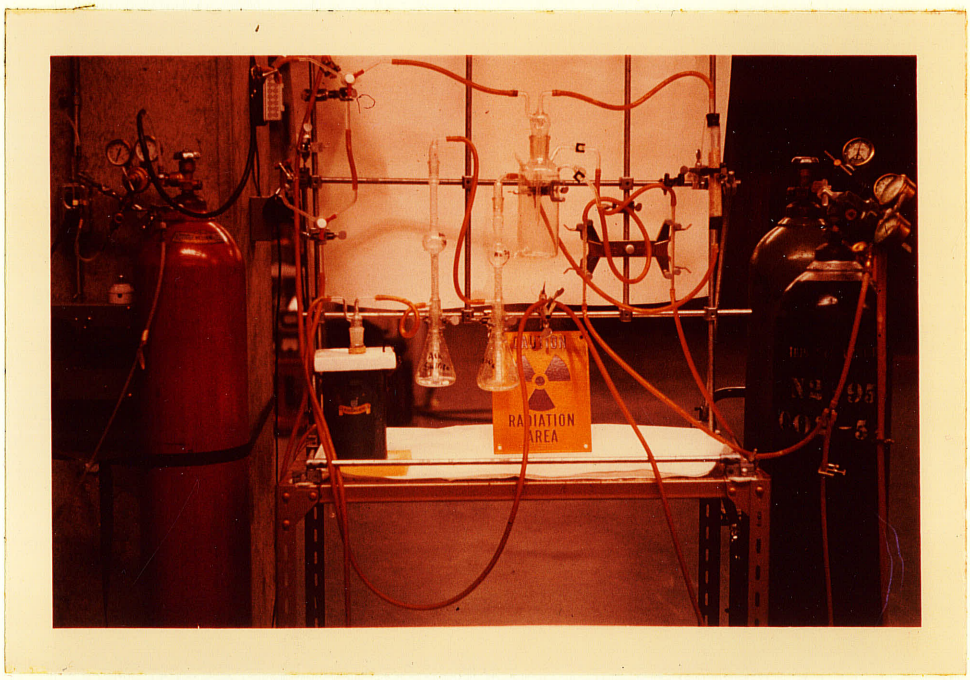
EXPERIMENTAL APPARATUS AND PRODUCTION OF RADIOISOTOPE

The radioisotope of nitrogen, $^{13}N_2$, used in these studies was produced by the bombardment of $^{14}N_2$ with 50 Mev protons. The nitrogen containing target material, melamine, under these bombardment conditions produces several other radioisotopes, notably carbon-11. A series of traps designed to remove ^{11}C and other unwanted reaction products was described along with details of the production process in a paper presented to the IXth International Congress for Microbiology (16). $^{13}N_2$ produced during the reaction was swept from the porous target by a stream of He gas at a rate of approximately 0.3 liters/minute which passed by means of narrow bore tygon tubing to the trap system and thence to a mixing bottle where other gases, $^{14}N_2$, CO_2 and O_2 were added at controlled rates (Fig. 7). The gas mixture was dispensed to a manifold which distributed it uniformly to a series of culture reaction vessels. After passage through the reaction vessels the gas was collected by manifold and conveyed to plastic reservoir bags.

A radioisotope decay curve (Fig. 12) was plotted from

FIGURE 7. Target assembly and trap components of $^{13}N_2$ production system.




RADIATION
AREA

readings taken on the level of cell-incorporated radioactive $^{13}N_2$. The procedure was carried out to check for the presence of cell-incorporated ^{11}C which may have escaped the trap system.

Following exposure, usually for 30 minutes, to the radioactive gas mixture the reaction flasks were outgassed for 5 minutes with a (cold) N_2-CO_2 mixture. The contents of each flask were tipped into individual aluminum foil dishes and the residual radioactivity, a measure of cell-incorporated $^{13}N_2$, was determined using a Nuclear Enterprises external sample scintillation counter. The pulse height analyzer circuit of the counting aparatus was so adjusted to count gamma energy level associated with positron decay of $^{13}N_2 \longrightarrow {}^{13}C$.

The sodium iodide, NaI, detection crystal was shielded with a series of concentrically fitted lead rings to a total thickness of two inches. Shielding lowered the background counting rate of the detector to 65 counts per minute.

SAMPLE PREPARATION

Whole cell samples for $^{13}N_2$ experimental purposes were prepared by harvesting 2-3 day old growing cell populations by centrifugation at 5000 x g for 15 minutes. The pellet was resuspended uniformly, in a fresh medium from

which energy substrate had been omitted and respired for approximately two hours under shaking conditions at room temperature. Approximately two hours prior to the exposure of the culture to $^{13}N_2$ energy substrate was added to the cell suspension and shaking was continued. For exposure to the radioisotope-gas mixture, 10 ml aliquots of the heavy cell suspension as described were dispensed to the reaction culture flasks and rubber stoppers holding the inlet and outlet gas tubes were tightly wired in place. The gas inlet tube was so adjusted as to extend to within 0.5 cm of the surface of the cultural suspension. The culture reaction vessel inlet and outlet tubes were then connected to their appropriate inlet and exhaust manifolds by equal lengths of rubber tubing. The reaction vessels were shaken by wrist action shaker throughout the duration of the gas treatment to provide for maximal gas exchange between the liquid culture medium and the atmosphere above it.

