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THE EFFECT OF NICKEL IONS ON ARTHROBACTER MARINUS, A NEW SPECIES

ANDRE BENOIT COBET

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Microbiology
THE EFFECT OF NICKEL IONS ON ANTHOPODACTER
PARINUS, A NEW SPECIES

by

ANDREW BENOLT CORFT

B.S., San Diego State College, 1959
M.S., San Diego State College, 1963

A THESIS

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This thesis is dedicated to my wife Helen, and sons Paul and Dean, for providing help, understanding and patience only a loving family can give.
I wish to express my thanks to the Chief, Bureau of Medicine and Surgery, Department of the Navy, for having provided me the opportunity to do this research.

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ABSTRACT

THE EFFECT OF NICKEL ON ARTHROBACTER MARINUS, A NEW SPECIES

by

ANDRE BENOIT COBET

A study was undertaken to determine the mechanism whereby nickel produced an unbalanced growth situation, resulting in large, plasmolyzed spheroplasts (megalomorphs) in the marine bacterium, *Arthrobacter marinus* nov. spec. The effect of increasing nickel concentration was gradual with a slight effect on the size in $1 \times 10^{-4}$ M NiCl$_2$ while the cell formed megalomorphs attaining a size of 10-15 μm in $4 \times 10^{-4}$ M NiCl$_2$ under the condition used. Division did not occur at $5 \times 10^{-4}$ M nickel or higher whereas growth was not inhibited until $3 \times 10^{-3}$ M nickel. The multiplication of the culture was not appreciably stimulated by any concentration of nickel.

At least $8 \times 10^3$ cells/ml of basal medium were necessary for multiplication at $4 \times 10^{-4}$ M nickel while as few as 10 cells/ml were able to multiply at $3 \times 10^{-4}$ M nickel. The concentration of proteinaceous nutrient was important in determining the concentration of nickel needed for inhibition of multiplication and followed a second order reaction. As the temperature of incubation was increased above 35 C the inhibitory effect of nickel increased while at 30 C the culture was able to
multiply at $5 \times 10^{-4}$ M nickel, but not at 25 C.

Osmotic support was needed by the megalomorphic cell which could be provided by either inorganic ions or sucrose. If the support was not provided the cells would form filaments rather than the globular shape and could not tolerate as high concentrations of nickel.

The formation of megalomorphic cells occurred at two periods, immediately on the addition of the nickel and after an extended lag period of some 70 hr. The first group of megalomorphs was probably from those cells actively metabolizing whereas the second group were probably in late stationary when the nickel was added requiring the time for accumulation of necessary growth factors.

The addition of $4 \times 10^{-4}$ M nickel to the culture at the late lag-early logarithmic stage of growth resulted in a greater increase in population than when added before or after this period. Addition at zero time or late logarithmic stage resulted in no cell division. The formed megalomorph was capable of normal division when the cells were returned to fresh basal medium or the nickel diluted to low levels.

_A. marinus_ was capable of some acclimatization to inhibitory concentrations of nickel as well as adjusting the morphological response to follow the increased level of nickel required for divisional inhibition.

The culture in late logarithmic stage released 260 and 280 μ absorbing material to the medium. The oxygen utilization of the endogenous metabolism of _A. marinus_ was not
affected nor was there an immediate effect on the addition of nickel to an actively metabolizing culture.

The production of DNA, RNA, and protein by *A. marinus* occurred at near normal ratios during the formation of the megalomorphic cells. Thus there was no discontinuance in the gross synthesis of the major cellular constituents even though cell division had ceased. The C:P ratios remained at levels similar to those at the time of addition of nickel while the N:P ratio increased slightly.

The concentration of nickel taken up and bound by the culture depended on the nickel concentration in the medium, being greatest with $6 \times 10^{-4}$ M nickel in the medium at 14.5 µg Ni/mg of cellular nitrogen. The uptake of nickel was rapid with almost half the total nickel absorbed being bound by the cell while the other half was easily removed by a single wash.

Electron microscopy of the cells at various times during the formation of the megalomorphic cell did not show gross dissimilarities from the normal cells except for the size, shape and plasmolysis in the megalomorph. The DNA, ribosomes, and cell wall appeared normal in the megalomorph.

The production of megalomorphs was not unique only to nickel as zinc, copper, cadmium, and cobalt also produced morphological alterations in the cell. Nickel, however, produced the most dramatic forms. Dithizone washing of the medium prior to addition of the nickel resulted in an alteration in the medium such that there was greater growth in
the control medium while the toxicity of nickel increased.

Sucrose without nickel at increased molarity produced a morphological alteration in the culture similar to those from nickel while the phase density was lighter with a textured appearance.

Hydroxyurea and caffeine produced filamentous cells at inhibitory concentrations while cycloserine and D-methionine produced megalomorphs of similar size and appearance as those from nickel.

The size of the megalomorph was dependent on the concentration of some component in the basal medium for its production. In a synthetic medium with glucose as an energy source and a medium with 100 ppm protein the megalomorphs were reduced in size to 2-3 and 5-8 μ respectively.

A two logarithmic increase in magnesium ion resulted in only a sparing of the toxicity of a tenth logarithmic increase in the level of nickel while being ineffectual in sparing the morphological effect of nickel. Calcium and iron were without effect in sparing the action of nickel while calcium at increased levels was antagonistic to the culture. The chelators EDTA and 8-hydroxyquinoline did not produce morphological variation in the culture at inhibitory levels.

Biotin, cobalamine and pantoyl lactone were without effect in preventing or correcting the resultant morphology from increased nickel concentration.

The abnormally enlarged growth of *A. marinus* in the
presence of nickel is caused by an alteration in the divisional processes without an appreciable effect on growth. This may be through a disruption of the septum formation mechanisms, cell wall associated and paraplasmatic enzymes, or the DNA-membrane interaction needed for cell division.
I. INTRODUCTION

The role of bacteria in the marine environment is concerned with the remobilization of organic nutrients. The details of how this is accomplished are not fully understood ecologically.

The inorganic requirements and relationships of the major bulk elements such as magnesium, sodium, potassium, and calcium with the marine microbial population have been studied in regard to osmotic support and a few major metabolic processes. The importance of the minor or non-essential trace elements in marine microbial processes and their relationship with the marine environment has not been investigated.

At present there are no satisfactory criteria for separation of the microbial population into fresh water or marine types other than the ability to grow or not to grow in marine media on initial isolation (ZoBell and Upham, 1944). The presence of heavy metals in the sea has been proposed as one of the principal inhibiting factors for the fresh water bacterial population in sea water (Jones, 1964).

This investigation was undertaken to determine the effect of a toxic trace element, found in the sea, on a marine bacterium. Cytological, physiological and biochemical studies were employed to determine the mechanism of nickel involvement on the life of a specific marine bacterium which greatly enlarged in the presence of increased nickel concentrations.
II. LITERATURE REVIEW

The ocean contains species of all the chemical elements. Only 14 elements are found in concentrations greater than 1 mg/liter by weight (Goldberg, 1965). These elements, oxygen, hydrogen, chlorine, sodium, magnesium, sulfur, calcium, potassium, bromine, carbon, strontium, boron, silicon, and flourine, are geochemically the more unreactive (Goldberg, 1965) and contain the group of elements known as the "bulk" elements, with the exception of iron, (Lehninger, 1950). The other "minor" elements are found in micromolar and sub-micromolar levels (Goldberg, 1965) and contain the essential trace and trace elements (Lehninger, 1950) which are included in some of the more important biological reactions.

The concentrations of the reactive metals, such as iron, vary in the ocean, being slightly higher near shore than in deeper waters, and appear in both the soluble and particulate fractions of sea water (Lewis and Goldberg, 1954). Nickel has been determined to exist in a range of 0.1 to 6.0 μg/liter (Black and Mitchell, 1952; Mason, 1952; Goldberg, 1961). Goldberg (1961) reports nickel to occur mainly as two species, Ni^{++} and NiSO_{4} at about 2 μg/liter. Corcoran and Alexander (1964) found nickel to exist in very low quantities in the particulate fraction with a minimum at the surface of 0.01 μg/liter and a maximum of 0.1 to 0.15 μg/liter at 300-600 meters in depth. The soluble nickel was maximal at 550 meters with a concentration of 3.7 μg/ml which remained
fairly constant at increasing depths.

The concentration of nickel in the sediments has a mean value of 364 mg/Kg in a Pacific Ocean core and 63 and 92 mg/Kg in 2 Atlantic cores (Hutchins et al. 1955). In the Atlantic Ocean the nickel content in the sediments increased with the distance from shore to the highest levels in the deep pelagic sediments (Wedepohl, 1960).

In biological systems the only report of an absolute requirement of nickel for growth was by Bartha and Ordal (1965). They found that for chemolithotrophic growth of Hydrogenomonas, strains H1 and H16, a concentration of $3 \times 10^{-7}$ M nickel was necessary. The role of nickel was not specified. It was suggested that nickel may have served as an enzyme cofactor.

Small amounts of nickel produce a 20-fold increase in production of 2, 3 butanediol by Aerobacter aerogenes (Ranganayaki and Rahadur, 1958). In vitro, nickel was able to act as the cofactor in place of the normally accepted metal to activate a few enzymes as: arginase (Stock, Perkins, and Hellerman, 1938); enolase (Wold and Ballon, 1957); carboxypeptidase (Coleman and Vallee, 1960); and phosphomonoesterase (Neumann, 1949). Whether these replacements occur in vivo is unknown.

Nickel in normal biological systems has been found to be associated with the RNA of the cell. In beef liver the nickel was at a concentration of 63 μg/g RNA, being firmly bound since dialysis was unable to reduce the content (Wacker
and Vallee, 1959). The phosphorus group of the nucleotide base was not considered the main point of reaction as the $E_{Me/RNA}$ phosphate was about 1:50 over a wide range of sources while for DNA the value was 1:150. Fuwa et al. (1960) found nickel to have a great stabilizing effect on the secondary structure of RNA even when H bonds are being disrupted. This was demonstrated by heating RNA to its melting point ($T_m$--the temperature when 50% of the H bonds holding the double strand are broken) and added nickel to $10^{-3}M$. The maximum absorbance at 258 m$\mu$ instantaneously dropped to that of RNA when the same nickel level was added prior to heating.

Huff et al. (1964) found that Tomato Mosaic Virus-RNA underwent stabilization of the secondary structure in the presence of nickel. However, the biological activity was rapidly lost as a first order reaction. The stabilization of the secondary structure was by metal bond formation between nitrogen bases while the loss in biological activity was due to metal ion-catalyzed hydrolysis of phosphodiester bonds.

In Great Britain, pulmonary cancer has become an occupational disease among workers exposed to gaseous nickel (Goldblatt, 1958). Gilman (1962) reported that nickel could induce the formation of rhabdomyosarcomas in rats. Sunderman (1963) working with rats found on administering nickel carbonyl by inhalation that nickel caused an alteration in RNA from the lungs. There was an increase in the RNA-Ni binding and disruption of the hydrogen bonding. Bassur and Gilman
(1967) working with tissue cultures of muscle found nickel to produce abnormal morphology in the normal cell line, while the rhabdomyosarcoma line was unaffected.

Wahlin and Almaden (1939) applied the term "Megalomorph" to those cells which were abnormally long and frequently swollen. There are a variety of agents which are capable of inducing the formation of such megalomorphic cells. These agents are generally stress producing with some damage to the cellular systems. Induction is possible by magnesium deficiency (Webb, 1949), deficiency in growth factors (Chaplin and Lochhead, 1956; Chan, 1964), metals (Rosenberg et al. 1967; Sobek and Talburt, 1968), antibiotics (Kantor and Deering, 1968), temperature (Terry, Gaffar and Sagers, 1966), and pressure (ZoBell and Cobet, 1962). For reviews of abnormal morphological effects, see Hughes (1956) and Duguid and Wilkinson (1961).

The rare earth metals between atomic number 57 and 71 were found to produce no change in the morphology of Escherichia coli or Saccharomyces cerevisiae. Morphological changes resulted in Streptomycetes and four fungi, while changes in cell wall and membrane damage led to death. The toxicity of the metals follows the order of decreasing atomic number and increasing atomic radii (Talburt and Johnson, 1967). Wurm (1951) suggested that Streptococcus faecalis failed to grow in the presence of lanthanum due to the depletion of phosphate from the medium by the metal as well as depleting the intracellular phosphate.
Renshaw and Thomson (1967) in fractionating filamentous cells of *E. coli* exposed to Pt\(^{191}\) as a neutral species found platinum distributed in the cytoplasmic proteins 45%, nucleic acids 30% and metabolic intermediates 19% while in *Bacillus cereus* and *Staphylococcus aureus* the distribution of platinum was cytoplasmic proteins 5%, nucleic acids 19% and metabolic intermediates 74%. There was greater retention of platinum in the intermediates of the gram-negative cells, possibly by metabolites complexing with the platinum.

Sobek and Talburt (1968) compared the distribution of \(^{14}C\) activity in the normal cell and those incubated in the presence of cesium. They found a lowering of the \(^{14}C\) activity from 47 to 19% in the cold TCA extract, an increase from 6 to 34% in the alcohol extract, an increase from 1.3 to 21% in the alcohol-ether extract, a decrease from 7 to 5% in hot TCA extract, and a decrease from 39 to 22% in the residue. There was alteration in the morphology of the cell with marked clumping.

The history of the genus *Arthrobacter* is described by Conn, Wolfe and Ford (1940) before being organized into a genus. The type species was originally described as a *Bacterium globiforme* in 1928. In the 4th edition of Bergey's Manual this species was placed in the genus *Achromobacter* and in the 5th edition was returned to its original position as *Bacterium*. Conn and Dimmick (1947) proposed that *Bacterium globiforme* be placed in the abandoned genus *Arthrobacter* as
the type species. No organisms had been assigned to the genus on its original creation by Fischer in 1894.

The morphology of the *Arthrobacter* genus is variable being rod shaped in young cultures and coccoid in older cultures. The Gram reaction also is variable with gram-positive, variable, and gram-negative organisms in the genus. The Gram reaction, however, may change with the stage of growth (Mulder, 1964). Mulder and Antheunisse estimated the number of *Arthrobacter* in the soil to be in the range of 70-80% of the bacterial population in arable soil and to 20-40% in prairie soil. They may be considered as part of the autochthonous population of the soil as proposed by Winogradsky (1949).

Because of the variability in morphology and Gram reaction the cultures have probably been distributed into a number of other genera in the course of characterizing various ecological populations.

The bacterial composition of the Narragansett Bay was found to be as high as 25% *Arthrobacter* with a mean of 8% (Sieburth, 1967). The numbers of *Arthrobacter* found in the oceans have not been well established.

Reviews for the genus *Arthrobacter* are presented by Mulder (1964) and Mulder and Antheunisse (1963).
III. MATERIALS AND METHODS

1. Organisms

A culture of a bacterium identified as *Arthrobacter* sp. producing unusually large megalomorphic forms when placed in high concentrations of nickel was isolated from Woods Hole sea water (Wirsen, 1966). When this bacterial culture was plated to determine its purity, two colony types were detected. One was a translucent white colony of gram-negative, non-motile rods and the second and predominant colony was more opaque with gram-negative motile rods. These two colony types were separated by repeated dilution and re-isolation. Both colony types produced megalomorphic forms in the presence of increased amounts of nickel. The second or motile organism was investigated in this thesis and identified as a marine *Arthrobacter*.

The type species of the genus *Arthrobacter* was obtained from the American Type Culture Collection as ATCC #8010, *Arthrobacter globiformis*.

The stock cultures of the marine *Arthrobacter* were maintained on slants of Marine Agar (Difco) and *A. globiformis* on slants of Trypticase Soy Agar (BBL). The cultures were transferred to fresh slants every 6 weeks, incubated at ambient temperature for 24 hr and placed in 4-6°C for storage.

2. Media

The basal medium used in most experiments had the following composition: peptone (Difco) 0.5 g, yeast extract
(Difco) 0.5 g, synthetic sea water (Lyman and Fleming, 1940) 1000 ml. The final pH of the medium was 7.8 - 8.0.

Synthetic medium was prepared with NH₄NO₃ 0.05%, K₂HPO₄ 0.002%, L-asparagine 0.001%, glucose 0.5% in 75% synthetic sea water.

If a particular cation was included in a medium the proper dilution was added prior to sterilization which was accomplished by autoclaving at 121 C for 15 min. Flasks were stoppered with non-absorbant cotton wrapped within a Kim-Wipe (Kimberly-Clark) to prevent unraveling of the cotton plugs.

Aqueous solutions of 1.0 M concentration of the following salts were prepared: CuCl₂, CoCl₂, ZnCl₂, CdCl₂, MnCl₂, Pb(NO₃)₂, NiCl₂, MgCl₂, FeCl₃, CaCl₂, AgNO₃, while HgCl₂ was prepared in 0.1 M solution. These salt solutions were diluted and used where indicated.

3. Inoculum

The inoculum for growth experiments was produced from 18 hr cultures grown at ambient temperature (23-27 C) on Marine Agar slants (Difco). The growth was washed from the surface with 75% synthetic sea water and diluted as described in Standard Methods for the Examination of Water and Wastewater (1965). Dilutions to achieve cell concentrations of 10⁵ cells/ml were used throughout.

4. Cell Density Measurements

Optical density measurements were performed using the half-inch test tube in the Bausch and Lomb Spectronic 20
colorimeter at 420 μm. Absorbance in the range of 230-320 μm was determined with matched one cm quartz cells in a Bausch and Lomb Spectronic 600 dual-beam spectrophotometer (Bausch and Lomb, Inc.).

5. Aeration of Cultures

The cultures were shaken at 300 rpm on a New Brunswick gyrotory shaker model G-33 or S-3. Temperature experiments were performed in a controlled environmental psychrotherm incubator-shaking machine, model G-26, (New Brunswick Sci.).

6. Glassware

Glassware was initially sulfuric acid-washed followed by multiple rinses in distilled water. At the completion of each experiment the glassware was washed mechanically in Heikl detergent (Heinicke Corp.) followed by distilled water rinse for 1.5 min in a Heinicke glassware washer (Heinicke Corp.).

7. Photomicrography

Photographs were taken with a Reichert camera (Reichert) attached to a Zeiss WL Research Microscope (Carl Zeiss). The cells were prepared as wet mounts, observed under phase contrast with photographs taken on Kodak Tri-X film at a quarter of a second exposure.

8. Species Identification of Arthrobacter marinus

Cultures of A. marinus species and A. globiformis (ATCC #8010) were plated to, Marine Agar and Trypticase Soy Agar, their respective growth media and four single colony isolates of A. marinus and two single colonies of the type
species were isolated, transferred to slants and main­
tained for use in the identification procedures to follow.

The cultural characteristics were determined using the
criteria set forth in the Manual of Microbiological Methods
(1957), Skerman (1967) and Steel and Cowan (1965). Growth
cycle pleomorphism was observed by phase contrast microscopy.
Flagella were demonstrated by electron microscopy of a 24
A° thick 30 degrees shadow cast cell preparations with tungstic
oxide.
Gram stain: by Hucker modification.
Spore stain: by Malachite green.

Prepared media used for growth characteristics were:
Extract Agar (BBL), Extract Broth (BBL), Tryptose-Glucose-
Extract Agar (Difco), and Potato-Dextrose Agar (BBL) all
prepared with 75% synthetic sea water. Marine Agar 2216
(Difco) was prepared in distilled water. Soil Extract-Trypticase
Soy Agar was obtained by Tryptase Soy Agar (BBL)
prepared with 50% soil extract (Pringsheim, 1948) and 50%
distilled water.
Nicotine agar: prepared according to Sguros (1955), nicotine
(Eastman) 0.4%, KH₂PO₄ 0.2%, KCl 0.5%, MgSO₄ 0.0025%,
FeSO₄ 0.0025%, yeast extract (Difco) 0.01%, Bacto-agar
1.5% in both distilled water and 75% synthetic sea water.
The following media were prepared in 75% synthetic sea water.
Gelatin hydrolysis was determined on plates of 0.4% gelatin
(Difco) in Extract Agar (BBL) by flooding the plates with
acid mercuric chloride solution. Stab cultures were also prepared with gelatin 12%, in Extract Broth (BBL). Incubation was at both 20 and 25 C. Starch hydrolysis was tested on plates of Starch Agar (Difco) at intervals up to 10 days by flooding plates, incubated at 25 C, with iodine solution. Reduction of nitrate was determined by cultivation in Trypticase Soy Broth containing 0.1% KNO₃. Nitrite was tested for by Griess-Ilosvay reagent. Production of indole: Kovac's reagent was used to determine the presence of indole in the Trypticase Soy Broth. Production of H₂S: by Lead Acetate Agar slants (Difco) and Extract Agar with 0.1 cysteine and 0.02% FeSO₄·7H₂O. Utilization of Citrate: Simmons Citrate Medium (BBL) and Koser's Citrate Broth (Difco). Urease production: Bacto-Urea Broth (Difco) in both distilled water and 75% synthetic sea water. Catalase: a loopful of the growth from the Marine Agar 2216E slant (Oppenheimer and ZoBell) was added to one drop of 3% H₂O₂. Litmus Milk: Litmus Milk (Difco) prepared in distilled water. Acetylmethylcarbinol: MR-VP Medium (BBL) was inoculated and acetylmethylcarbinol tested by Barritts method. Ammonium and nitrate utilization as sole source of nitrogen: Media of composition: NH₄Cl or KNO₃ 0.1%, glucose 1.0%,
Creatinine and creatine utilization for sole source of nitrogen and/or carbon: For carbon and nitrogen utilization the media was prepared according to Dubos and Miller (1937), creatinine or creatine 0.2%, $K_2HPO_4$ 0.005% in both distilled water and 75% synthetic sea water. For carbon source utilization, $(NH_4)_2SO_4$ 0.05% was added to the medium.