Initially, the fixation response by the culture to different energy substrates was measured by $^{13}N_2$ incorporation. Uniform aliquots from the original cell harvest suspension were distributed to the reaction flasks and the respective substrates were added. Subsequently cells were grown with different substrates, harvested, respired and resuspended in the reaction flasks in a medium containing, as energy source, the same substrate which

provided energy for original growth. In this instance, for example, cells grown on lactate were again fed lactate during the $^{13}N_2$ fixation interval while cells grown on glucose were fed glucose during exposure to the radioisotope.

Where pyruvate or succinate were to be used as energy sources during $^{13}N_2$ fixation the cell suspension was made up in equal parts of cells grown in glucose and cells grown in lactate. This change in procedure was necessitated by the practical difficulties involved in growing cells for extended periods of time (48-72 hours) with either pyruvate or succinate at substrate concentrations. Comparison of these data was made on the basis of counts per minute/mg protein. When the observed count was corrected to zero time (end of exposure to $^{13}N_2$) and the average background count value was subtracted.

The concentration of energy substrate, unless otherwise noted, was 10 mgm/ml in each case. The results of these studies are presented in Tables IV, V and VI.

PROTEIN ESTIMATION

Protein estimation on whole cells was made by first hydrolyzing the cells in 1N NaOH for 20 minutes at 100°C. The hydrolysate was neutralized with HCl and the protein estimated by the method of Lowry as reported by Layne in 1957 (30) using bovine albumin as the standard.

HYDROGENASE ASSAY

Two methods were used in an attempt to demonstrate hydrogenase activity in the culture. The first of these, measuring the evolution of H_2 gas manometrically, was proposed by Peck and Gest in 1956 (40) and is described as follows: cells from an actively growing population were washed twice with 0.1 M potassium phosphate buffer (pH 7) and frozen at -20°C. From the frozen pellet an extract was prepared by grinding the cells with an equivalent weight of levigated alumina at 4°C for 10-15 minutes following which sufficient 0.2% KCl was added to bring the volume to 4.0 ml/gm of cell paste. After centrifugation at 2000 x g to remove the alumina and any remaining whole cells the supernatant was dispensed to clean centrifuge tubes for further centrifugation at 28,000 x g for 15 minutes. The supernatant was discarded and the residual pellet was resuspended in 0.2% KCl and again centrifuged at 28,000 x g for 15 minutes. The washed pellet was uniformly suspended in 0.005 M potassium phosphate buffer (pH 6.0) and stored at -20°C under air.

For the Warburg assay 0.8 ml of crude 'hydrogenase' and 0.625 M potassium phosphate buffer (pH 6.5) were added to the main compartment. The side arm which had been fitted with a serum stopper received 0.2 ml of an 80 µmole/ml methyl viologen solution. The center well contained 0.1 ml

of 20% KOH and a small strip of fluted filter paper. The flask was sealed and flushed repeatedly with Argon. While the flushing process was being carried out, 0.2 ml of hydrosulfite reagent* was added to the side arm by syringe through the serum stopper. Flushing with Argon was continued for a further 15 minutes.

The hydrogenase assay method of Fredricks and Stadtmann (22) measures the H_2 uptake by pyruvate to yield lactate according to the following scheme:

$$H_2 + NAD^+ \xrightarrow{\text{hydrogenase}} NADH + H^+$$

$$NADH + H^+ + \text{pyruvate} \xrightarrow{\text{lactic dehydrogenase}} \text{Lactate} + NAD^+$$

$$\text{Total} \quad H_2 + \text{pyruvate} \longrightarrow \text{Lactate}$$

In this assay a cell sonicate was used as follows. To the main chamber of the Warburg vessels were added 60 μmoles of pyruvate, 15 μmoles NAD, 200 μmoles Tris-HCl pH 7.8, 60 μmoles of reduced glutathione and aliquots of the cell sonicate. The side arm contained two units of lactic dehydrogenase and the center well received 0.2 ml of 20% KOH and a strip of fluted filter paper. The final total volume (liquid) was 3.0 ml. The Warburg flasks were then gassed

*The hydrosulfite reagent was prepared immediately prior to the experiment as follows: 210 mg of $Na_2S_2O_4$ was placed in a serum stoppered bottle, air was evacuated and Argon gas flushed through several times, then 3.0 ml of H_2O and 2.0 ml of 0.125 N NaOH were added.

with H_2 for 5-10 minutes. Controls were as follows: complete system minus NAD, minus pyruvate, or minus lactic dehydrogenase and lastly, a boiled sonicate control.

RESULTS AND DISCUSSION

THE MEDIUM

Observations made on a number of cultural trials in which one or more of the components of the modified Burk's medium, as noted in Materials and Methods, Section II, were omitted suggested that calcium acetate, sodium citrate and biotin made no observable contribution to the ability of the medium to support good growth. The final composition of the medium judged best suited for this culture under the environmental conditions prevailing is as follows: in one liter of distilled water sodium lactate, 6.0 gm; K_2HPO_4, 0.1 gm; NaCl, 0.1 gm; $MgSO_4.7H_2O$, 0.3 gm; $(NH_4)_2SO_4$, 0.032 gm (8 p.p.m.); Na_2MoO_4, 0.001 gm and Larsen's trace elements.

The isolate (G-15) was propagated in both agar and liquid forms of the medium. Of the energy sources examined, i.e., glucose, sucrose, mannitol and lactate, the latter gave the best yield in terms of cell population for equivalent incubation periods. This evidence is presented in the form of a bar graph in Fig. 9. Wherever possible however, liquid cultures were preferred for experimental work because when grown on solid agar surface the culture tended

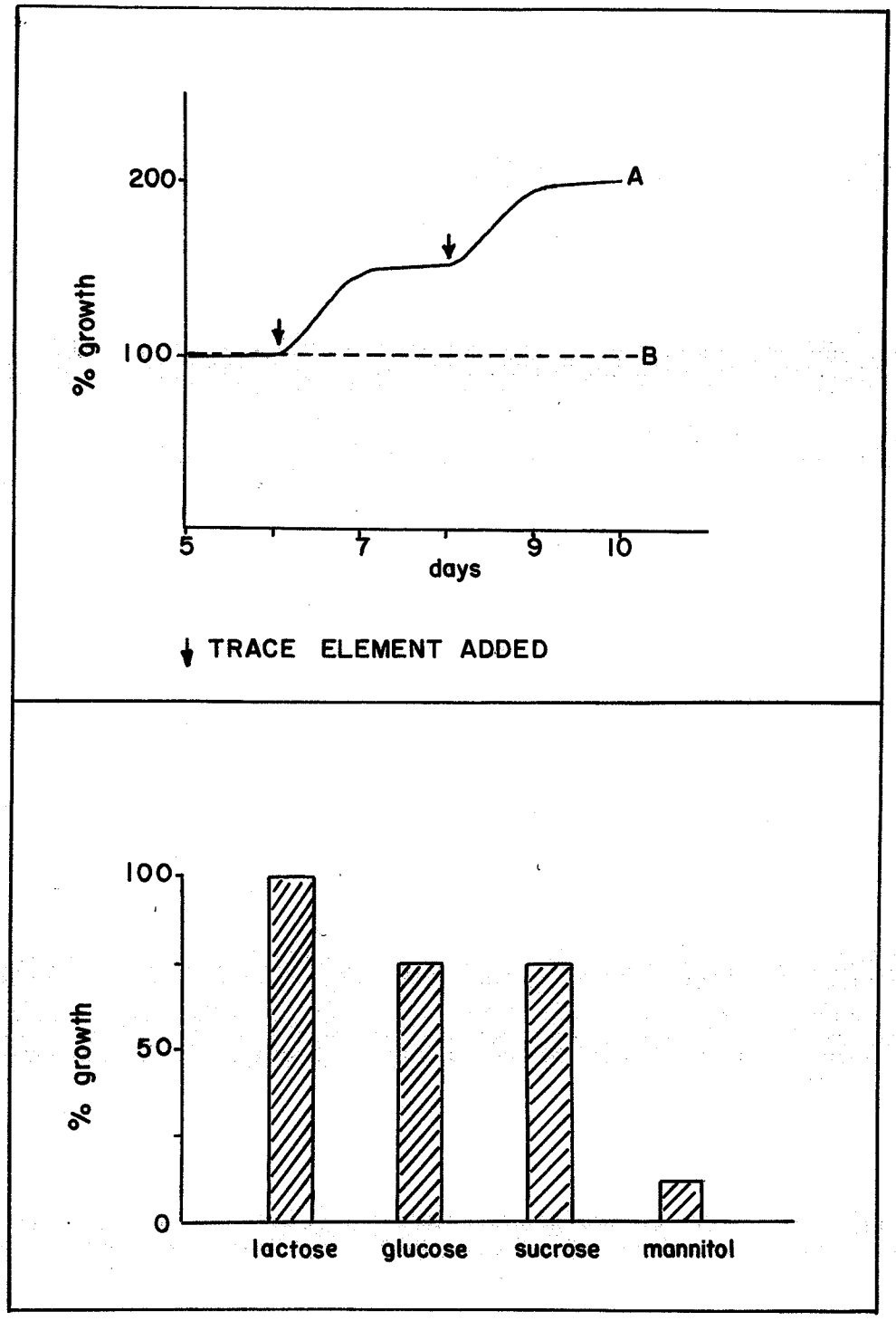
FIGURE 8. Stimulation of growth by added trace elements.