Carbohydrate utilization: Bacto-Phenol Red Broth (Difco) with 0.5% carbohydrate. The synthetic base was composed of $NH_4NO_3$ 0.1%, $K_2HPO_4$ 0.002%, phenol red 0.002%, asparagine 20 ug/ml and carbohydrate, 1.0%.

Cellulose utilization: Whatman #2 filter paper (W & R Balston, Ltd.) soaked in the synthetic base above without the phenol red indicator.

Ashby's nitrogen free medium was prepared: mannitol 1.5%, $MgSO_4\cdot6H_2O$ 0.02%, $K_2HPO_4$ 0.02%, NaCl 0.01%, $CaSO_4\cdot2H_2O$ 0.01%, $CaCO_3$ 0.5%, Noble Agar (Difco) 1.5% in distilled water.

Thiotone Broth: prepared according to Blankenship and Doetsch (1961), Thiotone (BBL) 0.1%, glucose 0.25%, $K_2HPO_4$ 0.25% in both distilled water and 75% synthetic sea water.

The growth response to temperature was derived from data obtained from growth in peptone 0.05%, yeast extract 0.05%, $MgCl_2\cdot6H_2O$ 0.3%, $CaCl_2$ 0.06%, KCl 0.04% and NaCl 7.02% (1.2M) in distilled water.
Heat shock of the culture was performed with a four day and two week growth from slants of TSA-soil extract agar and Marine Agar. The cells were suspended in 75% synthetic sea water to a level of $10^8$ cells/ml, heated to 70°C for 5, 10, 20, 30 min and plated to Marine Agar 2216E (Oppenheimer and ZoBell, 1952).

Deoxyribonucleic acid (DNA) was isolated by methods of Marmur (1961) and the guanosine + cytosine (G + C) molar concentration obtained by the method of Wang and Hashagen (1964). The cultures were grown in the basal medium at ambient temperature. The fish sperm DNA (Sigma) and calf thymus DNA (Calbiochem) were used as controls, A grade (Calbiochem) chemicals as follows: thymidine-5'-monophosphate, deoxyguanosine-5'-monophosphate, deoxyadenosine-5'-monophosphate, and deoxycytidine-5'-monophosphate were used in preparing the standards.

9. Oxygen Utilization

The oxygen utilization was measured using standard Warburg methods outlined in Umbriet, Burris, and Stauffer (1957). Double side arm flasks with a center well were used. The cell suspension was obtained from growth in double strength basal medium, washed twice in and finally resuspended in 75% synthetic sea water to an OD of 1.5 at 420 μm. The nickel solution was 0.1 M NiCl$_2$ in the side arm which on dilution in the flask produced a $6 \times 10^{-3}$M concentration. The nutrient solution was 0.85% each peptone and yeast extract in 75% synthetic sea water and produced a concentration of
nutrients comparable to the basal medium. The total volume of fluid was brought to 3.2 ml, including 0.2 ml of 20% KOH in the center well, with 75% synthetic sea water. Table 1 shows the proportions of the components of the procedure.

10. Protein, Ribonucleic Acid and Deoxyribonucleic Acid Production in the Presence of Nickel

The basal medium was inoculated with the culture to a level of about $5 \times 10^6$ cells/ml of medium. The culture was incubated for three hr at which time $4 \times 10^{-4}$ M nickel was added.

The cells were harvested by centrifugation at 7,000 rpm for 10 min at 4 C. The packed cells were washed twice with 75% synthetic sea water and an aliquot removed for nitrogen determination and direct cell count. The remaining cells were extracted by a ZoBell and Cobet (1964) modification of the Ogur et al. (1952) method. Determinations for protein were by micro-Kjeldahl using ammonium sulfate as a standard and 6.75 conversion factor from nitrogen to protein. RNA was determined by the method of Ceriotti (1955) using adenosine 2' + 3' monophosphoric acid, yeast (Sigma), and DNA determined by the method of Burton (1956) using fish sperm DNA (Sigma) as a standard. The direct cell count was with a Petroff-Hauser chamber under phase contrast microscopy.

11. Carbon, Nitrogen, Phosphorus Ratios

The basal medium was inoculated with $7 \times 10^6$ cells/ml and after four hr incubation the medium was divided into
three aliquots, one receiving $4 \times 10^{-4}$ M nickel, a second $6 \times 10^{-4}$ M nickel, and the third served as control. Periodically, portions were removed for plate counts to Marine Agar 2216E. Cells were harvested at these same times by centrifugation at 7,000 rpm for 10 min at 4 C. The cells were washed twice in and brought to an OD of approximately 0.500 at 420 nm with 75% synthetic sea water. From this cell suspension carbon was determined by the method of Menzel and Vaccaro (1964) using CO$_2$ as a standard, nitrogen by micro-Kjeldahl using ammonium sulfate as standard, and phosphorus by the method of Bartlett (1959) using a 0.8 mg/ml solution of KH$_2$PO$_4$ (Fisher) as a standard.

12. Quantitation of Nickel Uptake

The basal medium inoculated with $10^7$ cells/ml was incubated 3.5 hrs to bring the culture to early logarithmic phase at which time nickel was added. To determine the ability of the organism to concentrate nickel, increasing concentration at $2.0 \times 10^{-6}$, $1.0 \times 10^{-5}$, $1.0$, $2.0$, $4.0$, $6.0$, and $8.0 \times 10^{-4}$ M were added and the culture was allowed to incubate three additional hrs. The time relationship for uptake was determined by adding $4 \times 10^{-4}$ M nickel and incubating 0, 30, 60, 120, and 240 min. The retention of nickel by the cells was determined by multiple washing of the cell harvest from three hr incubation in $4 \times 10^{-4}$ M nickel. The zero wash was obtained by one wash of the cell harvest with 75% synthetic sea water containing $4 \times 10^{-4}$ M
nickel, the other preparations were washed with 75% synthetic sea water without nickel 1, 2, or 4 times.

The cells from the above procedures were harvested by centrifugation at 7,000 rpm for 10 min at 4°C and washed once in 75% synthetic sea water. The cell pack was freeze-dried, crushed, mixed and placed in a tared crucible. A weighed amount was kept for nitrogen analysis determined by micro-Kjeldahl. The remaining dry cells were weighed and ashed to constant weight at 650°C in a muffle furnace (Thermolyne type 1400, Thermolyne Corp.). The ash was dissolved in 3 ml of concentrated hydrochloric acid A.C.S. (Fisher) and brought to 15 ml with double distilled water. The concentration of nickel was determined on this material by the University of New Hampshire Chemical Engineering Department using atomic absorption spectroscopy.

13. **Respiratory Deficiency Induction by Nickel**

The method used is essentially that of Ogur, St. John and Nagai (1957) and Lindegren, Nagai and Nagai (1958). The growth resulting from $4 \times 10^{-4}$ M nickel in basal medium was plated on the surface of 2216E agar plates and incubated at ambient temperature. The resulting colonies were overlayed with 0.1% triphenyl tetrazolium chloride (TTC) in 1.5% agar and re-incubated an additional hr. A second procedure was to incubate a normal culture on the surface of agar plates of the basal medium containing increasing concentrations of nickel. The colonies formed on incubation at ambient temperature were overlayed with TTC as above.
14. **Electron Microscopy**

To the culture growing in the basal medium was added \(4 \times 10^{-4}\) M \(\text{NiCl}_2\) after three hrs incubation. Portions of the culture were removed just prior to the addition of the nickel and at 1, 2, 3 and 6 hrs after the addition. Each sample was brought to 0.76% glutaraldehyde (Eastman Chemical Co.), using the redistilled product, and allowed to fix for 3 hrs at 6 C. The partially fixed cells were centrifuged at 1500 rpm for 10 min, washed twice with 100% synthetic sea water and embedded in 1.0% Ionagar (Oxiod) prepared in 100% synthetic sea water. The embedded material was cut into 1-2 mm\(^3\) blocks and placed overnight in 1.0% osmic acid in veronal buffer pH 6.1 (Ryter and Kellenberger, 1958) prepared with synthetic sea water. The following morning the blocks were washed twice for 30 min periods in the veronal buffer, placed in 0.5% uranyl acetate (Reagent grade, Fisher) prepared in 100% synthetic sea water for two hrs, washed twice more in the veronal buffer and dehydrated through increasing concentrations of ethyl alcohol of 50, 70, 90, 95, and 100% for 30 min periods in an ice bath. The absolute alcohol was changed twice to assure complete dehydration.

The dehydrated material was placed in propylene oxide with two changes for 10 min each then placed overnight in propylene oxide:epon mixture (1:1) in an open vial. The epon mixture was prepared from equal volumes of mixture A and B, plus 1.5% 2, 4, 6-tri (dimethylaminomethyl) phenol, (Fisher).
Mixture A was prepared with 62 ml epon resin 812 (Fisher) and 100 ml dodecenylsuccinic anhydride (Polyscience), and Mixture B with 100 ml epon resin 812 and 89 ml malic methyl anhydride (Polyscience). The infiltrated agar blocks were transferred to #0 empty gelatin capsules (Park Davis) and filled to the top with the epon mixture. The filled capsules were placed in a 15 lb vacuum for two hrs at room temperature. After returning to ambient pressure, the agar blocks were arranged and replaced at 15 lb vacuum at 60 C for 2 days.

The blocks were sent to Dr. John Albright of Boston University for sectioning and microscopy using a Siemens Model #1 electron microscope. The section were post stained with lead citrate.

15. Electron Probe and Electron Diffraction Analysis for Nickel

The culture was inoculated to the basal medium and after 4 hr, $4 \times 10^{-4}$ M nickel was added and the culture allowed to incubate an additional 6 hr. The cells were centrifuged at 7,000 rpm for 10 min at 4 C. The cell pellet was carefully resuspended in 1.0% osmic acid in veronal buffer, pH 6.1 (Ryter and Kellenberger, 1958), prepared in 100% synthetic sea water for osmotic support. The cells were allowed to fix in the osmic acid for two hr after which they were washed twice with 75% synthetic sea water and embedded in 1% Ionagar (Oxoid) prepared in 75% synthetic sea water. The agar embedded material was cut into pieces of about 1-2 mm$^3$ and replaced into the osmic acid-veronal buffer overnight. The agar blocks were washed twice in 75% synthetic
sea water for 20 min periods and dehydrated in ethyl alcohol. Propylene oxide (Eastman) and epon 812 (Fisher) were used to infiltrate and embed the agar blocks using the procedure described previously.

The cell sections were cut 1.0 to 1.5 μ on an LKB ultramicrotome using a glass knife. The sections were placed on Mextaform H₃-copper grids (Ernest F. Fullam) containing a formvar support and carbon coated. A set of sections were sent to Advanced Metals Research (Burlington, Mass.) for electron probe analysis for nickel using a 2 μ laser beam. A second set of sections were kept for electron diffraction analysis.

Mr. Emory Clippert of the University of New Hampshire Microbiology Department performed the electron diffraction pattern using a Tronscope Model TRS-80 electron microscope (Akashi Seisakusho). The standardization of the electron scope was done by the method outlined by Hall (1953) using a gold diffraction pattern. The calculated values of the pattern from the enlarged cells were searched in Index (Inorganic) to the Powder Diffraction File (1966).

16. Dithizone Treatment of the Basal Medium

The basal medium was treated with dithizone as outlined by Albert (1958) to remove heavy metals. The medium was washed three times in a separatory funnel with 10 ml of 0.1% solution of dithizone (Eastman) in chloroform (Fisher) per liter of medium. The medium was washed with chloroform to remove any dissolved dithizone remaining in the medium. The
chloroform was added in 50 ml amounts until the last two washes were colorless, about 6 washes. The medium was heated to boiling twice to drive off the dissolved chloroform.

To this dithizone-treated medium was added various concentrations of nickel from $1 \times 10^{-4}$ through $5 \times 10^{-4}$ M.

17. Inhibitors

A variety of inhibitors including penicillin G (Sigma), chloramphenicol (Sigma), hydroxyurea (Sigma), cycloserine (Calbiochem), actinomycin D (Sigma), D-methionine (Calbiochem), and caffeine (Eastman) were prepared in basal medium and added to 10 ml portions of the basal medium to provide a series of inhibitor concentrations.

18. Amino Acids

The medium to determine the possibility for maintenance of the coccoid form was prepared by adding to the synthetic medium without L-asparaginase, singly, sufficient amounts of the amino acids L-proline (Calbiochem), L-tryptophan (Fisher), DL-serine (Nutritional Biochemical), DL-phenylalanine (Eastman), L-arginine (Eastman), L-asparagine (Eastman), L-lysine (Calbiochem), L-glutamic acid (Nutritional Biochemical), and DL-alanine (Nutritional Biochemical) for a final concentration of 40 mg/100 ml of medium.

19. Antagonistic Effects of Magnesium

The basal medium was prepared using an altered synthetic sea water. The sea water was altered by omission of MgCl$_2$ from the salts. No substitution was made to maintain con-
sistant osmotic support. The medium was divided into 5 aliquots and magnesium added at 5 different levels, $1 \times 10^{-4}$, $1 \times 10^{-3}$, $1 \times 10^{-2}$ and $1.2 \times 10^{-1}$ M MgCl$_2$ and the control with no added magnesium. Each aliquot was prepared with a series of nickel concentrations increasing from $9 \times 10^{-5}$ through $5 \times 10^{-4}$ M. The normal concentration of magnesium in sea water is 0.053 M (ZoBell, 1961.)

20. **Antagonistic Effects of Calcium**

The basal medium was prepared using an altered synthetic sea water. In this instance, the CaCl$_2$ was omitted. The medium was handled as described previously with the calcium content brought to $1 \times 10^{-4}$, $1 \times 10^{-3}$, $1 \times 10^{-2}$ and $1 \times 10^{-1}$ M and finally no calcium addition. The normal concentration of calcium in sea water is 0.01 M (ZoBell, 1961).

21. **Effect of Increased Iron Content**

Basal medium was prepared in 4 aliquots, to three FeCl$_3$ was added to contain $1 \times 10^{-6}$, $1 \times 10^{-5}$ and $1 \times 10^{-4}$ M over the amount already present as contaminants in the basal medium salts. The fourth was used as an untreated control. A series of nickel concentrations was prepared increasing from $1 \times 10^{-4}$ through $4 \times 10^{-4}$ at unit increments. The normal concentration of iron in sea water is $1.2 \times 10^{-7}$ M (Goldberg, 1965).

22. **Effect of Pantoyl Lactone**

The effect of pantoyl lactone was determined in two ways: on cells in the process of enlargement and secondly on cells
already enlarged.

The basal medium was inoculated with the culture and allowed to grow three hr. At this time $6 \times 10^{-4}$ M nickel was added, the culture shaken, DL-pantoyl lactone (Sigma) added to a level of 1.0, 0.5 and 0.025%, and the pH adjusted to 8.0 with NaOH. This treated culture was allowed to incubate with microscopic observations made periodically.

In the second case the culture was examined by allowing the logarithmic phase culture to grow in the presence of nickel for 3 hr before the addition of pantoyl lactone and pH adjustment. Thus, the cells were megalomorphic on addition of pantoyl lactone.

23. **Effect of Chelating Agents**

To basal medium was added increasing molar concentrations of 8-hydroxyquinolin sulfate, C grade (Calbiochem) or ethylenediaminetetraacetic acid (Fisher). The pH was adjusted to 7.8–8.0, the normal pH of the basal medium.
IV. RESULTS

A. Culture Identification

1. Arthrobacter marinus

The nickel-tolerant organism was previously identified as *Achromobacter* sp. by Wirsen (1966). In the course of working with the culture changes in cell size and staining characteristics of the organism during the various growth stages did not resemble that usually ascribed to the genus *Achromobacter*. These observations indicated the genus as *Arthrobacter* and a re-identification of the culture was undertaken.

a. Morphology

A striking characteristic of this organism is its extreme morphological variability during growth. The morphology of the genus *Arthrobacter* (Conn and Dimmick, 1947) was characterized as, "Varied, with a tendency to go through a more or less definite cycle, the most characteristic features of which are gram-negative rods in young cultures and gram-positive coccoid forms (arthrospores) in old cultures." Skerman (1967) stated definitely, "Ultimately the rods transform completely to cocci" and considered the transformation of the cocci through rods to cocci again as a prime feature of the genus.

The marine organism exhibited this cyclic growth pattern and therefore met the principal requirements for the definition of the genus. In a stationary culture the cells were single and were coccoid in shape about 0.6-0.8 x 1.0 μ. When
the cocci were inoculated to Marine agar (Difco) the cells elongated into large rods about 1.2-1.5 μ x 2.0-4.0 μ in the logarithmic phase of growth. As the culture reached early stationary phase the cells gradually became smaller ranging about 1.0 x 1.5 - 2.0 μ. With further incubation the size and shape of the cell completed the cycle requiring about five days. This is shown in the photomicrographs taken by phase contrast microscopy (Fig. 1). The rods were often slightly bent and appeared singly, more often in pairs and occasionally in short chains of 3-4 cells. The daughter cells of dividing rods often became arranged in V-form due to the snapped post-fission movement, a commonly seen characteristic of coryneform bacteria (Starr and Kuhn, 1962).

In the hanging drop preparations from young broth cultures motile cells were observed. Motility was restricted to the logarithmic or rod phase of growth. Electron photomicrographs showed the presence of a single, sub-polar flagellum appearing to consist of two thin and very long strands (Fig. 2). This position of the flagellum placed the organism in the "degenerate peritrichous" group of Conn and Wolfe (1938).

The Gram reaction of this organism varied with growth conditions. In very young cultures from Marine agar slants the rods were gram-negative. In older cultures the cocci gave an appearance of a gram-positive reaction but on closer examination many of the cells clearly have a gram-negative cytoplasm
with large positively stained granules. These granules are
evident in the phase contrast pictures (Fig. 1c). Possibly
the positively stained granules retained sufficient crystal
violet that the remaining negatively stained cytoplasm was
not easily observed. These gram-positive granules appear to
be similar to those observed by Jensen (1960). Cells from
young cultures on Trypticase Soy Agar-soil extract were gram-
positive, remaining as such in the coccoid form. There
was no evidence of spores by staining two week old cultures
from TSA-soil extract and Marine Agar slants with malachite
green stains.

b. Cultural Characteristics

**Extract Agar slant:** growth was luxuriant, filiform, glisten-
ing, butyrous, and cream colored. **Agar plate:** Colonies
were circular, 3-4 mm in diameter, convex, smooth, entire
margins, opaque, cream and butyrous in consistency.
**Broth:** growth exhibited a slight surface ring, strong
diffuse turbidity and a viscid sediment.

**Tryptose-Glucose-Extract Agar slant:** growth was luxuriant,
filiform, glistening, viscid, and ivory to cream in
color. **Plate:** colonies were circular, 4-5 mm in diameter,
convex, smooth, entire, opaque, and ivory to cream in
color.

**Marine Agar slant:** growth was luxuriant, filiform, glistening,
butyrous (becoming viscid on aging), opaque and cream
colored. **Plate:** Colonies were circular 2-3 mm in dia-
meter, convex, entire, butyrous becoming viscid on
aging, opaque and cream colored.
Potato Dextrose Agar slant: growth was luxuriant, filiform, glistening, butyrous, and translucent. Plate: Colonies were circular, 2-3 mm in diameter, convex, entire, smooth, opaque, translucent, and butyrous in consistency.

Trypticase Soy Agar-Soil Extract slant: abundant growth, same appearance as on Marine Agar slant.

Nicotine Agar: no pigment formed on the medium with synthetic sea water; no growth in the medium prepared with distilled water.

c. Physiological tests:

Hydrolysis of gelatin: negative

Hydrolysis of starch: negative

Reduction of nitrate: negative in both agar and broth

Production of indole: negative

Production of H₂S: positive on both Lead Acetate agar and cysteine medium.

Utilization of citrate: positive on Simons Citrate medium and Koser's Citrate Broth

Urease production: negative

Catalase: positive

Litmus Milk: good growth in litmus milk. The reaction was usually unaltered with distinct reduction of the litmus. The milk was not coagulated but was slowly cleared being complete within 3 weeks and producing an abundant viscid sediment.