Replicate cell populations were grown in nitrogen-free medium and designated A and B.

A - received Larsen's as indicated by arrows

B - control.

FIGURE 9. Substrate preference under nitrogen fixing conditions.


200
100
% growth
A
B
5
7
9
10
days
TRACE ELEMENT ADDED
100
50
0
% growth
lactose
glucose
sucrose
mannitol

to be strongly adherent, sticky and difficult to remove.

The importance of the trace element amendment to the medium is clearly shown in Fig. 8 where the comparison of the effect of various concentrations of the trace element solution are compared to a control system in which the trace elements are omitted. It is interesting to note that no trace element component when added by itself was sufficient to support growth adequately as shown by Table III.

TABLE III. The effect of various trace element additions on the growth of G-15 in a nitrogen-free medium.

Trace element addition	Growth response
Larsen's complete solution	+
B	-
Zn	-
Co	-
Cu	-
Mn	-
Fe	-
Control	-

An examination of the possible contribution of the nitrate moiety of the cobaltic component showed no observable stimulatory effect. When treated with 2,6-dimethyl phenol the culture medium was found to contain less than 2.0 p.p.m. nitrate in the final concentration. Despite this apparent inconsequential level of nitrate in an otherwise nitrogen-free medium it was decided to prepare the trace element

solution with $CoCl_2.6H_2O$ rather than with the nitrate form. No detectable change in growth response resulted from this substitution.

AMMONIUM AS A CATALYST FOR NITROGEN FIXATION

Stimulation in growth response when catalytic amounts (up to 8 p.p.m.) of NH_3 is shown in Fig. 10. When the culture was incubated at 37°C a stimulation effect was about four-fold while at 15°C this stimulatory effect was reduced to approximately 1.5 fold. Concurrently, it was observed that a slight decrease occurred in the length of the lag phase at both temperatures.

As might be expected the age and amount of inoculum played an important part in the successful initiation of cultural growth. Indeed, on a number of occasions, it was observed that inoculum volumes of less than 2% of the final volume of culture often resulted in failure of the culture to start growth. Similarly, cultures less than 4-5 days old proved to be much less suitable as sources of inoculum. Growth curve studies (Figs. 10 and 11) indicate a leveling off of the lag phase of growth about the age of 4-5 days. Subsequently, over a period of some weeks observations were routinely made on cell population densities in the culture. Unexpectedly, these cell population densities were found to increase over a period of eight weeks with turbidity

FIGURE 10. Effect of ammonium on growth when present at catalytic levels.

Growth response is expressed as percent of growth response in the absence of ammonium.

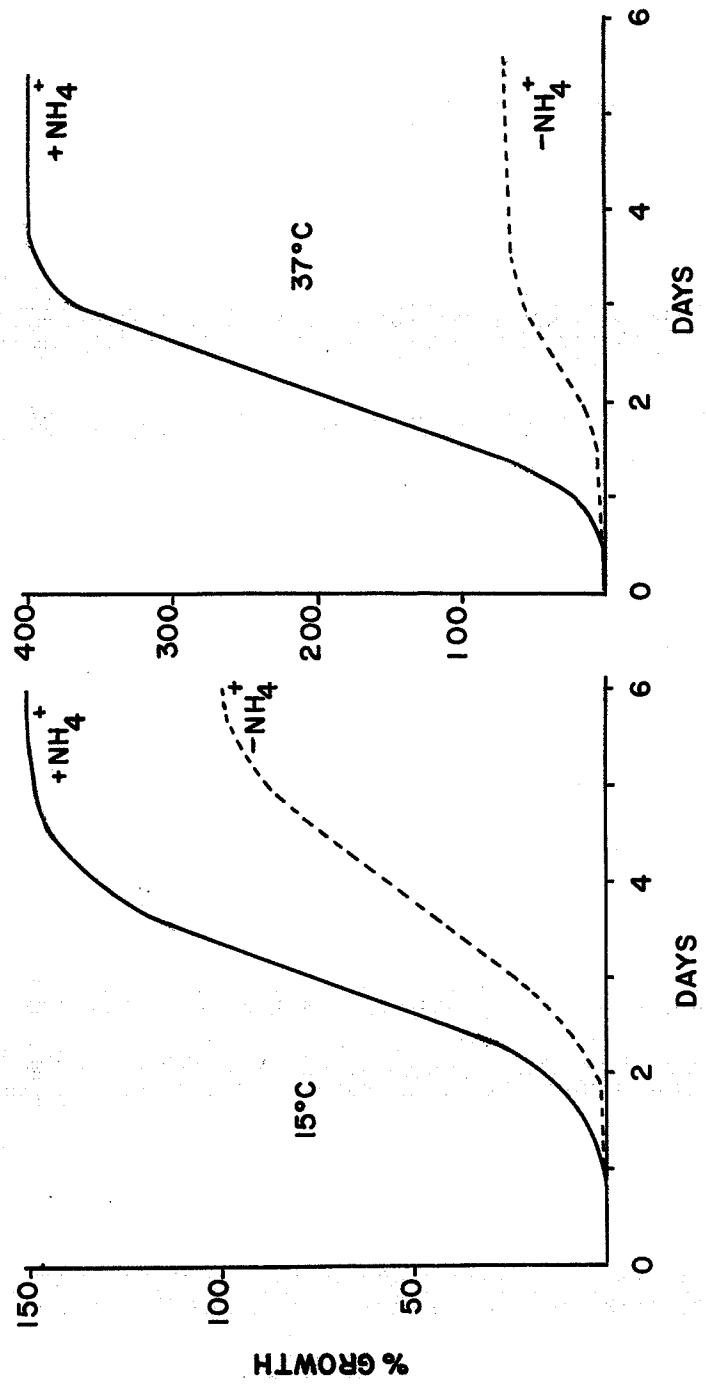
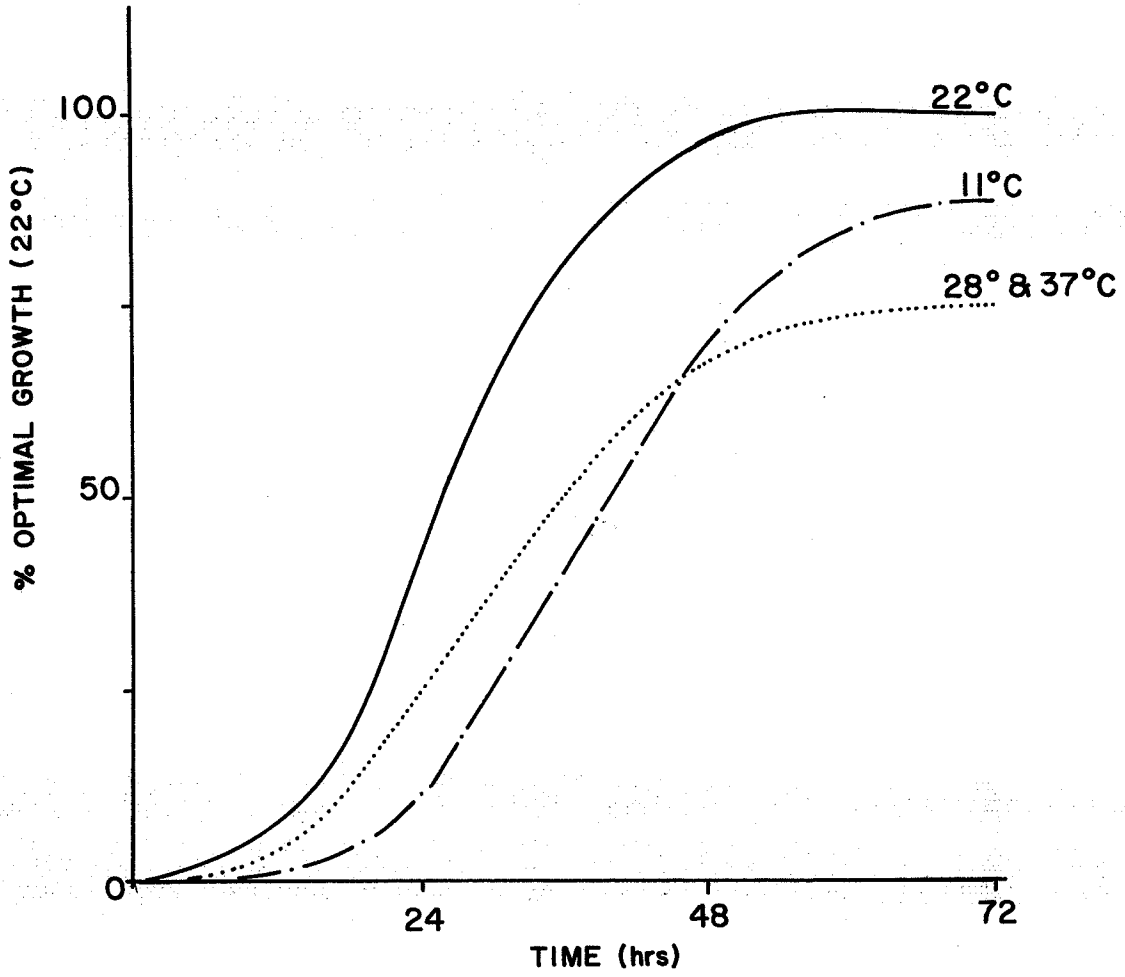

% GROWTH
150
100
50
0
2
4
6
DAYS
15°C
+NH4+
-NH4+
400
300
200
100
0
2
4
6
DAYS
37°C
+NH4+
-NH4+

FIGURE 11. Temperature growth responses for the isolate G-15.