Acetylmethylcarbinol: negative

Ammonium-sucrose broth: diffuse turbidity and slight sedimentation—was able to use ammonium ion as sole source of nitrogen.
Nitrate-sucrose broth: diffuse turbidity and slight sedimentation—was able to use nitrate as sole source of nitrogen.

Creatinine or Creatine Broth: no growth with or without added ammonium chloride—was unable to use creatinine or creatine as sole source of nitrogen and/or carbon.

Carbohydrate utilization: Bacto-Phenol red broth base with 0.5% carbohydrate produced acid but no gas from sucrose, glucose, galactose and weakly positive on mannitol. Alkaline reaction on arabinose, xylose, glycerol, lactose and dextrin. In the synthetic sea water medium, acid and no gas was produced in sucrose, glucose, galactose, glycerol, maltose, mannose, mannitol, inositol and ribose with slow acid production in lactose. No reaction with salacin, sorbitol, arabinose, rhamnose and xylose.

Cellulose: unable to utilize cellulose as carbon source.

Ashby's nitrogen free medium: no growth

Thiotone broth: the culture produced cells of normal morphology in medium with synthetic sea water; did not grow in distilled water prepared media.

d. Temperature Requirement

The range of temperature of growth is from 6 C, the lowest temperature checked, through 40 but not 42 C. Most rapid growth occurred at 40 C. However, maximum population occurred at 25 C. The response of the culture to heat shock in 75% synthetic sea water at 70 C for as little as 10 min was sufficient to sterilize the culture with a population of 10^8 cells/ml.
e. Guanosine plus Cytosine (G + C) Molar Concentration of the Deoxyribonucleic Acid

The indirect method of Wang and Hashagen (1964) produced the results in Table 2 of the G + C molar concentrations of A. marinus, A. globiformis, fish sperm and calf thymus DNA. These values are compared with those in the literature.

The G + C values found for A. marinus are in accord with the values found for the type species A. globiformis. They do not, however, correspond with that for Achromobacter fisherii. M% (G + C) = 40-42 (Marmur, Falkow and Mandel, 1963).

The use of a peptone medium for detection of acid production from carbohydrates was unsatisfactory as the alkaline reaction from the peptone masked the acid produced indicating a negative result. After 48 hr incubation, at ambient temperature, the phenol broth base showed three acid reactions of the 16 tested whereas 10 had occurred in the synthetic base by A. marinus. The type species produced none in the phenol broth base and 14 in the synthetic base during the same time. At the end of one week, the synthetic base increased only one carbohydrate for each organism: lactose for A. marinus and ribose for A. globiformis. The synthetic base, once acid, did not revert to a neutral or alkaline reaction as had occurred with the peptone containing medium. Conn and Dimmick (1948) found the same problem with the peptone medium and used a synthetic base to describe the species A. simplex.
The organism has a definite inorganic requirement which is easily satisfied by addition of the synthetic sea water. ZoBell and Upham (1944) studied several biochemical reactions of marine bacteria in both fresh water and seawater media and noted quantitative but no marked qualitative differences in the results. The use of synthetic sea water medium is justified since the marine organism failed to reproduce in the fresh water medium.

The morphological, cultural, and physiological properties of this marine organism place it in the genus *Arthrobacter*. In the 7th Edition of Bergey's Manual of Determinative Bacteriology, the genus has been divided into two groups on the basis of utilization of ammonium and nitrate salts and citrate as the sole source of nitrogen and carbon respectively. This marine organism is placed in the first group with those species able to utilize these compounds. It does not correspond to any species previously described in Bergey's Manual.

The characteristics of this organism also differ sufficiently from the published descriptions of *A. nicotianae* (Giovannozzi Sermanni, 1959), *A. crystallopoietes* (Ensign and Rittenberg, 1963), *A. duodecadis*, *A. flavescens* (Lochhead, 1958), *A. atrocyaneus* (Kuhn and Starr, 1960), *A. polychromogenes* (Schippers-Lammertse, Muijsers and Klatser-Oedekerk, 1963), *A. viscosus* (Gasdorf et al. 1965), and *A. ramosus* (Jensen, 1960).

It is justified to describe this culture as a new species.
for which the name *Arthrobacter marinus* is proposed because of its original isolation from the marine environment.

B. **Growth Conditions of A. marinus and Nickel**

2. **Growth Response to Nickel Concentrations**

The growth response of *A. marinus* to increasing concentrations of nickel as NiCl$_2$ was determined by the addition of increasing concentrations from $1 \times 10^{-4}$ through $5 \times 10^{-4}$ M in the basal medium. The sterile medium was inoculated, shaken, and OD readings made at 13, 24, 48, 72, 96, 120, and 144 hr.

The presence of increasing concentrations of nickel resulted in an increase in the lag phase from about three hr in the untreated control without added nickel to over 70 hr at a level of $4 \times 10^{-4}$ M nickel (Fig. 3). At a concentration of $5 \times 10^{-4}$ M nickel the culture did not grow during the period of 144 hr. The slope of the line during the logarithmic phase of growth was depressed with each increase in nickel concentration resulting in an increased time required for the attainment of maximum OD (population) at the higher concentrations of nickel. The OD of the culture are presented. Since the light transmittance depends on the size and shape of the particles, as well as their numbers, the values are relative and are presented to show the initiation of growth, general shape of the curve, and the time of attainment of maximum OD at each nickel concentration. The occurrence of the maximum OD coincided with the maximum population values.
of the culture. The populations normally associated with the maximum OD are in Table 3.

The formation of the megalomorphic cells was a gradient response and dependent upon the concentration of nickel, being most evident at $4 \times 10^{-4}$ M nickel. Fig. 4 presents photomicrographs comparing the maximum size of megalomorphs from the various nickel concentrations with the control culture. The most dramatic change in morphology occurred between $2 \times 10^{-4}$ and $3 \times 10^{-4}$ M nickel. Considerable vacuolation of the megalomorphs developed which increased with time and nickel concentration. The shape of the cells were generally round to oval with the cytoplasm pushed over to one side of the cell in a crescent shape. As the cell became older the cytoplasm became more vacuolated and the shape more irregular. In the stationary phase culture, the cells became smaller but retained the vacuoles and crescent-shaped cytoplasm.

When the cells are inoculated into the basal medium containing $4 \times 10^{-4}$ M nickel with samples removed periodically for observation by phase contrast microscopy, a majority of the cells (>95%) undergo the transformation to megalmorphs, not all cells becoming megalomorphic initially. The course of this enlargement is presented in Fig. 5 and compared with the inoculum in Fig. 5a. After 1.5 hr incubation the cells were somewhat larger than the normal logarithmic rod and more oval in shape. In some instances a small vacuole was formed at the end of the cell. An
occasional cell was seen to bulge in the mid-section as if the newly formed cell wall was weakened and unable to retain the rod shape. The cells at this stage have an even phase density (Fig. 5b). After 2.5 hr incubation the cells became larger with a wide variation in phase density within the cell (Fig. 5c). A tremendous change in size occurred during the subsequent 2 hr incubation in which cells were still somewhat oval, over half the volume appearing as vacuole and the size increasing by about 30 volumes (Fig. 5d) in relation to the control. After 6.5 hr incubation the cells increased to 70 volumes, and became multi-vacuolated (Fig. 5e). At 10 hr incubation the cells were 3.5 times larger than previously, multi-vacuolated and more irregular in shape (Fig. 5f). The cells after 10 hr incubation in $4 \times 10^{-4}$ M nickel were about 250 volumes larger than the untreated normal size rod with approximately 40 volume % of the cell being cytoplasm as a crescent against the cell wall and 60 volume % as vacuole.

The megalomorphic cells formed after 4.5 hr incubation were osmotically sensitive when placed in distilled water.

The response of the culture to the presence of nickel was graded and rather rapid with attainment of dramatic size in 4 hr. Nickel ions caused a decrease in the maximum population attained at stationary phase and an increase in the maximum size of individual cells. The lag phase of growth was extended with increasing concentrations of the cation.
3. **Stimulatory Response of A. marinus to Nickel**

The oligodynamic action or the stimulation of growth may be seen in certain metals at levels slightly lower than those toxic to the growth of the culture. Increasing concentrations of nickel from $1 \times 10^{-7}$ through $4 \times 10^{-4}$ M were added to the basal medium to determine if the cation was stimulatory to A. marinus. The inoculated treatments were shaken at 200 rpm with OD readings at 8, 14 and 24 hr.

The OD at 8 hr was slightly increased with a concentration of $9 \times 10^{-5}$ M nickel; at higher nickel concentrations the OD decreases (Fig. 6). The cells in the basal medium containing nickel concentrations below $9 \times 10^{-5}$ M were of normal morphology whereas greater nickel concentrations caused enlargement and rounding of the cells. At 14 hr, the approximate time of maximum OD for the culture through $9 \times 10^{-5}$ M nickel, slight increase in OD of the culture resulted in the presence of nickel compared to the control. Growth was little stimulated by nickel.

4. **Effect of Autoclaving of Basal Medium on Growth**

The medium contained various proteins and other compounds which are capable of forming complexes with the added nickel ions thereby reducing cation availability to the cell. Heat and pressure may be capable of increasing the amount of complexing in the system. Autoclaving of the medium provides both increased heat, to 121 C, and pressure, to 15 lbs.
The effect of autoclaving the nickel solution with the basal medium was determined under three conditions. The medium was autoclaved with $4 \times 10^{-4}$ M nickel in one case. The medium and nickel solution were autoclaved separately and combined to a level of $4 \times 10^{-4}$ M after cooling to ambient temperature. In the third condition, the autoclaved nickel solution was added to a level of $4 \times 10^{-2}$ M nickel 60 min after inoculation of the medium with _A. marinus_. The treated flasks were shaken at 200 rpm at 25 C with OD readings made at 24, 48, 72, 96, 120, and 144 hr.

The response of the growth in the medium autoclaved with the nickel and medium with the nickel added after cooling of the basal medium indicated the capability of complex formation under the influence of heat and pressure and is represented in Fig. 7. This difference was shown as an increase of about 70 hr in the lag phase of growth in the medium with nickel added after autoclaving over growth in the medium autoclaved with the nickel. This lag period was more than doubled that required for growth to occur in the medium autoclaved with the nickel.

The addition of nickel one hr after inoculation of the basal medium resulted in a decrease of the lag period of only 20 hr over the extreme time. Thus, the addition of the stresses, fresh medium and nickel ions, one at a time separated by one hr, resulted in a somewhat decreased lag period. However, the increased availability of the nickel
cation over the autoclaved treatment was still evident.

None of the treatments resulted in growth at $5 \times 10^{-4}$ M nickel or was there any difference in the size or shape of the resultant cells at $4 \times 10^{-4}$ M nickel regardless of the time of addition of the cation to the medium. Autoclaving of heavy metals with complex media appears to reduce the availability of the metal cations.

5. **Effect of Aeration on Growth**

An increase in the rate of shaking results in an increase in oxygenation of the medium to a maximum depending upon the volume of medium and the size and shape of the container. On the other hand, increased agitation of the medium to attain adequate oxygenation of the culture may, as a result of the shearing forces and turbulence of the liquid, rupture the megalomorphic cells formed in response to the increased nickel concentrations.

To determine the effect of gyrotory shaking on *A. marinus*, a series of basal medium containing nickel concentrations from $1 \times 10^{-4}$ through $5 \times 10^{-4}$ M was incubated under three conditions: static, shaken at 180 and 360 rpm. Optical density readings were made at 15, 24, 36, 60, 84, 108, and 160 hr.

The two rates of shaking compared with a static culture and the results for $4 \times 10^{-4}$ M nickel are presented in Fig. 8. An advantage of shaking the cultures over static incubation was evident. However, there was little difference between the two rates of shaking in the untreated basal medium.
In the nickel containing medium the shaking rate at 360 rpm showed a decided advantage over 180 rpm and static cultures with slight advantage of 180 rpm over the static treatment.

In the flasks containing nickel, the relationship was similar in that the culture at 360 rpm did not effect the cell size or shape. The cell morphology at $4 \times 10^{-4}$ M nickel was the same and had the same degree of vacuolation under the three conditions. The time required for attainment of maximum size was the principal variable.

6. Size of Inoculum

An alteration in the number of bacteria/ml in the inoculum with a constant level of nickel in the medium, resulted in a change in the amount of nickel available/cell. This ratio of Ni/cell increased at the same rate as the concentration of cells decreased with the lower cell populations facing a more toxic metallic environment.

A series of cell concentrations was prepared to produce from as little as 10 through $5.4 \times 10^4$ cells/ml of culture. The appropriate dilutions of cells were added to a series of nickel concentrations from $2 \times 10^{-4}$ to $5 \times 10^{-4}$ M. The cultures were shaken at ambient temperature (23-27 C) with OD readings at 12, 24, 36, 60, 84, 108, 228, 324, and 454 hr. The maximum OD for each culture and the time at which it occurred is presented in Table 4.

As expected, a decrease in cell numbers in the inoculum of any given nickel concentration resulted in an increase in time needed to attain maximum OD. Similarly, as the nickel
concentration was increased at a given cell level there was an increase in the time needed for attainment of maximum OD. The OD decreased with increases in nickel concentration at all cell levels.

At a concentration of $4 \times 10^{-4}$ M nickel there was a minimum required inoculum of between 800 and 8600 cells/ml to bring about measurable OD within the 454 hr of the experiment. The morphology of the cells grown in $4 \times 10^{-4}$ M nickel were the same at the two inoculum levels permitting growth whereas the cells grown in $3 \times 10^{-4}$ M nickel were similar with the exception of the 10 cell inoculum which appeared as if grown in $4 \times 10^{-4}$ M (Fig. 4). Since the morphology resulted from a graded response to the level of nickel, the inhibitory level for the two inocula 880 and 98 cells/ml was just shy of $4.0 \times 10^{-4}$ M nickel while at 10 cells/ml the inhibitory concentration is just over $3.0 \times 10^{-4}$ M nickel.

Nickel is dependent on the cell population for both its inhibitory and morphological effects. As few as 10 bacteria/ml are able to grow in the presence of $3 \times 10^{-4}$ M nickel while a population between 880 and 8600 cells/ml are needed for growth in $4 \times 10^{-4}$ M nickel.

7. **Protein-Nickel Relationship on Multiplication**

The concentration of nickel needed to inhibit the multiplication of the organism in the basal medium was dependent upon the interaction, in large measure, of the
nickel with other constituents in the medium. An alteration in the amount of organic portion of the basal medium resulted in an alteration of the concentration required for limitation of multiplication by nickel.

The concentration of protein containing organic nutrients in the medium was varied such that the yeast extract maintained its relationship of 1:1 peptone in 75% synthetic sea water. A series of increasing nickel concentrations was prepared for each concentration of protein as in Table 5. The OD of each culture was made at 12, 24, 48, 72, 96, 120, and 144 hr and in the case of 100 and 200 ppm protein again at 168 hr.

In Fig. 9 the relationship of nickel to protein was evident as the concentration of protein was increased from 100 to 4,000 ppm in the medium. This relationship was not linear and tended to fall-off at higher levels.

The morphology of the growth occurring at the limiting concentration of nickel was similar in size, shape and appearance regardless of the concentration of protein (Fig. 5e). At the lowest concentration of protein, 100 ppm, the cells are somewhat smaller about 5-8 μ in diameter.

The value obtained for zero protein was taken from another experiment where synthetic medium with glucose was used as the growth medium. The cells were somewhat smaller but round attaining a size of 2-3 μ in diameter with no vacuolation (Fig. 38a).
The concentration of protein in the medium affected the inhibitory concentration of nickel. This relationship was directly related, as the concentration of protein was increased the concentration of nickel needed for inhibition also increased. The protein concentration was involved in the maximum size attained by the cell in response to the nickel. The lethal action of the nickel resulted when the available nickel ions exceeded the chelating ability of the nutrients in the medium. This was difficult to calculate due to the complexity of peptone and yeast extract.

8. **Inorganic Concentrations vs. Nickel Concentration**

The osmotic support afforded by the inorganic content of the basal medium was sufficient to allow the production of osmotically sensitive forms. To determine at what salt concentration osmotic support became critical and how this might affect the megalomorphic formation the inorganic content of the medium was lowered progressively.

Various salinities were prepared by mixing in various ratios two media, basal medium in distilled water and basal medium prepared in 100% synthetic sea water. For each salinity a series of concentrations of nickel were prepared from $1 \times 10^{-4}$ to $5 \times 10^{-4}$ M. Optical densities were read at 12, 24, 36, 60, 84, 108, 132, and 144 hr.

A shift in the osmotic support by adjusting the concentration of synthetic sea water in the medium showed the inability of the culture to compensate for the reduced ionic environment (Table 6). The time required to attain
maximum OD in the control containing no nickel increased with decreases in salinity and was also accompanied by a decrease in maximum OD. With the addition of nickel concentrations to the culture, the time for maximum OD decreased to 60%. The time then increased in lesser concentrations of synthetic sea water. This was more pronounced as the level of nickel was increased. The inhibitory effect of nickel increased as the synthetic sea water was decreased below 60% and became limiting at $1 \times 10^{-4} \text{ M}$ at 5% synthetic sea water.

The morphology of the growth of *A. marinus* which resulted from concentrations of nickel reflected the effects of a decreased osmotic support. In 100 and 80% synthetic sea water the cells were as expected for this concentration of nickel (Fig. 4e). At 60% synthetic sea water, the size was decreased to 8-10 μ but the morphology was similar to that in the higher salinities. The cells in 40% synthetic sea water were filamentous rather than round with bulbs resembling false branching and large vacuoles evident in the cytoplasm (Fig. 10a). Cells in 20% synthetic sea water were similar with fewer bulbs and no false branching sites evident (Fig. 10b). In lower concentrations of synthetic sea water the filaments became more uniform in diameter, less vacuolated and more phase dense to the point where at 5% synthetic sea water the cells were of normal diameter and about 10-15 μ in length (similar to Fig. 35b). Thus, the salinity of the medium has a profound effect upon the morphology of *A.*
9. The Effect of Temperature on Growth

The change in the incubation temperature of a culture may result in an alteration to its response under stress. The temperature affects the growth rate, the rate of a variety of reactions both enzymatically mediated and not, the divisional rate, and many control and regulatory responses of the cell.

The basal medium was prepared with increasing concentrations of nickel from $8 \times 10^{-5}$ through $6 \times 10^{-4}$ M and the cultures were incubated at 10, 20, 25, 30, 35, and 40 C. Readings for OD were made at intervals to 144 hr in the temperature range 25 through 40 C, 168 hr at 20 C, and 260 hr at 10 C.

The optimal temperature for maximum populations is about 25 C, as observed by OD in the untreated controls (Table 7). Increased temperatures reduced the time required to attain maximum OD of the culture from 12 hr at 40 C compared to 120 hr at 10 C.

The inhibitory concentration of nickel was not changed at the lower temperatures (below 25 C) at which the limiting concentration of nickel was $4 \times 10^{-4}$ M. A temperature of 30 C allowed growth of the culture to occur at $5 \times 10^{-4}$ M nickel, albeit, to very low OD levels. As the temperature was increased the inhibitory concentration of nickel decreased, and especially between 35 and 40 C.

The morphology of the cells at the limiting concen-
tration of nickel was similar at all temperatures, although the size at 40 C was decreased to about 6-8 \( \mu \) in diameter.

10. **Maximum Nickel Concentration for Inhibition of Growth**

The ability of a cell to multiply may be affected without an effect on the growth processes itself resulting in an unbalanced growth condition. The necessary requirement must be that the threshold concentrations of nickel inhibiting the individual systems of replication and growth are sufficiently different that one can be inhibited without affecting the other.

The inoculated culture in basal medium was allowed to incubate for 3.5 hr at which point the cells were in early logarithmic growth phase. The nickel solution was added at increasing concentrations from \( 2 \times 10^{-4} \) through \( 5 \times 10^{-3} \) M and allowed to incubate at ambient temperature for an additional 24 hr. Microscopic observations were made periodically for morphological changes in the cells.

That the concentration of nickel inhibitory to growth was markedly different from that concentration needed to inhibit multiplication was demonstrated. As the nickel concentration was increased from non-toxic levels the divisional processes were inhibited at \( 5 \times 10^{-4} \) M nickel where the cell was unable to multiply under the nutrients levels in the basal medium. Microscopic observation of these cells, however, indicated the size as 12-15 \( \mu \) in diameter. Further increase in the nickel concentration resulted in continued unbalanced growth with the cells unable to divide.
but able to produce more protoplasm. An upper limit for cessation of growth was reached at 3 x 10^{-3} M nickel. In the levels of nickel where division was inhibited, the morphology of the megalomorph (Fig. 4e) remained constant but the size gradually reduced to 2-3 \mu \times 2-4 \mu at 2 x 10^{-3} M nickel.

The threshold concentrations inhibiting the 2 processes, multiplication and growth, are separable by almost one log difference in nickel concentration, 5 x 10^{-4} M for multiplication and 2 x 10^{-3} M for growth.

11. Distilled Water--Seawater Dilution Relationships of A. marinus Cells

Since the megalomorphic cells were osmotically sensitive they should burst when placed in distilled water leaving the "normal" cells and those cells not yet osmotically sensitive unaffected. Dilution of a culture in 75% synthetic sea water, osmotically similar to the conditions of growth, should not have affected the cells. Plating of culture material diluted in both manners on 2216E agar plates should have given three populations: (1) total population (75% synthetic sea water); (2) osmotically insensitive population (distilled water); and (3) osmotically sensitive (by difference).

Such an experiment was performed on a control containing no added nickel, 4 x 10^{-4} and 8 x 10^{-4} M nickel containing culture in basal medium. The cultures were incubated and 2, 4, 8, 20, 32, 48, 72, and 84 hr OD readings
and plate counts on marine 2216E agar were performed. The untreated control cells when diluted in distilled water consistently yielded higher plate counts, almost a log unit at 20 hr, than when diluted in 75% synthetic sea water (Fig. 11). The growth curve was normal as expected with a sustained stationary phase, which may have been related to its autochthonous role in nature (Winogradsky, 1949).

The growth of $4 \times 10^{-4}$ M nickel showed an initial higher count with dilution in distilled water over dilution in 75% synthetic sea water. As the cells became megalomorphic, between 2 and 4 hr (Fig. 5c,d), they also became osmotically sensitive as seen by a reversal of the distilled water-sea water dilution relationship. There was a decrease in both populations until after 50 hr incubation the culture commenced multiplication and the population curves rose. After 70 hr incubation a second process of megalomorph formation occurred and the curve diverged again. Thus, there were two general periods of megalomorphic formation, one upon inoculation of the medium probably by those cells metabolically active in the inoculum, and the second period after 70 hr incubation. This latter growth probably resulted from those cells in the inoculum that were in a late stationary phase of growth and needed the 50 hr to regain the physiological capability to overcome the stress of the added nickel. These two periods of
megalomorphic formation were confirmed by microscopic observations.

The distilled water-synthetic sea water dilution relationship was similar at $8 \times 10^{-4}$ M nickel (Fig. 12). The population continued to decline, the cells became megalomorphic and osmotically sensitive with no second growth occurring.

The OD of the cultures at the various sample times are shown in Table 8.

The decline in the OD of the control culture while the population was in the stationary phase agrees with the normal reduction in volume of the cell as it ages. The slight increase in OD of the $4 \times 10^{-4}$ M nickel culture during the first 30 hr reflects the initial megalomorphic formation followed by the second at 84 hr.

The second period of megalomorphic growth occurred in a manner similar to that in the first period. The cells undergoing multiplication in the 60-70 hr period were essentially normal in appearance becoming megalomorphic thereafter.

12. Cultural Stage of Growth vs. Nickel Effect

The physiological age of a culture may be a determinant in the response of that culture to an added stress. This effect of physiological age may reflect differences in the content of lipid, proteins, nucleic acids, growth factors, intermediates of these groups, permeability properties, and inorganic ion constituents of the cells (Lamanna & Malette,
The added stress in this experiment was $4 \times 10^{-4} \text{ M}$ nickel added to the culture during various stages in the life cycle with the changes in population followed by plate count. A single flask of basal medium was inoculated with $2.6 \times 10^6 \text{ cells/ml of A. marinus}$. At 0, 1, 2, 3, 4, 6, and 8 hr, 100 ml of the growing culture was removed, placed in a separate sterile flask, $4 \times 10^{-4} \text{ M}$ nickel added and the culture re-incubated. Population changes were followed in each flask by surface plating to Marine Agar 2216E at 0, 1, 2, 4 hr, and in some cases, 6, 8, 10 and 12 hr after addition of nickel. The control underwent a normal sigmoidal curve, the first 3 hr being lag phase and the stationary phase starting after 10 hr (broken line, Fig. 13).

Addition of the nickel to the culture at zero hr resulted in a slight drop in count followed by a doubling in count over the subsequent 8 hr. Addition at 2 hr produced a slight burst of division finally yielding a little over one doubling in count during the next 6 hr. Addition at 3 hr, the end of the normal lag phase, produced about a 5 times larger population during a 5 hr period. This represents about two divisions in the population. Additions at 4 and 6 hr produced a doubling in 4 hr. Finally, an addition at 8 hr failed to produced a division in the subsequent 4 hr.

The cells may need to accumulate some material needed for division. If so, the cells appear to have accumulated
this material at highest concentration by the end of the lag phase. If nickel was added before or after this time the cells were able to go through only one divisional cycle. The closer the addition of nickel was to the end of the lag phase the greater the population increased and the shorter the time needed to achieve this change.

The ability of the cells to grow was not impaired since the cells formed megalmorphs in all cases, with time in the presence of nickel being the determinant of size.

13. Subsequent Growth of the Megalomorphic Cells

The ability of the megalomorphic cells to divide and form colonies in the absence of nickel has been shown in previous experiments (topic 11). To determine if the divisional processes were partially impaired, on a limited number of divisions occurring, or if there was a sudden burst of divisions on removal of the nickel stress, since the protoplasm has been accumulating mass, the megalomorphic culture was transferred to fresh medium and the subsequent growth followed by plate counts.

Fig. 14 shows the normal growth curve in basal medium acquiring a maximum population of about $2.5 \times 10^8$ cells/ml in a 20 hr period. The other culture was allowed to grow for 4.5 hr before the nickel was added, at which point the cells started transformation to megalomorphic cells. At the end of 26 hr the culture was diluted 1:10 with fresh medium thereby lowering the nickel concentration to $4 \times 10^{-5}$ M, a level which allowed essentially normal growth. There was a
gradual lag phase of about two hr followed by a period of rapid division producing a population of $2.9 \times 10^8$ cells/ml similar to the control.

The megalomorphic cells were capable of division after 21.5 hr in the presence of nickel and the subsequent growth rate in logarithmic phase was similar to that in the basal medium both attaining the same ultimate population. The division limiting effect of $4 \times 10^{-4} M$ nickel was rapid in its action. This effect was overcome equally as rapidly, either by dilution of the nickel in the cells or renewed availability of some division requiring substance.

14. **Acclimatization to Nickel**

The ability of an organism on repeated subculture to become resistant to a stress is well known. To determine if the test organism was capable of acquiring an ability to divide in the presence of inhibitory concentrations of nickel and/or the morphology became normal at $4 \times 10^{-4} M$ nickel, the culture was repeatedly inoculated into nickel containing basal medium. Growth from a 96 hr culture in $4 \times 10^{-4} M$ nickel was inoculated to the surface of a plate of Marine Agar 2216E and a single colony isolated. The isolate was inoculated to a new set of basal medium containing increasing concentrations of nickel from $4 \times 10^{-4}$ through $7 \times 10^{-4} M$. This cycle was repeated three times, each time isolating a colony from the growth in $4 \times 10^{-4} M$ nickel broth.

The growth on the agar plates of the culture isolated from $4 \times 10^{-4} M$ nickel broth in all cycles was normal. The
growth resulting in the nickel containing broths are presented in Table 9.

The culture was able to gradually acquire the capacity to divide at higher concentrations of nickel with each cycle. The megalomorphic characteristic also progressed as the cell's ability to grow at higher nickel concentrations was acquired and tended toward reduction in size at $4 \times 10^{-4}$ M nickel with each cycle.

C. Physiological Response to Nickel

15. 260-280 μm Absorbance of Cultural Supernatant

The bacterial membrane functions in maintaining the integrity of the cell as well as being a metabolically active structure. When an inhibitor is added to the culture its first contact is with the cell surface. In small concentration, the inhibitor may accumulate at the cell surface and induce permeability changes in the membrane. Rather than study membrane permeability directly, the indirect assessment is made by leakage of compounds from the interior of the cell to the medium, particularly 260-280 μm absorbing material.

The synthetic medium containing glucose as an energy source, was prepared with $1 \times 10^{-5}$ through $1 \times 10^{-4}$ M nickel. At various times portions of the cultures were removed and centrifuged at 3,000 rpm for 10 min. The twice centrifuged supernatant was placed in match quartz cuvettes and the OD read against the uninoculated medium as a reference in the range of 230 to 320 μm. The results are presented in Fig. 15.
The values plotted as stationary cultures were from cultures at maximum OD. The growth for early logarithmic phase in $2 \times 10^{-5}$ M nickel and late logarithmic phase in $1 \times 10^{-5}$ M nickel is also presented.

The culture in the synthetic medium without nickel showed an increased absorbance over the uninoculated culture with a sharp drop from 230 to 250 μ and a more gradual drop thereafter to 320 μ. The supernatant from the early logarithmic culture in $2 \times 10^{-5}$ M nickel showed a response similar to that of the control. The late logarithmic culture supernatant, $1 \times 10^{-5}$ M nickel, showed a decrease in absorbance to 245 μ to 320 μ. The stationary culture of both $1 \times 10^{-5}$ M and $2 \times 10^{-5}$ M nickel demonstrated this same response with greater absorbance across the curve. There was very slight growth at $4 \times 10^{-5}$ M nickel, the supernatant producing a high absorbance at 230 μ followed by a rapid drop to 245 μ where it remained essentially negative.

During the early stages of growth the supernatant from nickel containing cultures produced an ultraviolet absorption pattern similar to the untreated control culture. As growth progressed there was release of absorbing material in the 260 μ area becoming marked by the stationary phase. The time of release of the 260 μ material coincided to some degree with the formation of the megalomorphic cells, mid-logarithmic phase. How much of this absorbance was due to release from membrane permeability and disruption and how much was due to cytolysis, was difficult to assess without further analysis.
Release of 230 μm absorbing material in all the phases and conditions of growth was attributed to carbohydrate release.

The cells in the synthetic medium grown in the presence of a limiting concentration of nickel were not as large as those from basal medium and were in the range of 2-3 μ in diameter.

16. **Effect of Nickel on Oxygen Utilization**

On the addition of nickel ions to a culture the cells commenced almost immediate transformation to megalomorphic cells. If this were a result of slight changes in permeability of the membrane, resulting in increased nutrient flow by disorganization of structural integrity, or by nickel interference with metabolic systems the oxygen uptake should reflect a change on nickel addition. The results from a Warburg respirometry study are presented in Fig. 16. The experimental procedure was illustrated in Table 1. The endogenous control gradually used 46 μl of oxygen during the 225 min. The addition of $6 \times 10^{-3}$ M nickel at 105 min did not effect oxygen uptake in the endogenous treatment as it continued at an unchanged rate finally utilizing 39 μl. The addition of both nickel and nutrients at 45 min resulted in a sharp increase in oxygen uptake with a slight decline after 100 min finally reaching 330 μl. When the nutrients alone were added at 45 min there was a rapid increase in oxygen uptake, greater than when added with the nickel. The addition of nickel at 105 min in this treatment did not cause an
immediate change in the rate of uptake. There was a gradual leveling off of the curve after 180 min reaching 424 µl of oxygen at termination.

The nickel addition did not affect the endogenous respiration. The addition of nickel and nutrients caused an initial rise in oxygen uptake similar to that of a nutrient addition alone. After 45 min there was a gradual reduction in the oxygen utilization. On the addition of nickel to an actively metabolizing culture, a gradual decrease was noted after 60 min. Whether these two additions are interrelated is probable but not established without comparison to a nutrient control containing no nickel. The nickel did not affect a sudden change in oxygen utilization in an actively metabolizing system nor does it change the initial utilization of substrate in the presence of nickel. The morphological alterations started at the addition of nickel and size increase was noted in 60 min. Possibly the decline in oxygen utilization was related to the increased size since diffusion problems of both nutrients and waste could not be affected. This experiment was carried out in peptone-yeast extract medium since it had been established that the cells would be unable to divide at the concentration of nickel and nutrients used. The nickel concentration was increased to meet the increased concentration of cells.

Nickel affected neither the permeability nor metabolic systems immediately as measured by oxygen uptake on addition of the cation.
17. Protein, RNA, DNA Production in the Presence of Nickel

Production of the nucleic acids and protein are indispensable for continued growth and multiplication of the cell. If nucleic acid production is stopped with only that amount present at the time of nickel addition or if completion of the rounds of nucleic acid replication begun is allowed, the cell will have two to possibly eight chromosomes (Maaloe & Kjeldgaard, 1966) which could account in part for the data in Fig. 13. If nucleic acid synthesis is not affected the nucleic acids would continue to increase in the cell.

The concentration of RNA, DNA, and protein per cell during the subsequent 12 hr after addition of nickel is presented in Fig. 17. In the controls, which were about to come out of the lag phase at the time analysis started, the protein and RNA rose to almost double in amount at 3 hr then gradually decreased. The DNA on the other hand fell to a level of about an eighth that found at zero time. The cell with the threshold concentration of nickel demonstrated a much greater initial increase in the 3 components at 3 hr after which they tended to remain constant, ultimately attaining levels about 36 times higher in RNA, and 10 times higher in DNA than in the untreated cells.

Even though there is a great fluctuation in the concentrations of the components analyzed during the course of study of two test populations, when the protein:DNA, protein:RNA
and the RNA:DNA ratios are plotted they appear very similar (Fig. 18). The protein:DNA ratio climbed to about a 4-fold increase followed by a gradual divergence with the control ratio being higher than the nickel ratio. The protein:RNA ratio increase was greater in the nickel-treated cells at 3 hr while at 12 hr the control ratio was higher. The DNA:DNA ratio was higher for the control at 3 hr, reversing at 7 hr but still very close at 12 hr.

The data indicate the continued production of the nucleic acids and protein per cell in the presence of 4 x 10^{-4} M nickel. The respective levels per cell in the three components decreased with different patterns in the untreated controls to lower levels as expected from the size relationships in the nickel-treated cells during the normal growth cycle (Fig. 1).

The ratios of the division components indicate patterns similar to the control culture, which should be under full operational control. Thus, some semblance of operational control must have been present in the nickel-grown cells. Any operational control in the nickel-grown cells may have resulted from the large size of the cells creating diffusion problems of nutrients and wastes. The comparison being made for metabolic control is between two cell populations, one with divisional problems becoming larger in size and a second population progressing normally through the growth cycle to stationary phase.

When the concentrations of the cell components are
plotted as a ratio with zero time equal to one, the data show a general decrease in DNA, RNA, and protein values of the control culture whereas the nickel-grown culture shows a decided increased ratio (Fig. 19). This pattern of these components was expected and is supported by the cell size relationships (topic 2).

18. C:N:P Ratios in the Presence of Nickel

The ratios of carbon, nitrogen and phosphorus vary through the normal growth cycle as the cells change in composition. If the megalomorphic cells undergo an extended lag phase until the size imposes diffusion problems, the ratios of the three elements should remain at levels formed at the time of addition of the nickel.

The ratio of carbon to phosphorus and nitrogen to phosphorus through a portion of a growth cycle is presented for the control as well as two other cultures, one with $4 \times 10^{-4}$ $\mu$ nickel and the other with $6 \times 10^{-4}$ $\mu$ nickel (Fig. 20B). The plate count population of the cultures is also presented for reference (Fig. 20A).

The growth was as expected with the control culture going into lag phase, logarithmic phase, and progressing into early stationary phase. The addition of $4 \times 10^{-4}$ $\mu$ nickel resulted in a reduced rate of cell division as seen previously while $6 \times 10^{-4}$ $\mu$ nickel inhibited multiplication completely, while growth continued. The carbon to phosphorus ratio of the control gradually dropped to a low, coincident with early logarithmic phase then gradually rose to the
original level. The addition of both levels of nickel resulted in a continued low ratio similar to that at the time of addition.

The nitrogen to phosphorus ratio of the control also gradually dropped to a low coincident with the early logarithmic phase gradually rising to the original level at stationary phase. The addition of $4 \times 10^{-4}$ M nickel caused an increase in the ratio for the following 3 hr where it remained. The addition of $6 \times 10^{-4}$ M nickel showed the same initial increase. However, it returned to the lower level and stabilized.

The C:P and N:P ratios for the controls were as might be expected with the highest amount of phosphorus relative to carbon and nitrogen occurring during the early logarithmic stage of growth. The addition of nickel resulted in a maintenance of the C:P ratio indicating continued high phosphorus content. The N:P ratios, however, indicated an increased nitrogen to phosphorus ratio on addition of $4 \times 10^{-4}$ M nickel. This may have resulted from an increase in nitrogen while the carbon and phosphorus remained constant. The major nitrogen containing compounds are proteins, nucleic acids, and the polypeptide of the cell wall. However, they also contain carbon which should have increased in relation to phosphorus, but did not. Nickel is capable of forming coordination compounds with ammonia. The principal nutrients in the medium are protein in nature which may release ammonia on their degradation thus providing the material for formation
of the complex molecule. This type of reaction would yield an increase in the nitrogen without increase in carbon or phosphorus. A second possibility is that nitrogen was constant and carbon and phosphorus were decreasing. The control culture, containing no nickel, produced an extracellular substance not seen in nickel-grown cells. If this material was a phosphorylated carbohydrate its absence in the nickel grown cells could account for these results. Care was not taken to remove the extracellular material from the cells which was evident during the latter stages of the logarithmic stages of growth in the control. The results are difficult to interpret strictly on the basis of relative ratios of C:N:P but indicate some metabolic imbalance under nickel stress.

19. Quantitation of Nickel Uptake

The uptake of nickel by A. marinus was determined on a nitrogen basis of the cells analyzed for nickel.

The conditions of growth produced approximately the same concentration of cells at the time of addition of the nickel as contained in the inoculum. At the end of 3.5 hr incubation the cell population was $1-2 \times 10^7$ cells/ml and at the first stages of logarithmic growth.

The uptake of nickel by the culture was found to correlate with the concentration of nickel in the basal medium (Table 10).

As the concentration of nickel in the medium was
increased the concentration taken up by the cells also increased. At the higher level of nickel the concentration taken-up fell, which may be related to a depressed physiological response at $8 \times 10^{-4}$ M nickel as indicated by the cells at the end of 3 hr being not quite as large as those from $6 \times 10^{-4}$ M nickel.

When the concentration of nickel was maintained at a constant level of $4 \times 10^{-4}$ M and the time of exposure varied an unusual result is found (Table 11). The 2-3 min sample was obtained by adding the nickel to the growing culture and immediately centrifuging the cells from the medium. In the other cultures the cells were removed at the times indicated. As the time was increased to 60 min the uptake of nickel was seen to decrease and then increase with further incubation. This anomolous condition is difficult to relate to a physiological response as the chemical data indicated the cells metabolizing and producing new cellular material rather rapidly. The high N:P ratios in Fig. 20 may explain this since the nickel values are based on nitrogen.

The amount of nickel bound to the cell substance and removed by washing in synthetic sea water was investigated. The zero wash was obtained by picking up the cells in $4 \times 10^{-4}$ M nickel in 75% synthetic sea water once in the concentrative steps. The other cultures were washed the indicated numbers of times with 75% synthetic sea water only (Table 12).
After one wash the amount of nickel retained has dropped by 47%, whereas after 4 washes the retained portion was 43%. About half the nickel originally associated with the cells was bound more or less fixedly to the cellular substance on the basis of cell nitrogen.

All the cell samples analyzed had to be washed to rinse the cells and the interstitial spaces of the medium since the dried cell material was analyzed for nitrogen content.

On the basis of cellular nitrogen, using the conversion factor of 6.7 for nitrogen to protein, if the amount of nitrogen per cell at the end of 3 hr incubation in the presence of 4 x 10^{-4} M nickel was taken as 6.7 x 10^{-12} g (from Fig. 17) then the concentration of nickel per cell was in the order of 6.6 x 10^{-14} g using 1 mg N per 10 μg nickel (Table 12).

The cells were able to take up the nickel rather rapidly as 2.7 μg/mg of cell nitrogen was taken up within about 2-3 min. This represents bound nickel since the cells had been washed once with 75% synthetic sea water.

20. **Respiratory Deficiency Induction by Nickel**

A number of metals including nickel have been shown to induce respiratory deficiency in yeast (Lindegren, Nagai and Nagai, 1958). The testing of respiratory deficiency is scored by the inability to reduce triphenyltetrazolium chloride (TTC); the colonies remain white, while respiratory-sufficient colonies turn red from the reduced formazan.
Colonies from a culture of *A. marinus* grown in the presence of $4 \times 10^{-4}$ M nickel for 6 hr and subsequently grown in 2216E medium without nickel produced all red colonies when overlayed with TTC, that is, they were all respiratory sufficient. Colonies formed on agar plates containing increased concentrations of nickel also did not produce respiratory-deficient colonies. Sectoring of the colonies was also absent.

Nickel was not able to induce respiratory deficient cells at least at the concentrations used. Lindegren (1958) stated that in yeast the respiratory-deficiency was due to destruction or inactivation of cytoplasmic granules (mitochondria) rather than the genome.