The culture was propagated in a liquid nitrogen-free medium at the indicated temperatures.


% OPTIMAL GROWTH (22°C)
100
50
0
22°C
11°C
28° & 37°C
24
48
72
TIME (hrs)

readings ranging from a low of 50 Klett units at 4 days to approximately 350 in eight weeks. No attempt was made, in view of time available, to examine the phenomenon of slow, prolonged growth in the culture. It may well be, however, that this observation can be accounted for by a stored food utilization program carried out by the cells. This would seem to be an interesting aspect of cultural behavior and awaits further study.

EFFECT OF TEMPERATURE ON CULTURE RESPONSE

Fig. 11 presents the data derived from growth curve studies at 11°C, 22°C, 28°C and 37°C in the nitrogen-free liquid medium. As indicated, optimal growth seems to occur at 22°C. The curves were plotted on the basis of unshaken cultures because, although total growth per unit time was somewhat less than under shaking conditions a greater uniformity of treatment was possible. The most noticeable visual observation was that incubation at 22°C gave the best results. Accordingly, the results of all other temperature treatments are presented as percentages of the optimal response at 22°C.

CULTURAL EXCRETIONS

The examination for nitrogenous excretory products gave negative results. No detectable quantities of either

amino acids or ammonia could be demonstrated in the culture medium after the growing population had been removed by centrifugation; in this respect this culture differs markedly from the nitrogen fixing Clostridium. It would seem, therefore, that the contribution by this organism in its natural habitat arises only on the death and autolysis of the cells.

EXPERIMENTAL APPARATUS AND PRODUCTION OF THE RADIOISOTOPE $^{13}N_2$

The major advantage gained by the use of the radioactive tracer technique lies, unquestionably, in the relatively short amount of time required to complete the assay. The problems of preparing the cultures for assay, however, are little different from those occasioned where nitrogen fixation is to be measured by more conventional means. There is good reason to believe that lower levels of nitrogen fixation can be detected by $^{13}N_2$ exposures than can be obtained by the use of the heavy nitrogen isotope $^{15}N_2$. In this instance, however, it should be pointed out that $^{13}N_2$ measurements thus far are qualitative and at best only semi-quantitative.

The principal disadvantage of the assay system used in this study is due to the short 10.05 minute half-life of the radioisotope. Care must be taken in the design of the experiments to provide a reasonable margin of working time

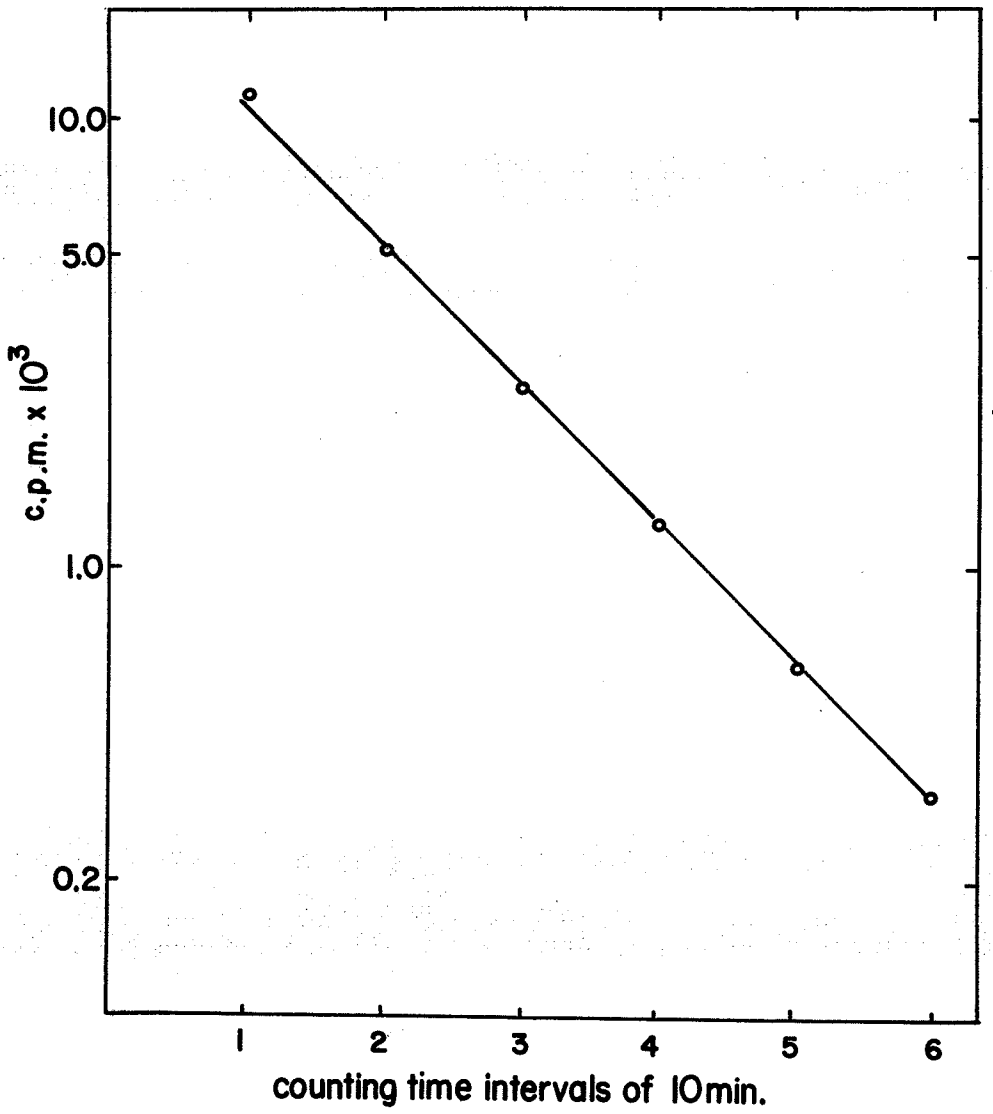
in view of this limitation. Assessments of substrate comparisons during fixation on a 'go-nogo' basis are easily made and similarly, experiments to determine the effect of various environmental conditions in fixation may be carried out without difficulty.