The type species of the genus was found by Morris (1960) to depend on the cytochrome system which mediated the ultimate transfer of electrons from respiration. If there were any alteration in the cytochrome system of *A. marinus*, growth and division would not occur. Consequently all colonies formed must be TTC positive, respiratory-sufficient. The organism either did not have a fermentative pathway or was unable to utilize it if nickel did indeed affect the cytochrome system.

D. **Electron Microscopy**

21. **Morphology**

The internal structure of the cells undergoing megalomorphic formation was examined by the technique of electron
microscopy. Preservation of the large cell vacuoles necessitates great care in providing osmotic support of the cell during fixation and other steps prior to dehydration and embedding.

The normal cells after 3 hr incubation in the basal medium are presented in Fig. 21. The nuclear material is characterized by fine, evenly-dispersed and uniformly-sized filaments. The ribosomes are densely packed within the cytoplasm and dispersed at random. The plasma membrane closely applied to the cell wall can be distinguished in cross sections of the cell. The entire outer membrane consists of 7 layers, a double track cell wall, and a double track plasma membrane. About the periphery of the cell are small globular structures approximately 0.05-0.1 μ in diameter delineated by a double track. These structures appear to be originating from the outer membrane and cannot be traced to the plasma membrane (Fig. 22).

At times the globules taken on an extended appearance protruding about 0.15-0.3 μ in length being made up of a series of the globules in tandem (Fig. 23). Two cells about to divide are shown in Fig. 24 with the mesosome evident at the center of the cytoplasm with a septum developing at the point of division of the daughter cells.

After one hr incubation in the presence of nickel the 7 layered outer membrane is still evident and does not appear to have changed structurally. The nuclear material and
ribosomes also appear unchanged in consistency. However, the nuclear material appears to be located at more sites in the cell (Fig. 25). The shape of the cell is somewhat irregular while an occasional cell contains a vacuole; this is also evidenced in the phase micrographs taken after 1.5 hr incubation in nickel.

The globular structures are still seen to be mainly as individual units with an occasional extended form protruding from the cell.

The presence of a bulge in the mid section of a few cells is noted (Fig. 26). There is a septum formed between the bulge and one of the normal diameter daughter cells while within the bulge is a vacuole appearing to be associated with the cytoplasmic membrane and a mesosome extending into the cell from the outer membrane.

After two hr incubation in the presence of nickel the cells and vacuoles become larger, and the cells are more irregular in shape (Fig. 27). The nuclear material is more peripherally located with the ribosomes still prominent and evenly dispersed. The term vacuole denotes a hole or cavity in the cytoplasm of the cell. The "vacuole" seen in Fig. 25 resembles a cell undergoing plasmolysis. This is more evident in Fig. 27 as the plasma membrane does not encircle the vacuole but has actually pulled away from the cell wall as seen in plasmolysis. The outer membrane is unchanged in structure and appearance. However, the
vacuoles are surrounded by the plasma membrane on one side and the cell wall on the other. The globular structures are still evident but reduced in number.

The cells after three hr incubation are little changed from those of two hr except the cells are slightly larger (Fig. 28).

After 6 hr in the presence of nickel the cells are greatly plasmolyzed with the space occupying a major portion of the total volume (Fig. 29). The outer membrane still is similar to the control cells with its 7 layers where the plasma membrane and cell wall are in contact. The nuclear material is dispersed through the cytoplasm in a large number of locations with much irregularity. The ribosomes are still prominent and evenly dispersed within the cytoplasm. Vacuoles in the cytoplasm are delineated by a single membrane. Along the inside of the cell wall away from the cellular material are a number of round structures probably extensions of the cytoplasmic membrane adhering to the cell wall and containing cytoplasm (Fig. 29). The globular structures are evident around the entire cell wall even in areas where membrane is not in contact with it. An enlargement of Fig. 29 (Fig. 30) shows the membrane separation from the cell wall with the membrane remaining in contact with the cytoplasm. The extensions of the cytoplasmic membrane are also evident along the cell wall of the plasmolyzed space. There is no evidence of polyphosphate
granules in Fig. 31 which shows a cell with a large number of small vacuoles surrounded by a single membrane. The nuclear material has maintained a fair volume of the cytoplasm and the ribosomes again appear unchanged. There are a large number of formed reticulations along the periphery of the cytoplasmic membrane associated with the cell wall. These reticulations are not seen where the cytoplasmic membrane is associated with the plasmolyzed space.

No alteration in appearance was noticed from the normal of the cell wall of the organism as it grows in the presence of nickel. The plasmolysis starts at the poles between the double tracks of the plasma membrane and the cell wall continuing to enlarge as growth of the cell progresses. The ribosomes remain dense and evenly distributed during the formation of the megalomorph. The fine uniform filamentous structured appearance of the nuclear material remains constant. However, the areas of concentration of nuclear material increase, becoming more peripheral at two hr than finally with numerous locations that appear randomly through the cytoplasm at 6 hr. The amount of nuclear material and ribosomes increase in amount as the size of the cell enlarges. This is in accord with the chemical data presented previously (Fig. 17). The globular structures are probably derived from the cell wall rather than the plasma membrane since they are found associated with the cell wall at 6 hr where the plasma membrane is not in contact with the cell wall.
They are much more numerous where the two membranes are in contact and may be related to the cell wall synthesis at that site. The nature of this globular material has not been determined. It is believed to be polysaccharide in substance. The organism produces large amounts of polysaccharide during culture in broths or on surfaces.

The one unusual point as seen in these sections is the formations of the reticulated material along the periphery of the cells at 6 hr. Whether they are mesosomal in nature is difficult to determine. The cell has not lost its capability to form mesosomes in the presence of nickel.

22. Electron Probe Analysis and Electron Diffraction

In electron probe analysis a laser beam 2.0 μ in diameter was focused on a cell and the resulting gaseous material was analyzed by an atomic spectrograph for any one of a number of elements, in this case nickel. The purpose of the experiment was to make sections of the megalomorphs, 1.5 μ, and use the probe to analyze for nickel in various areas to determine where in the cell the nickel was concentrated. The sensitivity of the probe has a lower limit of \(10^{-14}\) g under the laser beam. The amount of nickel in each megalomorphic cell was beyond the limit of detection of the system, even when a cluster of as many as 6 sections were analyzed simultaneously. The entire cell has on the order of \(2 \times 10^{-14}\) g of nickel. Sectioning and observing a small portion further reduced the amount possible to be detected.
These sections were also set up for electron diffraction of the various areas of each cell to determine if any compounds of nickel were evident in the cell and in what form they existed. The very nature of biological material with all its impurities and many compounds as well as the fixation and handling procedures of the megalomorphs made the interpretation of the results impossible. The calculated diffraction ring values for the nuclear area of the cell were 1.1, 2.0 and 3.1 with relative brightness of 70:100:40 respectively which corresponded to a Ni-Al compound, amongst a number of non-nickel compounds.

The electron probe was not sufficiently sensitive to show where the cells were concentrating the nickel and to what extent. The procedure of electron diffraction was not capable of distinguishing the various constituents of a cell.

E. Other Responses

23. **Effect of Some Heavy Metals Other than Nickel**

To determine if the effect of nickel on the morphology of this organism was unique or whether other heavy metals may also have a similar effect, the divalent cations, Co\(^{++}\), Cu\(^{++}\), Cd\(^{++}\), Zn\(^{++}\), Pb\(^{++}\), Mn\(^{++}\), and Hg\(^{++}\), as chlorides, and the monovalent cation Ag\(^{+}\) were individually added to the basal medium, in various concentrations and treated as described.

Table 13 shows the concentrations of the various metals in basal medium to which the organism was able to grow and
was inhibited.

The metal concentrations at which inhibition of growth occurred, as measured by OD, was in the range of $2-8 \times 10^{-4}$ M except for the highly toxic silver and mercury ions. This study of the metals suggests that they are affecting the same mechanisms within the cells. Manganese, being biologically somewhat similar to magnesium, is non-toxic to concentrations reaching saturation levels. Silver and mercury being quite toxic to most living systems were probably affecting the cell at a larger number of vital positions or had greater avidity for specific cell sites. The reduced toxicity of copper, when compared to its position on the avidity series, may be related to the pH of the medium, 7.8-8.0, providing a greater possibility of hydroxide formation thereby lessening the free ions available in the medium.

When the organisms were placed in the medium containing saturation levels of manganese chloride, the culture, during the early stages of growth, cleared the medium of the suspended particles to what appeared to be $\text{MnO}_2$. The bacterial cells were white in the normally clear straw colored medium with floculations and small balls of $\text{MnO}_2$ on the bottom of the flask.

Fig. 32 shows the effects of the various metals on the morphology of the cells. Of the metals examined zinc (Fig. 32 a and b) and copper (Fig. 32c and d) were more dramatic in
producing megalomorphs. Copper had a tendency to round the cells more than elongate them. Zinc simply increased the volume of the cells with the length-width ratios remaining similar to normal cells and enhanced the number remaining attached to form long chains. Cadmium (Fig. 32e) was least effective in megalomorph formation of the metals with about 5-10% of the cells affected. Those cells affected formed filaments of normal diameter. Cobalt (Fig. 32f) rounded the cells but did not change the cell diameter. More noticeable was a change in phase density to very opaque. The other cations, Mn++, Pb++, Ag+ and Hg++, did not produce morphological alterations in the culture, the cells remaining normal at the limiting concentration of the metals. None of these metals produced megalomorphic cells as large as those produced by nickel.

Morphological variation was not unique to nickel which causes the more dramatic morphological effect. Plasmolysis was produced by only one other metal, cobalt.

The result that a number of metals are capable of producing morphological alteration, but not the same type, may indicate similar sites of action by the metals. The sites of action may be affected to different degrees to produce the variety in morphology.

24. Dithizone

Since the production of morphologically altered cells was not specific with nickel but occurred with other divalent cations, it was of interest to determine if the heavy metals
occurring in the artificial sea water were contributing to the effect attributed to nickel. Dithizone (diphenylthiocarbozone) in an alkaline pH removed by chelation Bi^{3+}, Cd^{3+}, Cu^{3+}, Pb^{3+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, and probably Co^{2+}. Ferric iron, however, was not removed (Sandell, 1944).

The results of the growth response in dithizone-treated medium in the presence of nickel is presented in Fig. 33. The removal of metals by dithizone resulted in a two-fold greater OD than in untreated basal medium in those cultures of comparable nickel concentration (Fig. 3).

There was inhibition of the culture at $4 \times 10^{-4}$ M nickel in dithizone-treated medium where previously growth and division had occurred. The size and shape of the resulting megalomorphc cells were unchanged but displaced by a unit of dilution. The largest and most plasmolyzed cells were now at $3 \times 10^{-4}$ M nickel becoming smaller as the nickel concentration became progressively more dilute.

The apparent increased toxicity of nickel as a result of the dithizone treatment may be a consequence of the divalent ions imparting a protective effect against the uptake or activity of the nickel by competition for the reactive sites in the sea water medium. A second possibility is that the dithizone-chloroform treatment removed some other divalent cations besides those mentioned, particularly magnesium, producing a physiological stress on the culture. The third, and most likely possibility, is that the treatment
was such that the cells appeared yellowish with a rough texture. When the sucrose level was placed at 0.5 M, the cells after 15 hr incubation were similar to those grown in the presence of nickel (Fig. 34c). After 15 hr incubation the cells were about 10 μ in diameter with slight plasmolyzation in the cytoplasm. The phase appearance of the cytoplasm was grey with a few yellow rough textured areas. After 24 hr incubation, the cell was irregular in shape, plasmolyzed and the cytoplasm had a rough yellowish texture in phase density (Fig. 34d). At 0.7 M sucrose (Fig. 34e) the cells were about 10-13 μ in diameter and the cytoplasm filled much of the cell after 15 hr incubation. With further incubation the cells decreased in size and the texture of the cell became very rough with a yellowish phase contrast appearance (Fig. 34f).

The cells were affected by high concentrations of sucrose and nickel to cause megalomorphic cells. The plasmolyzation was greatly reduced in those cells from increased sucrose levels compared to nickel derived megalomorphs. The phase density was peculiar with the rough yellowish texture of the cells after 25 hr incubation in sucrose.

To examine the possibility that the sucrose effect on the morphology of the cells may result from the same or similar mechanism as nickel, sucrose and nickel were studied together in combination. On the addition of various concentrations of nickel to the different levels of sucrose listed above, the cells were seen to undergo the same transformation
attributed to nickel. Fig. 35a shows the control cells in the 15% synthetic sea water alone. Fig. 35b shows the effect of $1 \times 10^{-4}$ M nickel in the sea water with no sucrose added at 24 hr. The filaments then underwent a rounding and balling of the cytoplasm within the cell wall. This was similar to observations in reduced concentrations of synthetic sea water described previously (topic 8). Fig. 35c indicates the effect of the addition of $3 \times 10^{-3}$ M nickel in 0.3 M sucrose after 96 hr incubation. These cells were much larger than their control cells containing no added nickel and contained the large plasmolyzed spaces normally seen in the nickel grown cells. Fig. 35d and 35e show cells with $3 \times 10^{-4}$ M nickel in 0.7 M sucrose after 96 and 144 hr, respectively. Where the cells in Fig. 35a get progressively smaller; the cells at increased sucrose levels continue to enlarge becoming more plasmolyzed and somewhat irregular.

These cells grown in the presence of nickel did not have the rough texture found in their respective sucrose cultures alone and the phase density was that normally associated with the nickel-grown cells. The added size of the sucrose-grown cultures in response to nickel may be a result of the greater amount of plasmolyzation.

On the basis of the morphology of the sucrose and nickel grown cells, the two treatments appeared to act synergistically, suggesting that certain steps in the action of the two treatments
on the cells were similar.

The chelating ability of disaccharides at high concentrations has been described by Saltman (1963) and Charley and Saltman (1963). The greater the concentration of sucrose the greater the morphological response, indicating a possibility of chelation by sucrose. The concentration of synthetic sea water was reduced to 10% in the medium with sucrose at 0.5 M. Magnesium and calcium were individually added to the medium to 0.1, 0.01, and 0.001 M concentrations. To compare ionic strengths, sodium and potassium were added to 0.3, 0.1, 0.03, 0.01, and 0.003 M concentrations. The morphology of the various cells are represented in Fig. 36a, 36b, and 36c derived from 0.1, 0.01, and 0.001 M magnesium containing media respectively, and the control culture in Fig. 36d. The ionic concentrations are shown in Table 14, with the morphology of the cells produced by the addition of the cations related to the pictures in Fig. 36. For example, cells grown in the 0.5 M sucrose medium with 0.03 M sodium resulted in cells with morphology similar to that in Fig. 36b.

Normal-sized cells were produced by the higher concentrations of the cations with the morphology becoming progressively abnormal as the level of the cations was decreased. There was correlation in the ionic strengths at increased molarity which gradually diminished at lower concentrations with a Mg>Ca>Na>K ordering produced. The 0.1 M concentrations of magnesium and calcium had the same effect
and ionic strength as 0.3 M sodium and potassium. Magnesium at 0.001 M was as effective as 0.01 M sodium or potassium but was not comparable in ionic strength. The chelating ability of sucrose may have been saturated at the higher inorganic cation molarities allowing the cell to grow at normal size. The shape of the cells was still round at the higher molarities indicating that chelation was not the entire problem produced by sucrose on the cells.

Sucrose with its imposed conditions was still capable of supplying osmotic support to preserve the effect of nickel. The nickel itself effected the culture to produce megalomorphc cells rather than interacting with the inorganic environment to produce this effect. A portion of the inorganic environment was needed for physiological support by the cell, 5-10% synthetic sea water, while the remainder was osmotic support which can be replaced by sucrose.

26. Inhibitors

Inhibitors at increasing concentrations to bacteriostatic levels were used of three types: inhibitors affecting protein synthesis, cell wall synthesis, and nucleic acids. By inhibiting the various systems of the cell and observing for morphological changes possibly the site or sites affected by nickel may be suggested.

Chloramphenicol, an inhibitor of protein synthesis, was bacteriostatic at 40 μg/ml. The resulting growth from 30 μg/ml of chloramphenicol was normal in shape and other observable characteristics.
Actinomycin D, an inhibitor of DNA dependent RNA synthesis, was applied to the inoculated basal medium through a level of 20 µg/ml and produced no effect on the cell. Possibly the increased ionic concentration in the medium inactivated the antibiotic allowing normal growth at all concentrations tested.

Hydroxyurea, an inhibitor of DNA synthesis, was bacteriostatic at 10 mg/ml, the lowest concentration tested. However, the inoculum grew in an unbalanced condition through 50 mg/ml to produce long filaments of increased diameter (Fig. 37a). The cells have a large amount of granulation in the cytoplasm, and averaged 20-30 µ x 4.5 µ in diameter.

Caffeine, known to cross link the DNA in eucaryotic cells, produced an elongation of the cell into filaments about 20-40 µ in length with little or no change in diameter at a concentration of 4 mg/ml (Fig. 37b). The cytoplasm varied in phase density giving a granulated appearance. No plasmolysis was evident in the filamentous cells.

Penicillin, an inhibitor of cell wall synthesis, was added up to 1300 units/ml with no effect on growth. At 16 units/ml growth occurred in 24 hr. With increase in penicillin concentration the time for growth to become evident lengthened requiring 8 days at 1300 units/ml. The resulting cells at all concentrations appeared normal.

D-methionine, involved in alteration of cell wall synthesis, produced an effect similar to that of nickel
with enlarged, round and somewhat irregular shaped, plasmo-
lyzed cells with displaced cytoplasm (Fig. 37c). The size
was in the range of 5-8 μ in diameter at a concentration of
200 mg/ml.

Cycloserine, an inhibitor of cell wall synthesis, allowed
growth of the culture with up to 800 μg/ml. However, the
growth in the range of 800 to 70 μg/ml produced cells with
a morphology similar to those derived from nickel (Fig. 37d).
These cells were plasmolyzed with a crescent-shaped cytoplasm
in a circular cell wall. The size was in the same range as
those megalomorphs resulting from nickel.

Cycloserine and D-methionine produced results similar
to that of nickel with large round plasmolyzed cells with
the cytoplasm displaced to one side. This suggests that an
agent interrupting cell wall synthesis can produce a megalom-
orph of the same characteristics as nickel. Hydroxyurea and
caffeine, however, produced elongation of the cell with
slight or no increase in diameter. If nickel also produces
an effect on the DNA system as does hydroxyurea or
caffeine, the cell wall would have to be involved as well in
order to produce the rounding of the cell observed with
nickel.

27. **Amino Acids**

Ensign and Wolfe (1964) using a number of amino acids
individually added to a synthetic medium were able to keep
the cells of *A. crystallopoietes* in a coccoid form during
the growth cycle rather than producing rods during the logarithmic growth phase. If an amino acid could prevent rod formation in \textit{A. marinus}, amino acids may be a factor in controlling megalomorphic formation in the presence of nickel.

Of the 9 amino acids examined none were able to prevent the formation of rods during the logarithmic phase of growth. However, this led to a synthetic medium containing L-asparagine, the amino acid producing the greatest growth.

When nickel was added at varying concentrations to the synthetic medium containing asparagine at a level of 1 to 400 µg/ml increased amounts of nickel was required to limit growth as the concentration of asparagine was increased (Table 15). This was compatible with the results found with increasing concentrations of peptone and yeast extract (Fig. 9).

The morphology of the cells at the limiting concentration produced rather than the large megalomorphs, rounded cells without plasmolysis in a size range of 2-3 µ in diameter (Fig. 38a). This is compatible with previous results in media with low protein concentrations producing the smaller round cell (topics 7 and 15).

Nickel thus induced a rounding of the cell with another agent(s) involved in the enlargement process. This agent was available in the basal medium but not in the synthetic medium. The work of Landman and Halle (1963) implicated D-methionine in preventing repairs to the cell wall required for continued
rod shape after lysozyme treatment. Since D-methionine has already shown to produce megalomorphic cells (topic 26) 5 µg/ml of asparagine and methionine singly and in combination were prepared in the synthetic medium with various concentrations of nickel. Five µg/ml of asparagine produced results as above with the limiting concentration of nickel at $3 \times 10^{-5}$ M producing cells in Fig. 38a. The control cells without nickel were of normal morphology.

Five µg/ml of D-methionine resulted in a limiting concentration of nickel at $4 \times 10^{-5}$ M. The morphology of these cells was round, similar to Fig. 38a in the size range of 2-3 µ in diameter. D-methionine control cells without nickel produced some megalomorphic cells with irregular shape, plasmolyzed and enlarged to 4-6 µ in diameter (Fig. 38b). This was an anomalous result with nickel producing smaller cells than cells in the presence of D-methionine alone. All the nickel grown cells were affected similarly whereas less than half of the D-methionine control cells were enlarged.

When the two amino acids were combined at the same levels, the limiting concentration of nickel increased to $8 \times 10^{-5}$ M and produced cells typical of those associated with nickel but smaller, 4-6 µ in diameter (Fig. 38c). The amino acids alone containing no nickel produced cells of normal morphology. D-methionine in the presence of asparagine produced results comparable, with the exception of size, to the basal medium containing yeast extract and peptone.
D-methionine was capable of acting as the agent needed for enlargement of the cell. Other D-amino acids found in the protein portion of the basal media may be supplying this function for the formation of megalomorphs.

28. Antagonistic Effects of Magnesium

Abelson and Aldous (1950) demonstrated a sparing effect of magnesium on the inhibitory effects of nickel on E. coli. Magnesium, if able to produce such sparing effect on the growth of A. marinus might have been able to spare the effect of nickel on the morphology of the cell.

Table 16 presents the results of the maximum OD of the culture and the time at which it occurred with increasing concentration of magnesium.

In the control and at each concentration of nickel an increase in magnesium resulted in a greater OD. At all magnesium concentrations increased nickel resulted in a lower OD to levels where no division occurred. The inhibitory effect of nickel was greater at low magnesium levels with sparing action at increasing levels.

This sparing action of nickel inhibition by magnesium was rather feeble where a doubling of the nickel concentration from $2 \times 10^{-4}$ to $4 \times 10^{-4}$ M necessitated a two logarithmic increase in magnesium from $1 \times 10^{-4}$ to $1 \times 10^{-2}$ M.

Magnesium was unable to spare the morphological change by nickel, with the growth at the limiting concentration of nickel similar in size and other characteristics regardless of the magnesium concentration. Thus, magnesium ions
enhanced the growth, as measured by OD, and was able to spare the toxic activity of nickel but was not able to alter the morphological effect of nickel.

29. **Antagonistic Effects of Calcium**

The ability of magnesium to spare the toxicity of nickel may be nothing more than competition for active sites on the *A. marinus* cell surface by mass action of the magnesium ion. Calcium was used in a similarly designed experiment to assess this possibility.

Table 17 presents the maximum OD of the culture and the time at which it occurred for the various concentrations of calcium. In the controls an increase in the calcium concentration from zero to $1 \times 10^{-2}$ M produced no significant change in OD. However, on increase to 0.1 M there was a drop in the OD. On the addition of nickel with an increase in calcium, the OD increased then decreased at 0.1 M to a point where division was inhibited at $4 \times 10^{-4}$ M nickel. At all concentrations of calcium an increase in nickel resulted in a decrease in OD with inhibition at $5 \times 10^{-4}$ M nickel. Again megalomorph formation resulting from nickel was not spared by the increase in calcium.

There was a slight protective effect afforded by the calcium but not comparable with that seen with magnesium (Table 16). The decreased OD and inhibition at $4 \times 10^{-4}$ M nickel at 0.1 M calcium was possibly a result of the antagonism between calcium and magnesium compounding the
effect of nickel. There was an association of the nickel with magnesium which is closer than simple competition by mass action. If it were simple mass action calcium should have produced the same sparing effect as observed with magnesium.

30. **Effect of Increased Iron Content**

Cobalt has been implicated in a derangement of the iron metabolism of *Neurospora crassa* (Healy, Cheng, and McElroy, 1954). Sivarama et al. (1960) have shown that in *N. crassa* the growth inhibition caused by cobalt and nickel could be competitively overcome by an adequate concentration of iron in the culture medium.

To determine if the inhibitory effect of nickel on *A. marinus* could be overcome by an increased level of iron, a study was performed as for magnesium and calcium. Table 18 presents the maximum OD and the times at which they occurred. There is no significant difference on the addition of ferric iron in the cultural or morphological response of the cells to increased concentrations of nickel. The addition of $4 \times 10^{-4}$ M nickel to $1 \times 10^{-4}$ M iron did result in a decrease time for attainment of maximum OD. The OD at any level of nickel was essentially the same at all concentrations of iron. Nickel did not produce an effect on ferric iron similar to that of *N. crassa* as measured in this manner.

31. **Chelators**

If the effect of nickel is to prevent the uptake
of magnesium resulting in a magnesium deficiency the use
of chelating agents in the basal medium should produce
a similar effect.

8-hydroxyquinoline sulfate when added to the medium
produced an inhibition of the culture at $1 \times 10^{-4} \text{ M}$, however, the inoculated cells had normal morphology.
At $1 \times 10^{-5} \text{ M}$ 8-hydroxyquinoline the culture grew with
normal morphology with a great tendency toward cell clumping.

Ethlenediaminetetraacetic acid (EDTA) was inhibitory
at a concentration of $2 \times 10^{-2} \text{ M}$ again without effect on
the morphology of the cell.

The two chelators are unable to produce an alteration
in the morphology of the culture.

32. **Biotin and Cobalamine**

A megalomorphic response was found associated with
both biotin deficiency in *A. globiformis* (Chan, 1964) and
cobalamine deficiency in an *Arthrobacter* sp. (Chaplin and
Lochhead, 1956). Addition of the respective growth factors
allowed normal growth in the cultures. These megalomorphs
were not similar to those produced by nickel. To show that
this effect of nickel was not a similar response the
growth factors were tested for their sparing activity.

Biotin (Nutritional Biochemical Corp.) was added
to $2 \times 10^{-4}$ and $4 \times 10^{-4} \text{ M}$ nickel containing basal medium
at a level of 1 and $5 \mu\text{g/ml}$. Cobalamine (Nutritional
Biochemical) was supplemented to a level of 2 and 10 μg/ml in the basal medium and nickel. Both growth factors were ineffective in preventing the formation of megalomorphic cells.

Nickel was not connected with a deficiency in either biotin and cobalamine by A. marinus which was relieved by their addition to the culture.

33. Pantoyl Lactone

Grula and Grula (1962) found pantoyl lactone, among a number of compounds, able to reverse division inhibition of a species of Erwinia caused by D-serine, penicillin and ultraviolet light.

Pantoyl lactone proved to be of no value in preventing or correcting the megalomorphic cells produced in basal medium in response to 4 x 10^{-4} M nickel. The high levels of pantoyl lactone produced pH changes in the medium. If the pH was not readjusted to 7.8-8.0 the growth of the culture on addition of nickel was only slightly enlarged, 5-7 μ in diameter at 4 x 10^{-4} M nickel where the usual response is 10-15 μ in diameter. Thus any effect of the compound was attributed to change in pH. A sparing effect by pH has been noted on other occasions and is independent of the agent used. An acid pH reduced the toxicity of the nickel with a change in the size of the megalomorph, the largest size being at the limiting concentration of nickel.

The altered morphology is not a result of a deficiency in biotin, cobalamine or pantoyl lactone. These three
agents are unable to correct or prevent the altered morphology produced by nickel.
DISCUSSION

The removal of marine organisms from their natural habitat for study in the laboratory does present an artificial environment for the culture. Consequently, the results derived from laboratory studies are at times difficult to relate to natural ecological relationships.

The salinity of sea water, temperatures, and increased levels of nickel, and other conditions studied in this thesis, are found in the marine environment. The temperature of the surface waters in shallow marginal waters may go as high as 40-42 °C (Gunter, 1957) in the summer. The concentration of nickel found in the ocean is about $3 \times 10^{-8} \text{ M}$ (Johnston, 1964). The organic content of sea water is low when measured on the basis of mg/liter. Most marine bacteria are found in close association with particulate matter (Jones and Jannasch, 1959) thus, the concentration of organics may be quite high in the microenvironment of the bacteria. A. marinus was capable of measurable growth on as little as 10 ppm (protein), an ecologically significant concentration of organic matter found at local sites. Elevated temperatures to as high as 56 °C on the bottom, and increased nickel levels are found to occur in the Red Sea (Densmore, 1968), where heavy metal concentrations are 1,000 - 50,000 fold higher than normally found, producing at least a level of about $3 \times 10^{-5} \text{ M}$ nickel, placing the level at the lower limits of the range found to be inhibitory to the bacteria in low organic concentrations. With an increase in temperature the effect of nickel would
be intensified. In the area of the Red Sea studied the
temperature itself would be inhibitory to *A. marinus* and
the lower waters are anaerobic. Nevertheless, the concen­
trations of nickel studied are realistic particularly in
conjunction with the 1000 ppm organic matter necessary for
batch culture study. These levels may be found under
certain conditions at another site with further investigation
in the oceans.

When nickel is introduced to a cell its effect depends
in part upon the physiological state of the organism, temper­
ature, pH, organic substrate and amount in the medium, its
inorganic composition, the accumulation of metal by the
cell, compounds in the cell that bind nickel and the
permanence of the binding by the cell. These considerations
make the concept of a single biochemical attack on the cell
produced by nickel unlikely, but rather that the cation is
involved in a large number of interactions.

Lankford et al. (1957, 1966) asserted that all
microbial cells have involved intimately with the initiation
of the divisional processes a chelating substance which may
be endogenous or exogenous. This "Schizokinen" is also
needed for continued maintenance of cell division. This
chelating material is involved in essential-metal inter­
actions similar to the siderochromes (Burnham and Neilands,
1961). The "Schizokinen" was found to be a secondary hydro­
xamate involved in iron chelation and able to stimulate the
siderochrome auxotroph Arthro. JG-9 (Byers, Powell and Lankford, 1967).

Many species of the genus Arthrobacter have been known to require siderochromes for proper growth. Nickel may inactivate any "schizokinen-like" compounds produced by the cell thus producing an effect on the divisional process. The inactivation may be by attachment at the reactive site or on the molecule affecting its reactivity. The synthesis of this compound would probably be greatest during the lag phase of growth, concentration increasing with time. Nickel is least toxic at the end of the lag phase when the "Schizokinen" should be maximal (Fig. 13). If this were the main avenue of attack by the nickel, the cell wall system would also have to be involved in the megalomorphic cell formation otherwise filamentous growth should result.

Adiga et al. (1961) suggested that zinc and nickel induced a magnesium deficiency in the cell since magnesium was able to reverse the effects of the two metals on growth. Shankar and Bard (1952) observed filament formation in cultures of Clostridium perfringens when grown in suboptimal concentrations of magnesium. Webb (1944) noted that a deficiency of magnesium produced filamentous growth in certain gram-positive bacteria.

Magnesium is the most common metal cofactor for enzymes which have had their metal requirements determined (Vallee, 1960). However, in a number of these enzymes other
metals are able to replace magnesium, i.e. Mn, Co, Cu, and Zn \textit{in vitro} (Starkey, 1955). Some of these metals simultaneously increase the magnesium requirement for growth, stabilize the cell and prevent leakage of magnesium from the cell (Webb, 1968).

There are similarities in the effect of magnesium deficiency in other organisms and the effect of nickel on 	extit{A. marinus}.

\textit{A. marinus} produces copious amounts of extracellular material, presumably polysaccharide, which assumes about half the volume of the centrifuged cell pellet. On addition of nickel to the medium the extracellular material is not produced. Extruded polysaccharides may be an extension of and derived from the cell wall synthesizing system (Salton, 1964). In magnesium deficiency a lowered polysaccharide content in the cell wall was produced in \textit{Aerobacter aerogenes} (Tempest, Hunter, and Sykes, 1965).

The stability of the plasma membrane may be affected with 260-280 m\textmu absorbing material being released during their stationary phase in \textit{A. marinus} (Fig. 15). The permeability and stability of the membrane are related to the availability of magnesium to the cell (Brock, 1962; Webb, 1968).

There was no gross limitation on the synthesis of the nucleic acids on a cellular basis (Fig. 17). However, there was a limitation by $4 \times 10^{-4}$ M nickel of further increase in population to 3-4 fold with maximal levels occurring when the nickel was added during the late lag phase (Fig. 13).
Kennell and Kotoulas (1967a) found DNA synthesis to continue during magnesium deficiency while the population and RNA was limited to a 3-4 fold increase. There was also qualitative differences in the RNA with a net effect of lowering of the content of functional ribosomes (Marchesi and Kennell, 1967). The RNA in A. marinus was not fractionated in this study.

The electron micrographs (Fig. 21-31) show the ribosomes to be large, evenly distributed through the cytoplasm and the number increasing with the enlarged size of the cell thus in agreement with the chemical data on synthesis of RNA (Fig. 17). Magnesium is important in ribosomal stability in the growing cell (McCarthy, 1962) and electron micrographs of cells grown under magnesium deficiency show a reduction in size and abundance of ribosomes (Kennell and Kotoulas, 1967b; Morgan et al. 1966).

At 4 x 10^{-4} M nickel half of the nickel absorbed by the cell is firmly bound while the other half is easily removed by washing (Table 12). A portion of this bound nickel may be replacing magnesium in the ribosome thus making the released or replaced magnesium available for other processes in the cell. The structure of the ribosome would thus be maintained as seen in the electron micrographs (Fig. 31).

Tissieres, Schlessinger and Gros (1960) demonstrated that manganese was about 50\% as effective as magnesium in the activation of protein synthesis by E. coli ribosomes.
Nickel with its higher stability constant is less likely to allow dissociation of the ribosome thus reducing the percentage of functional ribosomes present. This would produce a mimicking of magnesium deficiency.

This effect on the ribosomes should reflect in the protein system and does in magnesium deficiency (Marchesi and Kennell, 1967). In *A. marinus* the protein:RNA ratio was slightly lower when the nickel-treated cells were compared to the normal cells in basal medium (Fig. 18). This does not rule out qualitative differences in the protein, and does not indicate the rate differences for synthesis of the protein.

Inhibition of protein synthesis by chloramphenicol was without effect on the morphology of the cell (topic 26) as were the chelating agents EDTA and 8-hydroxyquinoline which should have induced magnesium deficiency (topic 31) if in effective concentrations.

These experiments were performed in sea water media containing about $1.8 \times 10^{-2}$ M magnesium and in one instance $1.2 \times 10^{-1}$ M magnesium where megalomorph cells were formed. Nickel would thus have to cause almost complete cessation of magnesium transport into the cell. Nickel could, however, have competed for reactive sites.

There are a few similarities between magnesium deficiency effects in other organisms but not sufficient to support this as a cause of megalomorph formation in *A. marinus*. It is also observed that magnesium deficiency in other organisms results in filament formation thus necessitating involvement
of the cell wall by other mechanisms in the case of *A. marinus* for production of the globular megalomorph.

The involvement of DNA in the formation of filaments resulted from ultraviolet radiation (Adler and Hardigree, 1965) with dimer formation in the DNA strands, and increased hydrostatic pressure with reduced synthesis in the DNA (ZoBell and Cobet, 1964).

Caffeine in eucaryotic cells (Kihlman, 1966) and hydroxyurea in procaryotic cells (Rosenkranz et al. 1966) were found to produce alterations in the activity of DNA. These agents are capable of forming filamentous growth but not the globular form in *A. marinus*, even at increased osmotic support levels. Mercury and copper ions, the latter capable of megalomorphic formation in *A. marinus* (Fig. 32) can bind to the nucleoside bases in such a manner to produce cross-linking of the two DNA strands (Thomas, 1954; Dore and Yamane, 1960; Eichhorn and Clark, 1965). If nickel acts to cause crosslinking of the DNA then the nickel, and copper, also have to function at another site, probably the cell wall system, since caffeine and hydroxyurea produce filaments while copper and nickel form the globular form.

Cell division in yeasts has been shown to involve sulphydryl groups with dependence on their functional maintenance (Nickerson and Van Rij, 1949; Nickerson and Falcone, 1956, 1957; and Wolpert, 1963). The addition of sulphydryl poisons result in sulphydryl inactivation leading
to divisional inhibition (Nickerson, 1949; Wolpert, 1963). The functional material was shown to be an enzyme, protein disulfide reductase, involved in cell wall expansion (Nickerson and Falcone, 1957). The two metals, mercury and silver, which are extremely toxic to sulfhydryl groups were without effect on the morphology of the cell at limiting concentrations. Either the sulfhydryl groups are not involved, or the toxicity of the two metals is such that growth was inhibited as well.

The shape of the bacteria is determined by the mechanical rigidity of the cell wall (Salton, 1964). During balanced growth the plasma membrane is in contact with the cell wall and they both expand and change shape.

The genus **Arthrobacter** has a propensity for an unusual growth response under usual methods of culture. The organisms are characterized and the majority stain as gram-negative, however, they have the chemical composition of gram-positive cell walls (Cummings and Harris, 1959; Krulwich et al., 1967; and Gillespie, 1963a, b).

The Gram reaction change of *A. marinus* from gram-negative in the marine medium to gram-positive in the TSA-Soil Extract medium may indicate some factor not presented to the cell in the sea water medium is presented, or presented in a form utilizable by the cell, in the soil extract. This may be a terrigens factor or similar factor needed to mobilize the metal ion (Morrison, Antoine, and Dewbrey, 1965). Thus
the cell is already under stress as a result of the deprivation of this material when grown in the sea water medium before exposure to nickel.

The maximum size a cell may attain is determined by the space within the cell wall. The osmotic pressure within the protoplast is at least as high as the surrounding medium so that the cell membrane is pressing against the cell wall with a turgor pressure (Mitchel and Moyle, 1956). If the cell wall is weakened its protective action is lost and the protoplast is forced to regulate its own volume unless protected by osmotic support in the medium. Otherwise the cell will burst (McQuillen, 1960; Wiebull, 1953).

The graded effect of nickel was influenced by the osmotic support of the medium (Fig. 10). At low osmotic support levels, 5% synthetic sea water containing medium, the _A. marinus_ grew as a filament at $1 \times 10^{-4}$ M nickel while at increased levels of synthetic sea waters these cells were round. This may also reflect the degree of weakening of the cell wall where 60% synthetic sea water is needed to provide osmotic support before growth in the medium is evident at $4 \times 10^{-4}$ M nickel. The need for greater osmotic support in the medium for globular plasmolyzed megalomorph (Fig. 5e) at 60% synthetic sea water (topic 8) while filamentous forms are produced in 20% synthetic sea water with plasmolysis (Fig. 10b) indicates a greater dependence by the cell wall on osmotic support than the protoplast since the cell is plasmolyzed in both cases. There may be a dependence on an increase
in some ions to provide (1) co-factors for the rounding process; (2) magnesium to meet the increased requirement of the cell as a result of nickel stress; (3) increased osmotic support due to greater damage to the cell wall.

Tomasz and Borek (1959, 1960) found in cells treated with 5-fluoracil when placed in hyperosmotic media that 95% of the inoculated cells were capable of growth whereas 95% failed to grown in the conventional nutrient agar. Cell wall synthesis was also suspected to be involved in the process.

The major portion of the volume regulation in the cell is due to the inorganic fraction, particularly sodium and potassium (Rothstein, 1964). An osmotic problem is seen to develop in the megalomorph as a result of nickel stress. The cells grown in the absence of nickel are seen to have normal morphology whereas in the same medium with $4 \times 10^{-4}$ M nickel the cell wall expands away from the protoplast to produce an appearance of being plasmolyzed (Fig. 5e, f). Hence, the cell has become hypotonic in relation to the exterior environment causing shrinking of the cell membrane away from the wall. The plasmolysis is evident in the electron micrographs with the cytoplasmic membrane definitely separated from the cell wall (Fig. 29, 30). An alternate possibility is that the cell wall synthesizing system has become uncontrolled producing cell wall while the protoplast, not being able to keep up the pace because of diffusion problems associated with the increased size, has lagged behind,
retracting from the wall.

These plasmolyzed cells, megalomorphs, when placed in or diluted with the same medium minus the nickel undergo rapid filling of the plasmolyzed space prior to commencement of the divisional processes.

A variety of spheroplasts formed as a result of cell wall inhibiting agents are seen to produce the plasmolyzed appearance of the cell (Lark, 1958; Thorsson and Weibull, 1958).

Montgomerie et al. (1968) have shown that penicillin causes an imbalance in the sodium-potassium accumulation resulting in an increased sodium:potassium ratio in Streptococcus faecalis. Cooper (1955) found penicillin produced a loss of both sodium and potassium in Staphylococcus aureus. Whether this electrolyte imbalance is a result of the penicillin itself or an alteration in a cell wall-membrane interaction is unknown.

Since walled cells have little need to control their volume by sodium-potassium discrimination (Schultz and Soloman, 1961), the membrane alone is unable to cope with the situation of volume control.

Since the megalomorphs are not very different chemically or as may be seen by electron micrographs (Fig. 21-31) from the normal cells, mechanisms controlling cell division may have been damaged by exposure to nickel. The failure to form cross walls and divide may be the result of inactivation of
the cross wall forming or cell wall polymerizing enzymes in
the presence of nickel rather than interference with the
synthesis of nuclear material which controls the synthesis
of enzyme proteins. However, the involvement of DNA cannot
be totally disregarded.

There is a large volume change in the normal cells from
the coccoid shape in stationary phase to the rod shape in
logarithmic phase. This is not the result of change in gross
chemical composition in the cell wall but rather changes in the
degree of polymerization of the polysaccharide backbone of the
peptidoglycan (Krulwick et al., 1967a) and degree of cross
linking of the peptides (Krulwick et al., 1967b), both being
greater in the rod form of *A. crystallopoietes*. Thus the
organism alters the structure of the outer envelope in the
course of its growth. In the formation of the megalomorph
the swelling and osmotic sensitivity of the cell suggests
that the cell wall components which are responsible for
cellular structure have been altered.