To be certain that only $^{13}N_2$ was being introduced into the samples a decay curve of the incorporated radioactivity was plotted (Fig. 12). The possibility of $^{11}CO_2$ fixation being incorporated was considered but the decay curve indicates only $^{13}N_2$ since any appreciable amount of $^{11}CO_2$ would, if included, give a decay curve strongly influenced by the 20 minute half-life of ^{11}C. The decay curve is a straight line indicating a half-life value close to 10 minutes. If any ^{11}C were present the line would not be straight and the slope would be decreasing with time.

ENERGY SUBSTRATES SUPPORTING N_2 FIXATION

The results presented in Table IV support the fact that lactate is the preferred energy source of those tested for purposes of driving the fixation reaction. This finding is in agreement with the observations noted in Fig. 9 on cultural growth responses. It is interesting to note that pyruvate did not appear to support fixation well although it might be reasonably expected that the initial fate of lactate would involve its oxidation to pyruvate. This finding may

FIGURE 12. Decay curve of radioactivity incorporated by G-15.


10.0
5.0
1.0
0.2
c.p.m. x 10^3
1
2
3
4
5
6
counting time intervals of 10min.

simply reflect a problem of transport of the pyruvate across the cell membrane. On the other hand, the failure of pyruvate in this experimental series might be due to its relative instability in solution at substrate levels under our environmental conditions. It has been stated (14) that pyruvate in the Clostridial system is involved in a phosphoroclastic split to provide the reducing power for ferredoxin and it is also generally assumed that no ferredoxin system functions in the case of aerobic nitrogen fixation. Whether, in the case of G-15, a ferredoxin-like component is involved is still a matter of conjecture and remains to be examined.

TABLE IV. The effect of various substrates on $^{13}N_2$ incorporation using cells initially grown with lactate and with glucose.

Initially grown in	Reaction substrate	Substrate Concentration	mg Cell protein	CPM/mg protein at To
Lactate and glucose	Pyruvate	10 mg/ml	13.6	6.1
Lactate	Lactate	10 mg/ml	15.7	618.0
Glucose	Glucose	10 mg/ml	12.3	84.5
Lactate and glucose	Succinate	10 mg/ml	13.6	4.2

Cells that were initially grown with lactate were subsequently found to fix nitrogen, as $^{13}N_2$, at a faster

rate when supplied with lactate during exposure interval than when supplied with glucose. Where glucose formed the initial energy substrate during growth, it was found that fixation occurred best when glucose was supplied in the reaction vessel. This evidence is given in Tables V and VI.

TABLE V. Comparison of energy substrate effect on $^{13}N_2$ fixation by lactate-grown cells.

Substrate treatment*	CPM at To
Pyruvate	423
Glucose	3,526
Lactate	6,683
Control**	69

*At a final concentration of 10 mg/ml.
**Boiled cells in lactate medium.

TABLE VI. Comparison of energy substrate effect on $^{13}N_2$ fixation by glucose-grown whole cells.

Substrate treatment*	CPM at To
Pyruvate	230
Glucose	26,775
Lactate	5,331
Control**	129

*At a final concentration of 10 mg/ml.
**Boiled cells in glucose medium.