Cycloserine is known to affect the cell wall synthesizing
mechanisms by inhibiting the enzymes responsible for race-
mization of D-alanine and dimerization of the D-alanyl-D-
alanine (Strominger, Ito and Threnn, 1960; Neuhaus and
Lynch, 1962). This produces inhibition of cross linking of
the peptide of the peptidoglycan layer.

D-amino acids are capable of inducing morphological
variation in bacteria (Tuttle and Gest, 1960; Grula, 1960b).
D-methionine affects the cell wall synthesizing mechanism in
a manner similar to penicillin (Lark and Lark, 1959) resulting in plasmolyzed megalomorphic cells. The D-amino acid is involved as a "cell wall primer" inhibitor (Lark and Lark, 1961).

These two compounds cause plasmolyzed megalomorphic forms of _A. marinus_ identical with those produced as a result of nickel (Fig. 37). Megalomorphic formation is thus possible by disrupting the cell wall synthesizing mechanisms. The D-methionine may cause transport reduction for L-methionine (Lark, 1959), thereby involving protein synthesis in that particular instance.

Webb (1951) found filament formation to occur in response to magnesium deficiency in a complex medium but not in a simple nutrient solution unless supplemented with L-amino acids. This was attributed to intensification of the magnesium requirement by certain amino acids in the simple medium (Webb, 1951, 1966).

The effect of nickel on the morphology of the cell was also found to depend on the composition of the medium with the cells merely rounding in synthetic media or very low levels of protein. In the presence of D-methionine in the synthetic medium or increased levels of protein and nickel the cells form the typical megalomorphic cell. Increasing the concentration of protein in the media results in concomitant increase in D-methionine or other D-amino acids.

Depolymerization of the cell wall itself does not result in commitment to the megalomorph. There is some
factor, probably a D-amino acid(s), in the protein fraction of the medium which prevents repair to the lesion.

Miller, Zsigray, and Landman (1967) found that D-methionine as the amino acid or casein hydrolysate was responsible for the formation of "quasi-spheroplasts" as a result of chloramphenicol. The D-amino acid prevents repair of the damaged cell walls at a site involved in priming of cell wall formation and septation (Landman and Halle, 1963).

A number of D-amino acids are capable of producing this same result (Grula and Grula, 1962; Lark and Lark, 1959; Grula, 1960b), however, some amino acids are more active than others. The megalomorphic result of D-methionine is not readily reversible and produces a committed state of the cell (Miller, Zsigray and Landman, 1967; Lark and Lark, 1959). Other D-amino acids may not produce such an irreversible commitment thus allowing repair of the cell wall still providing a basis for the formation of the megalomorph as a result of nickel.

Rather than being a result of plasmolysis, the plasmolyzed appearance of the cell may be the result of the filling of the plasmolyzed space by a polymerized compound produced by the cell. This material would have to be greater than 30,000 molecular weight in order not to diffuse through the cell wall. The cell wall system would be affected in order for the cell to have the capacity of expansion producing the spherical shape. This may be by disruption of a membrane-cell wall relationship, or a polymerizing-depoly-
merizing enzyme in the paraplasmatic space may be inactivated by the presence of nickel. This may result in a production and accumulation of one of the wall constituents, i.e. teichoic acid, in the space. The accumulation would produce the filling of the paraplasmatic space providing pressure on the cell membrane resulting in the crescent shape of the cytoplasm as well as producing pressure on the cell wall to maintain the spherical shape of the cell. The material is not electron dense as it is not evident on the electron photomicrographs.

Filament formation in bacteria caused by other methods can be reversed by exposure to various agents. Cell division can be initiated in UV induced filaments in *E. coli* by incubation at 42 C or addition of pantoyl lactone (Walker and Pardee, 1967), mitomycin C induced filaments of *Erwinia* by divalent cations or pantoyl lactone (Grula and Grula, 1962). Pantoyl lactone was unable to spare the morphological effect of nickel on *A. marinus* or induce cell division (topic 33). Platinum induced filaments of *E. coli* were also not induced to divide by pantoyl lactone (Rosenberg et al., 1967). Thus these heavy metal induced aberrations in morphology may affect the cell in a manner different from those aided by pantoyl lactone. In *A. marinus* cell division was induced in the megalomorphic cells only by dilution of the medium to a low nickel level, complete removal of nickel or addition of a chelating agent to the medium.

Autolytic enzymes are associated with the cell wall of
a number of organisms (Shockman, Pooley and Thompson, 1967; Mohan et al., 1965 and Shockman, 1965). The autolysin studied by Shockman et al. (1967) was most active on the newly synthesized cell wall suggesting localization of the enzyme at or near the site of new cell wall growth. There was also a latent autolysin located at other sites on the cell wall probably acting in a closely controlled manner with the cell. Krulwich et al. (1967) described an autolysin of *A. crystallopoietes* associated with the cell wall and probably responsible for the less polymerized state of the peptidoglycan of the coccoid cell wall than that of the rod-shaped cell wall. The coccoid cell wall depended more critically on the peptide cross-linkage for its integrity (Krulwich et al. 1967a). The activity of the autolysins may be affected by alteration in the composition of the medium to maintain the coccoid state (Ensign and Wolfe, 1964) or by the normal events of the growth cycle producing coccoid cell in the stationary phase. Nickel may be affecting their activity, decreasing the integrity of the cell wall to allow the formation of the globular megalomorph rather than a filamentous state. The nickel may directly affect these autolysins or indirectly by disruption of a membrane-wall interaction. The involvement of a potentially lethal autolysin is an essential factor in the biosynthetic elongation of wall peptidoglycan and demands a close integration of its action with the biosynthetic process, at least during balanced exponential growth (Shockman et al. 1967).
Alternately, the polymerizing enzymes produce less polymerized material as a result of the nickel. The site where the septum is formed within the cell may be the most active site for cell wall synthesis. Since there is a suppression of septum formation in the cell there may also be an alteration in the other enzymes associated with the site.

Sucrose has been used at increased concentrations in a range of 0.3 to 0.7 M as a means of attaining osmotic support for sensitive cells (McQuillen, 1960). Grula (1960a) found a number of carbohydrates including sucrose capable of inducing long filamentous growth as a result of unbalanced growth. Hober (1946) reviews some of the literature on the effects of sucrose and other non-electrolytes on red blood cells. The compounds appear to produce their effect by a leakage of salts, possibly through an exchange of ions as well as by their dehydrating power.

The fact that the consequences of sucrose can be overcome by the cations of magnesium, calcium, sodium and potassium points to a chelating or ion exchange relation with the cell. The stability of cell walls has been shown to depend a great deal in its organic ion content (Asbell and Eagon, 1966).

The main point of attack by nickel is probably the cell wall synthesizing system. However, the involvement of the nuclear material cannot be totally excluded. The plasmolysis, physiological and metabolic disruption are secondary effects
of nickel or a result of cell wall-cell interactions.

There are at least five ways nickel may be affecting the cell wall. (1) It may inactivate the enzymes in the paraplasmonic space (Heppel, 1967) some of which may be involved in cell wall maintenance or synthesis. (2) Inhibition of those enzymes or DNA-membrane interactions associated with septum formation. (3) Inactivation or activation of the polymerizing enzymes and autolysins. This may occur at the cell wall or the site of cell wall synthesis. (4) An effect on the "Primer site" of cell wall synthesis (McQuillen, 1958; Lark and Lark, 1961; Miller, Zsigray and Landman, 1967) such that a modification of the cell wall at a critical site affects its further synthesis. (5) There may be an effect of nickel on the membrane such that the DNA-membrane interaction needed for cell division is disrupted (Lark, 1966; Ryter, 1968).

The interaction of nickel and cell to form megalomorphs is not a permanent effect as removal of the metal does allow reversal of the process and division can then occur. Nickel alone is not capable of inducing megalomorphic formation (topics 7, 15). Some constituent(s) in the organic nutrient fraction is necessary to produce the megalomorphic stage of growth. This may rely on a similar effect produced by the D-amino acids which, however, is not reversible.

In the course of cellular enlargement the surface: volume ratio will be greatly reduced thereby creating problems of nutrient and waste distribution. This may result in a sodium-potassium imbalance producing plasmolysis of the
cell.

The portion of the nickel taken up by the cell which is effective in division inhibition is probably loosely bound. On dilution or removal of the nickel the cell rapidly undergoes deplasmolysis and proceeds to divide. The firmly bound nickel is probably more involved in the process of growth inhibition, requiring a higher concentration.

Those cells in a dormant stage at the time of addition of nickel to the culture require an extended time to accumulate or produce the necessary material to proceed through a limited number of divisions and also are ultimately affected.

The formation of the megalomorph may thus be the result of a number of problems imposed on the cell. Not all of these may be attributed to nickel directly but may provide conditions for other stresses to be produced or become effective.
Table 1. Warburg

Components of Warburg respirometry experiment. (Reference for Fig. 16).

<table>
<thead>
<tr>
<th>Flask</th>
<th>Synthetic sea water, ml</th>
<th>KOH, ml</th>
<th>Suspended cells, ml</th>
<th>First Addition, ml</th>
<th>Second Addition, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45 min</td>
<td>105 min</td>
</tr>
<tr>
<td>Thermobarometer</td>
<td>3.0</td>
<td>0.2</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Endogenous</td>
<td>2.2</td>
<td>0.2</td>
<td>0.8</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Endogenous - Ni</td>
<td>2.0</td>
<td>0.2</td>
<td>0.8</td>
<td>---</td>
<td>0.2 Ni</td>
</tr>
<tr>
<td>Nutrient + Ni</td>
<td>1.8</td>
<td>0.2</td>
<td>0.8</td>
<td>0.2 Nut. Mix</td>
<td>---</td>
</tr>
<tr>
<td>Nutrient - Ni</td>
<td>1.8</td>
<td>0.2</td>
<td>0.8</td>
<td>0.2 Nut. Mix</td>
<td>0.2 Ni</td>
</tr>
</tbody>
</table>
Table 2. G plus C % M

Guanosine plus cytosine (G + C) percent molarity of *A. marinus* using the method of Wang and Hashagen (1964), three controls and their values from the literature.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(G + C) % M</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arthrobacter marinus</em></td>
<td>63.3</td>
<td>---</td>
</tr>
<tr>
<td><em>Arthrobacter globiformis</em></td>
<td>64.3</td>
<td>62-64 (1)</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>40.7</td>
<td>40 (2)</td>
</tr>
<tr>
<td>Fish sperm DNA</td>
<td>41.9</td>
<td>---</td>
</tr>
</tbody>
</table>

Ref: (1) Marmur, Falkow and Mandel, 1963.  
(2) Schildkraut, Marmur and Doty, 1962.
Table 3. Population and size in relation to nickel

Plate count populations and size associated with various molar concentrations of nickel at 25 C.

<table>
<thead>
<tr>
<th>Conc. of Ni, M</th>
<th>Plate Count, cells/ml</th>
<th>Maximum Ave. size, µ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>5 - 8 x (10^8)</td>
<td>2 x 4</td>
</tr>
<tr>
<td>1 x 10^{-4}</td>
<td>3 - 6 x (10^8)</td>
<td>2 x 4</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>3 - 6 x (10^7)</td>
<td>2-3 x 4-6</td>
</tr>
<tr>
<td>3 x 10^{-4}</td>
<td>3 - 5 x (10^6)</td>
<td>7 - 10, dia.</td>
</tr>
<tr>
<td>4 x 10^{-4}</td>
<td>4 - 7 x (10^5)</td>
<td>10 - 15, dia.</td>
</tr>
</tbody>
</table>
Table 4. Inoculum size

Time of maximum OD and the maximum OD at 420 μm (x 10^3) for A. marinus grown in basal medium with increasing levels of nickel ions at 25 °C in the presence of different inoculum size.

Concentration of NiCl$_2$, M

<table>
<thead>
<tr>
<th>Cells/ml, inoculum</th>
<th>No added nickel</th>
<th>2 x 10$^{-4}$</th>
<th>3 x 10$^{-4}$</th>
<th>4 x 10$^{-4}$</th>
<th>5 x 10$^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hr  OD</td>
<td>hr  OD</td>
<td>hr  OD</td>
<td>hr  OD</td>
<td>hr  OD</td>
</tr>
<tr>
<td>54,000</td>
<td>24  620</td>
<td>24  420</td>
<td>84  100</td>
<td>108  140</td>
<td>454  NG</td>
</tr>
<tr>
<td>8,600</td>
<td>24  620</td>
<td>24  380</td>
<td>60  180</td>
<td>228  100</td>
<td>454  NG</td>
</tr>
<tr>
<td>880</td>
<td>24  620</td>
<td>24  390</td>
<td>108  195</td>
<td>454  NG</td>
<td>454  NG</td>
</tr>
<tr>
<td>98</td>
<td>24  620</td>
<td>36  390</td>
<td>108  205</td>
<td>454  NG</td>
<td>454  NG</td>
</tr>
<tr>
<td>10</td>
<td>36  600</td>
<td>36  340</td>
<td>228  125</td>
<td>454  NG</td>
<td>454  NG</td>
</tr>
</tbody>
</table>

NG - No Growth
Table 5. Protein-Nickel

Nickel concentrations applied to various levels of protein, as 50% peptone and 50% yeast extract, to determine protein-nickel relationship. (Reference for Fig. 9).

<table>
<thead>
<tr>
<th>Protein, ppm</th>
<th>Range of nickel conc., M, NiCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>$1 \times 10^{-5}$ to $1 \times 10^{-4}$</td>
</tr>
<tr>
<td>200</td>
<td>$1 \times 10^{-5}$ to $1 \times 10^{-4}$</td>
</tr>
<tr>
<td>300</td>
<td>$1 \times 10^{-5}$ to $1 \times 10^{-4}$</td>
</tr>
<tr>
<td>600</td>
<td>$6 \times 10^{-5}$ to $4 \times 10^{-4}$</td>
</tr>
<tr>
<td>1,000</td>
<td>$1 \times 10^{-4}$ to $6 \times 10^{-4}$</td>
</tr>
<tr>
<td>2,000</td>
<td>$6 \times 10^{-4}$ to $4 \times 10^{-3}$</td>
</tr>
<tr>
<td>4,000</td>
<td>$4 \times 10^{-3}$ to $2 \times 10^{-2}$</td>
</tr>
</tbody>
</table>
Table 6. Salinity

Time of maximum OD and the maximum OD at 420 mu (x 10^3) with increasing molar concentrations of nickel and different percentages of synthetic sea water.

<table>
<thead>
<tr>
<th>Percentage synthetic sea water</th>
<th>100</th>
<th>80</th>
<th>60</th>
<th>40</th>
<th>20</th>
<th>10</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Ni, M (x 10^-4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No added nickel</td>
<td>24</td>
<td>600</td>
<td>24</td>
<td>580</td>
<td>24</td>
<td>580</td>
<td>24</td>
</tr>
<tr>
<td>1 x 10^-4</td>
<td>36</td>
<td>530</td>
<td>36</td>
<td>530</td>
<td>24</td>
<td>520</td>
<td>36</td>
</tr>
<tr>
<td>2 x 10^-4</td>
<td>24</td>
<td>410</td>
<td>36</td>
<td>400</td>
<td>36</td>
<td>380</td>
<td>36</td>
</tr>
<tr>
<td>3 x 10^-4</td>
<td>84</td>
<td>290</td>
<td>60</td>
<td>150</td>
<td>60</td>
<td>240</td>
<td>84</td>
</tr>
<tr>
<td>4 x 10^-4</td>
<td>132</td>
<td>130</td>
<td>108</td>
<td>135</td>
<td>84</td>
<td>175</td>
<td>144</td>
</tr>
<tr>
<td>5 x 10^-4</td>
<td>144</td>
<td>NG</td>
<td>144</td>
<td>NG</td>
<td>144</td>
<td>NG</td>
<td>--</td>
</tr>
</tbody>
</table>

NG = No Growth
Table 7. Temperature

Times of maximum OD and the maximum OD at 420 μm (x 10^3) for A. marinus grown in basal medium with nickel ions at various temperatures.

<table>
<thead>
<tr>
<th>Temperature, C</th>
<th>10</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Ni, M</td>
<td>hr</td>
<td>OD</td>
<td>hr</td>
<td>OD</td>
<td>hr</td>
<td>OD</td>
</tr>
<tr>
<td>No added nickel</td>
<td>120</td>
<td>480</td>
<td>48</td>
<td>550</td>
<td>24</td>
<td>650</td>
</tr>
<tr>
<td>8 x 10^{-5}</td>
<td>120</td>
<td>530</td>
<td>48</td>
<td>530</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>9 x 10^{-5}</td>
<td>120</td>
<td>510</td>
<td>48</td>
<td>520</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>1 x 10^{-4}</td>
<td>120</td>
<td>480</td>
<td>48</td>
<td>520</td>
<td>24</td>
<td>580</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>144</td>
<td>310</td>
<td>48</td>
<td>360</td>
<td>24</td>
<td>400</td>
</tr>
<tr>
<td>3 x 10^{-4}</td>
<td>144</td>
<td>230</td>
<td>96</td>
<td>160</td>
<td>72</td>
<td>190</td>
</tr>
<tr>
<td>4 x 10^{-4}</td>
<td>260</td>
<td>85</td>
<td>96</td>
<td>95</td>
<td>96</td>
<td>180</td>
</tr>
<tr>
<td>5 x 10^{-4}</td>
<td>260</td>
<td>NG</td>
<td>168</td>
<td>NG</td>
<td>144</td>
<td>NG</td>
</tr>
</tbody>
</table>

NG = No Growth
Table 8. OD during growth

Optical density ($x 10^3$) at 420 µm of the *A. marinus* cultures during the growth cycle in basal medium and in the presence of two high concentrations of nickel at 25 C. (Reference for Fig. 11 and 12).

<table>
<thead>
<tr>
<th>Incubation Time, hr</th>
<th>No Addition of Ni</th>
<th>$4 \times 10^{-4}$ Ni, M</th>
<th>$8 \times 10^{-4}$ Ni, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>300</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>590</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>580</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>540</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>490</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>84</td>
<td>440</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 9. Acclimatization

Acclimatization of *A. marinus* to increasing concentrations of nickel at ambient temperature.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Ni. Conc., M</th>
<th>Remarks</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$4 \times 10^{-4}$</td>
<td>Good Growth</td>
<td>4+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>Slight Growth</td>
<td>4+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$6 \times 10^{-4}$</td>
<td>No Growth</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$7 \times 10^{-4}$</td>
<td>No Growth</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>$4 \times 10^{-4}$</td>
<td>Good Growth</td>
<td>4+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>Good Growth</td>
<td>4+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$6 \times 10^{-4}$</td>
<td>No Growth</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$7 \times 10^{-4}$</td>
<td>No Growth</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>$4 \times 10^{-4}$</td>
<td>Good Growth</td>
<td>3+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$</td>
<td>Good Growth</td>
<td>4+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$6 \times 10^{-4}$</td>
<td>Slight Growth</td>
<td>4+ Morphology</td>
</tr>
<tr>
<td></td>
<td>$7 \times 10^{-4}$</td>
<td>No Growth</td>
<td></td>
</tr>
</tbody>
</table>

3+ = morphology resembling Fig. 4d
4+ = morphology resembling Fig. 4e
Table 10. Uptake of nickel from the medium

Uptake of nickel per mg cell nitrogen by A. marinus from basal medium containing increasing concentrations of nickel in 3 hr at ambient temperature.

<table>
<thead>
<tr>
<th>Concentration of Ni, in Medium, M</th>
<th>( \mu g ) Ni/mg Cell N</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1 \times 10^{-6} )</td>
<td>not detected</td>
</tr>
<tr>
<td>( 1 \times 10^{-5} )</td>
<td>0.4</td>
</tr>
<tr>
<td>( 1 \times 10^{-4} )</td>
<td>2.4</td>
</tr>
<tr>
<td>( 2 \times 10^{-4} )</td>
<td>5.1</td>
</tr>
<tr>
<td>( 4 \times 10^{-4} )</td>
<td>8.4</td>
</tr>
<tr>
<td>( 6 \times 10^{-4} )</td>
<td>14.5</td>
</tr>
<tr>
<td>( 8 \times 10^{-4} )</td>
<td>6.9</td>
</tr>
</tbody>
</table>
Table 11. Uptake of nickel in relation to time.

Uptake of nickel per mg cell nitrogen by *A. marinus* in relation to time of incubation in basal medium containing 4 x 10^{-4} M nickel at ambient temperatures.

<table>
<thead>
<tr>
<th>Time of Exposure, min</th>
<th>μg Ni/mg Cell N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 - 3</td>
<td>2.7</td>
</tr>
<tr>
<td>30</td>
<td>1.3</td>
</tr>
<tr>
<td>60</td>
<td>0.9</td>
</tr>
<tr>
<td>120</td>
<td>2.8</td>
</tr>
<tr>
<td>240</td>
<td>3.1</td>
</tr>
</tbody>
</table>
Table 12. Permanence of nickel uptake

Effect of washing on the retention of nickel taken up per mg cell nitrogen by A. marinus from basal medium containing $4 \times 10^{-4}$ M nickel in 3 hr at ambient temperature.

<table>
<thead>
<tr>
<th>Number of Washes</th>
<th>μg Ni/mg Cell N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.0</td>
</tr>
<tr>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>4.9</td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
</tr>
</tbody>
</table>
Table 13. Heavy metals

Growth of *A. marinus* in basal medium containing various concentrations of heavy metals at maximum time with incubation at 25 C.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Concentration of Cation Yielding:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth, M</td>
</tr>
<tr>
<td>Cobalt</td>
<td>$3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Copper II</td>
<td>$4 \times 10^{-4}$</td>
</tr>
<tr>
<td>Cadmium</td>
<td>$1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Zinc</td>
<td>$1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Lead</td>
<td>$1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Manganese</td>
<td>$3 \times 10^{-3}$</td>
</tr>
<tr>
<td>Silver</td>
<td>$1 \times 10^{-5}$</td>
</tr>
<tr>
<td>Mercury II</td>
<td>$1 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
Table 14. Cations and sucrose effect

Composition of the various cations in basal medium for correlation with the morphological changes in *A. marinus* caused by 0.5 M sucrose. (Reference for Fig. 36).

Fig. 36 symbols: a b c d

<table>
<thead>
<tr>
<th>Cation</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium</td>
<td>0.1</td>
<td>0.01</td>
<td>0.001</td>
<td>----</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.1</td>
<td>0.01</td>
<td>---</td>
<td>0.001</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.3,0.1</td>
<td>0.03</td>
<td>0.01</td>
<td>0.003</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.3</td>
<td>0.1,0.03</td>
<td>0.01</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Table 15. Asparagine

Concentration of asparagine in synthetic medium and the corresponding limiting concentration of nickel and effect of asparagine concentrations on growth of *A. marinus*.

<table>
<thead>
<tr>
<th>Concentration of asparagine, µg/ml</th>
<th>Limiting Concentration of nickel, M</th>
<th>Max. OD at 420 μm, No Ni Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3 \times 10^{-5}$</td>
<td>0.450</td>
</tr>
<tr>
<td>5</td>
<td>$3 \times 10^{-5}$</td>
<td>0.520</td>
</tr>
<tr>
<td>20</td>
<td>$1 \times 10^{-4}$</td>
<td>0.630</td>
</tr>
<tr>
<td>100</td>
<td>$3 \times 10^{-4}$</td>
<td>0.850</td>
</tr>
<tr>
<td>400</td>
<td>$7 \times 10^{-4}$</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Table 16. Magnesium

Time of maximum OD and the maximum OD, \((x \times 10^3)\), at 420 μμ for A. marinus grown in basal medium with increasing levels and combinations of magnesium and nickel at 25 C.

<table>
<thead>
<tr>
<th>Concentration of magnesium, M</th>
<th>No Addition</th>
<th>(1 \times 10^{-4})</th>
<th>(1 \times 10^{-3})</th>
<th>(1 \times 10^{-2})</th>
<th>(1.2 \times 10^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Ni, M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No added nickel</td>
<td>24</td>
<td>580</td>
<td>12</td>
<td>620</td>
<td>12</td>
</tr>
<tr>
<td>(9 \times 10^{-5})</td>
<td>24</td>
<td>320</td>
<td>24</td>
<td>530</td>
<td>12</td>
</tr>
<tr>
<td>(1 \times 10^{-4})</td>
<td>48</td>
<td>280</td>
<td>24</td>
<td>460</td>
<td>24</td>
</tr>
<tr>
<td>(2 \times 10^{-4})</td>
<td>96</td>
<td>150</td>
<td>24</td>
<td>145</td>
<td>24</td>
</tr>
<tr>
<td>(3 \times 10^{-4})</td>
<td>144 NG</td>
<td>144 NG</td>
<td>72</td>
<td>140</td>
<td>72</td>
</tr>
<tr>
<td>(4 \times 10^{-4})</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144</td>
<td>144</td>
<td>144</td>
</tr>
<tr>
<td>(5 \times 10^{-4})</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144</td>
<td>144</td>
<td>144</td>
</tr>
</tbody>
</table>

NG = No Growth
Table 17. Calcium

Time of maximum OD and the maximum OD (x 10^3) at 420 μm for *A. marinus* grown in basal medium with increasing levels and concentrations of calcium and nickel at 25 C.

<table>
<thead>
<tr>
<th>Concentration of calcium, M</th>
<th>No Addition</th>
<th>1 x 10^{-4}</th>
<th>1 x 10^{-3}</th>
<th>1 x 10^{-2}</th>
<th>1 x 10^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Ni, M</td>
<td>hr  OD</td>
<td>hr  OD</td>
<td>hr  OD</td>
<td>hr  OD</td>
<td>hr  OD</td>
</tr>
<tr>
<td>No added nickel</td>
<td>24  570</td>
<td>24  560</td>
<td>12  570</td>
<td>24  560</td>
<td>24  490</td>
</tr>
<tr>
<td>1 x 10^{-4}</td>
<td>24  490</td>
<td>24  490</td>
<td>24  520</td>
<td>24  540</td>
<td>24  390</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>48  170</td>
<td>24  270</td>
<td>24  360</td>
<td>24  310</td>
<td>72  280</td>
</tr>
<tr>
<td>3 x 10^{-4}</td>
<td>120 170</td>
<td>72  110</td>
<td>72  120</td>
<td>72  160</td>
<td>144 90</td>
</tr>
<tr>
<td>4 x 10^{-4}</td>
<td>144 50</td>
<td>144 145</td>
<td>144 150</td>
<td>144 125</td>
<td>144 NG</td>
</tr>
<tr>
<td>5 x 10^{-5}</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144 NG</td>
</tr>
</tbody>
</table>

NG = No Growth
Table 18. Iron

Time of maximum OD and the maximum OD ($x \times 10^3$) at 420 μm for A. marinus grown in basal medium with increasing levels and concentrations of iron and nickel at 25°C.

<table>
<thead>
<tr>
<th>Concentration of iron, M</th>
<th>No Addition</th>
<th>$1 \times 10^{-6}$</th>
<th>$1 \times 10^{-5}$</th>
<th>$1 \times 10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Ni, M</td>
<td>hr</td>
<td>OD</td>
<td>hr</td>
<td>OD</td>
</tr>
<tr>
<td>No added nickel</td>
<td>24</td>
<td>650</td>
<td>24</td>
<td>650</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>24</td>
<td>580</td>
<td>24</td>
<td>580</td>
</tr>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>24</td>
<td>400</td>
<td>24</td>
<td>370</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$</td>
<td>72</td>
<td>190</td>
<td>48</td>
<td>180</td>
</tr>
<tr>
<td>$4 \times 10^{-4}$</td>
<td>96</td>
<td>180</td>
<td>120</td>
<td>180</td>
</tr>
<tr>
<td>$5 \times 10^{-4}$</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144 NG</td>
<td>144 NG</td>
</tr>
</tbody>
</table>

NG = No Growth
Fig. 1  
Cellular development of normal cells of *Arthrobacter marinus* in the basal medium.  

a. Inoculum, 18 hr culture from surface of Marine Agar 2216E slant.  
b. Logarithmic phase, 5 hr after inoculation in basal medium and incubated at 25 C.  
c. Late stationary phase, 8 days after inoculation in basal medium and incubation at 25 C. Photomicrographs by phase contrast.
Fig. 2  Electron photomicrograph of flagellum. The culture was incubated at 25 °C for 12 hr in basal medium. The cells were shadow cast with tungstic oxide at an angle of 30 degrees and 25 Å thick.
Fig. 3 Growth response to nickel. The basal medium was prepared with increasing molar concentrations of nickel, inoculated with an 18 hr culture and shaken at 300 rpm at 25 C.
Fig. 4 Morphology of cells grown in the presence of nickel. The basal medium was prepared with increasing molar concentrations of nickel and incubated at 300 rpm at 25 C. Photomicrographs by phase contrast.

a. Inoculum, 18 hr culture from surface of Marine agar 2216E slant.

b. Culture incubated 24 hr in basal medium containing $1 \times 10^{-4}$ M nickel.

c. Culture incubated 30 hr in basal medium containing $2 \times 10^{-4}$ M nickel.

d. Culture incubated 96 hr in basal medium containing $3 \times 10^{-4}$ M nickel.

e. and f. Culture incubated 120 hr in basal medium containing $4 \times 10^{-4}$ M nickel.
Morphological changes of a culture grown in the presence of $4 \times 10^{-4}$ M nickel. The culture was inoculated to basal medium containing $4 \times 10^{-4}$ M nickel and incubated at 25 C. At indicated times aliquots were removed, wet mounts prepared and photomicrographs by phase contrast made.

a. Inoculum, 18 hr culture from Marine Agar 2216E slant.
b. After 1.5 hr incubation.
c. After 2.5 hr incubation.
d. After 4.5 hr incubation.
e. After 6.5 hr incubation.
f. After 10 hr incubation.
Fig. 6  Stimulation of growth by nickel. The basal medium was prepared with increasing molar concentration of nickel, inoculated at 25°C and shaken at 200 rpm. OD readings taken after 8 and 14 hr incubation are plotted and compared with a control culture containing no nickel. (at left).
OD AT 420 MUL

0 1X10^-7 1X10^-6 1X10^-5 1X10^-4

NI CONCENTRATION, M

14 hr

8 hr

.600

.400

.200
Effect of autoclaving on growth. The basal medium was prepared with $4 \times 10^{-4}$ M nickel in 4 ways. The inoculated culture was shaken at 200 rpm and incubated at 25 C.

- Control culture; no added nickel.
- $4 \times 10^{-4}$ M nickel added to the basal medium before autoclaving at 15 lbs pressure for 15 min.
- The basal medium and nickel solution autoclaved separately and combined to provide $4 \times 10^{-4}$ M nickel after cooling to ambient temperature.
- The basal medium and nickel solution were autoclaved separately. The basal medium was inoculated with the culture and incubated. After 60 min incubation the nickel was added to the culture.
Fig. 8

Rate of shaking. Inoculated basal medium prepared with and without $4 \times 10^{-4}$ M nickel were incubated at ambient temperature. The cultures were shaken at 360, 180 rpm, and under (S) static conditions. The upper three curves are from basal medium prepared without nickel and the lower three are from basal medium prepared with $4 \times 10^{-4}$ M nickel.
Fig. 9  Protein - nickel concentrations relationship. The medium was prepared with increasing concentration of protein from 100 to 4,000 ppm as 50% peptone and 50% yeast extract. Nickel was added to the medium as presented in Table 5. The plotted values are combinations of nickel and protein allowing multiplication of the culture, the next higher concentration of nickel tested was inhibitory to cell division.
Morphology of nickel grown cells in reduced inorganic environment. The nickel-containing basal medium was prepared with reduced concentrations of synthetic sea water, inoculated and incubated at ambient temperature.

a. 40% synthetic sea water and $3 \times 10^{-4}$ M nickel after 96 hr incubation.
b. 20% synthetic sea water and $2 \times 10^{-4}$ M nickel after 120 hr incubation.
Fig. 11 Distilled water - sea water dilution relationship. Basal medium was prepared with and without $4 \times 10^{-4}$ M nickel. Periodically, from the inoculated medium, aliquots from each flask were diluted in two ways: distilled water (D, $H_2O$) and 75% synthetic sea water (SSW) dilution blanks. The dilutions were plated to 2216E medium and incubated 48 hr. Incubation was at ambient temperature. The upper pair of curves represent the control containing no nickel while the lower pair represent the cultural response to nickel.
Distilled water - sea water dilution relationship, $8 \times 10^{-4}$ M nickel. Basal medium was prepared with $8 \times 10^{-4}$ M nickel. Periodically, from the inoculated medium, aliquots were diluted in two ways: distilled water (D. H$_2$O) and 75% synthetic sea water (SSW) dilution blanks. The dilutions were plated in 2216E medium and incubated at ambient temperature for 48 hr.
Fig. 13 Cultural stage of growth vs nickel effect. Basal medium was inoculated and aliquots were removed at intervals and brought to $4 \times 10^{-4}$ M nickel. Periodically all samples were plated to 2216E medium for viable population determination. Broken line is the control containing no nickel, solid lines are the aliquots removed at various times in hr.
Subsequent growth of the megalomorphic cells. After 4.5 hr incubation at 25 C the culture was adjusted to $4 \times 10^{-4}$ M nickel and incubated an additional 21.5 hr. The culture containing megalomorphic cells was then diluted 1:10 with fresh basal medium and reincubated. The control containing no nickel is also presented. Plate counts were made on the surface of 2216E plates. Incubation was at ambient temperature.
Fig. 15

260-280 μ absorbance of culture supernatant. Inoculated synthetic medium containing increasing concentrations of nickel were centrifuged twice and the supernatant examined for absorbance in the 230-320 μ range. The numbers denote molar concentrations of nickel in the medium at 25°C. Control: no added nickel, the culture was in the stationary phase after 36 hr incubation. 1 x 10⁻⁵ L: late logarithmic phase of growth after 36 hr incubation. 1 x 10⁻⁵ S: stationary phase of growth after 44 hr incubation. 2 x 10⁻⁵ S: stationary phase of growth after 68 hr incubation. 2 x 10⁻⁵ L: early logarithmic phase of growth after 44 hr incubation. 4 x 10⁻⁵: after 68 hr incubation.
Fig. 16 Effect of nickel on oxygen utilization. The procedure was as outlined in Table 1. Nut-Ni: nutrients added at 45 min followed by the addition of $6 \times 10^{-3}$ M nickel at 105 min. Nut + Ni: addition of nutrients and $6 \times 10^{-3}$ M nickel at 45 min. Endog: endogenous respiration control, no additions. Endog-Ni: endogenous respiration control with $6 \times 10^{-3}$ M nickel added at 105 min at 25 C.
DNA, RNA and protein concentration/cell. The culture was incubated at ambient temperature both with and without $4 \times 10^{-4}$ M nickel. The cellular material was extracted and analyzed for the constituents. Ni: nickel containing culture. C: control culture containing no nickel. Cell count by phase contrast microscopy using a Petroff-Hauser cell chamber.
Fig. 18 Relative ratio concentration of the nucleic acids and protein. The relative ratios of the data presented in Fig. 17 for cultures grown in the presence of $4 \times 10^{-4}$ M nickel (Ni) and the absence of nickel (C) are plotted. Zero time represents the ratios of the constituents before addition of nickel to the cultures.
Relative concentration of the nucleic acids and protein. The relative concentrations of the constituents presented in Fig. 17 for cultures grown in the presence of $4 \times 10^{-4}$ M nickel (Ni) and absence of nickel (C) are plotted. Values are based on the concentration of the constituents before addition of nickel to the culture, equal to one.
Fig. 20 C:N:P ratios in the presence of nickel. A. Viable cells/ml determined by plating to 2216E medium. The cultures were grown without added nickel (C), 4 x 10^{-4}M (4) and 6 x 10^{-4} M nickel (6). B. The upper group of curves represent the C:P ratios and the lower groups represent the N:P ratios of the cultures shown in Fig. 20 A. The nickel was added after 4 hr incubation at ambient temperature.
Normal A. marinus cells grown in the absence of nickel. The nuclear material (N) is evident as are the globular structures (G) attached to the outer cell wall. The sections were fixed in glutaraldehyde-osmium and stained with uranyl acetate and lead citrate.
Fig. 22 Normal cells with the globular structure (G) appearing to eminate from the cell wall.
Fig. 23  Normal cells with an extended globular structure (G) and mesosome (M).
Fig. 24 Normal cells showing mesosome (M) and septa (S) between daughter cells.
Fig. 25  

*A. marinus* grown in $4 \times 10^{-4}$ M nickel for one hr. The extended globular structure as well as single units (G) are seen on the surface of the cells. Vacuoles (V) are seen at the polar ends. Mesosomes (M) are still formed with normal appearance.
Fig. 26 One hr growth in the presence of $4 \times 10^{-4}$ M nickel mesosome (M), vacuoles (V), and septum (S) are formed with the cell.
Fig. 27  Two hr growth in the presence of $4 \times 10^{-4}$ M nickel. The vacuole (V) is seen to be present between the cell wall (CW) and the plasma membrane (PM). Globular structures (G) are seen to extend from the cell.
Fig. 28 Three hr growth in the presence of $4 \times 10^{-4}$ M nickel.
Six hr growth in the presence of $4 \times 10^{-4}$ M nickel. The plasmolized space (PS) is seen between the cell wall and the plasma membrane, with an occasional cytoplasmic vacuole (V) surrounded by membrane. The nuclear material (N) is localized through the cytoplasm at numerous locations. Along the cell wall are found extrusions (E) of the cytoplasm contained in a membrane. Along the periphery of the cytoplasm are found reticulations (R).
Fig. 30  Enlargement of Fig. 29 showing the cell wall (CW), cytoplasmic extrusions (E) and plasma membrane (PM).
Fig. 31 Six hr growth in the presence of $4 \times 10^{-4}$ M nickel. The reticulation (R) is seen along the periphery of the cytoplasm as well as a mesosome (M).
Fig. 32 Morphological changes as a result of other heavy metals. The cultures in basal medium incubated at ambient temperature in the presence of heavy divalent metals.

a. \(8 \times 10^{-5}\) M zinc after 38 hr incubation.
b. \(8 \times 10^{-5}\) M zinc after 62 hr incubation.
c. \(4 \times 10^{-4}\) M copper after 38 hr incubation.
d. \(4 \times 10^{-4}\) M copper after 62 hr incubation.
e. \(8 \times 10^{-5}\) M cadmium after 86 hr incubation.
f. \(2 \times 10^{-4}\) M cobalt after 96 hr incubation.
Fig. 33 Dithizone. To basal medium extracted with 0.1% dithizone in chloroform was added increasing molar concentrations of nickel. The control flask did not contain added nickel. The cultures were incubated at ambient temperatures.
Morphology variation as a result of sucrose. The synthetic sea water was reduced to 15% in the basal medium and sucrose added to increased molar levels. Incubation was at ambient temperature. Photomicrographs by phase contrast.

a. 0.3 M sucrose after 14 hr incubation.
b. 0.3 M sucrose after 24 hr incubation.
c. 0.5 M sucrose after 15 hr incubation.
d. 0.5 M sucrose after 24 hr incubation.
e. 0.7 M sucrose after 15 hr incubation.
f. 0.7 M sucrose after 24 hr incubation.
Fig. 35

Combined sucrose and nickel effect on morphology. The synthetic sea water was reduced to 15% in the basal medium with sucrose and nickel added at increasing molar concentrations. Incubation was at ambient temperature. Photomicrographs by phase contrast.

a. After 18 hr incubation without nickel or sucrose added to the medium.
b. After 24 hr incubation with $1 \times 10^{-4}$ M nickel and no sucrose added to the medium.
c. After 96 hr incubation with $3 \times 10^{-4}$ M nickel and 0.3 M sucrose.
d. After 96 hr incubation with $3 \times 10^{-4}$ M nickel and 0.7 M sucrose.
e. After 144 hr incubation with $3 \times 10^{-4}$ M nickel and 0.7 M sucrose.
Cationic alterations in the effect of sucrose on morphology. The synthetic sea water was reduced to 10% in the basal medium and the sucrose brought to 0.5 M. Magnesium was added to the medium at increasing concentrations. Other cations, calcium, potassium and sodium were added at increasing molar concentrations as referenced in Table 14. Incubation was at ambient temperature and photomicrographs by phase contrast.

a. Magnesium at $1 \times 10^{-1}$ M.
b. Magnesium at $1 \times 10^{-2}$ M.
c. Magnesium at $1 \times 10^{-3}$ M.
d. Medium without the added cation.
Fig. 37  Inhibitors. The inhibitor was added to the basal medium and incubated at ambient temperature. Photomicrographs by phase contrast.
a. Hydroxyurea at 50 mg/ml after 144 hr incubation.
b. Caffeine at 4 mg/ml after 96 hr incubation.
c. D-methionine at 2 mg/ml after 48 hr incubation.
d. Cycloserine at 500 μg/ml after 48 hr incubation.
L-Asparagine and D-methionine effect on morphology. The two amino acids L-asparagine and D-methionine were added to the synthetic medium at 5 μg/ml together and singly with increasing molar concentrations of nickel. Incubation was at ambient temperature. Photomicrographs by phase contrast.

a. L-asparagine, 5 μg/ml; nickel $3 \times 10^{-5}$ M; incubation 36 hr.
b. D-methionine, 5 μg/ml; no added nickel; incubation 120 hr.
c. L-asparagine and D-methionine, each at 5 μg/ml, nickel $8 \times 10^{-5}$ M, incubation 120 hr.