The obvious explanation for this observation was that a period of time was required for enzyme induction. Thus, cells cultured on glucose initially would require time for induction of the lactate utilizing system. Attractive as this speculation may be, the question as to whether the culture's pre-exposure interval of two hours in the presence of substrate would be adequate to provide time for enzyme induction. It should be remembered that enzyme induction under relatively poor nitrogen conditions prevailing during fixation might well result in a slower synthesis program and consequently longer induction intervals. The failure of glucose-grown cells to respond to lactate might, on the other hand, be due to some inhibitory effect of glucose arising as a result of some glucose carry-over from the growth medium. When glucose and lactate were made available to lactate-grown cells there was strong evidence of glucose suppression of the fixation process (Table VII).

TABLE VII. The effect of added glucose on $^{13}N_2$ fixation by lactate-grown cells.

Reaction substrate	Concentration	CPM/mg protein at To
Lactate	10 mg/ml	618.0
Lactate and glucose	10 mg/ml 5 mg/ml	3.5

This finding is apparently in agreement with the report by McNary and Burris (33) that glucose inhibits nitrogen fixation by depleting the ATP reserve in the direction of hexose phosphorylation rather than for nitrogen fixation. It should be remembered, however, that observation was based on studies of the fixation pathway in Clostridium butylicum.

It might be expected that an aerobe such as G-15 could utilize a part or all of the TCA cycle to provide energy for growth purposes but the question of the origin of the reducing power of reduction of N_2 to NH_3 has not been resolved. As will be noted later, attempts to demonstrate an active hydrogenase in a cell sonicate preparation were unsuccessful. The whole question of the participation of ATP-dependent hydrogenase in aerobic fixation is still in dispute although Burris (14) feels that the actual ATP-dependent hydrogenase is an integral part of nitrogenase and has a site for H_2 separate from that for N_2.

HYDROGENASE

Virtanen and Miettenin (50) in 1963 reported that Wilson and associates believed that all free-living nitrogen fixing microorganisms possess the enzyme hydrogenase. Thimann (49), on the other hand, points out that the importance of hydrogenase may have been overrated since the

normal oxidation-reduction reactions operating in a cell for growth purposes could supply the necessary hydrogen for reduction of N_2 to NH_3. In G-15, no hydrogenase could be demonstrated even though both directions of the reaction were assayed.

This finding would seem to substantiate Thimann's view, however, while it may be true that 'normal' hydrogenase may not have a place in nitrogen fixation neither can hydrogenase, per se, be dismissed. Burns and Bulen in 1965 (11) reported an ATP-dependent hydrogenase present in Azotobacter vinelandii which was different from the 'normal' hydrogenase in that it followed nitrogenase in purification procedures and that it had the same conditions for optimal activity as did nitrogenase. Burns (10) also obtained an ATP-dependent hydrogenase from Clostridium pasteurianum and found it to be similar to that of Azotobacter vinelandii in that it also paralleled the nitrogenase system in purifications and activity. Consequently, we have from Burris (14) "There is good reason to suggest that nitrogenase and ATP-dependent hydrogenase may be the same enzyme". The question as to whether hydrogenase forms an integral part of the N_2 fixing system has been in doubt. Interest now seems to be centered on the role of ATP-dependent hydrogenase rather than 'normal' hydrogenase.

Until 1960 when Carnahan et al (17) first reported a

method of preparing reproducible cell free extracts capable of fixing nitrogen, observations of cell free fixation were rare and experimental findings could seldom be replicated. With G-15, a sonicate was prepared in the usual manner and was found, not only to be capable of fixation, but to do so more readily than the whole cells (Table VIII). This could be explained on the basis of permeability or on the basis that enzymes involved in the process may be bound in the cell in some way that limits their activity.

TABLE VIII. A comparison of $^{13}N_2$ fixation in whole cells and in a sonicate of the isolate G-15.

Cell preparation*	Reaction substrate	Substrate concentration	CPM/mg protein at To
Whole cells	Lactate	10 mg/ml	112
Sonicate	Lactate	10 mg/ml	364

*A population of lactate-grown cells suspension was divided equally. One part constituted the whole cell preparation; the second part was sonicated 25 minutes using 10 Kc Raytheon sonic oscillator.

As can be determined from the foregoing description of the behavior of isolate G-15, a number of questions remain unanswered. First, the peculiar interference by brom cresol purple with the growth of the organism in diagnostic media should be reexamined since a finding of this type has not been reported for N_2 fixing organisms. Secondly, the failure to

demonstrate directly the lactic dehydrogenase system by conventional means strongly suggests this question should be reopened. The suspicion that an interfering compound other than NADH oxidase seems likely and identification of this substance should be undertaken. Thirdly, the failure to demonstrate ATP-dependent hydrogen evolution (hydrogenase) may have been due to the particular dithionite preparation used in the assay for it has become known recently that the dithionite used must not only be fresh but should be stored under an inert atmosphere.

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