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Title: ION AND SALINITY EFFECTS ON INDUCTION AND FUNCTION OF GALACTOSE PERMEASE IN AN ANTARCTIC PSYCHROPHILIC MARINE BACTERIUM

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A number of Antarctic marine psychrophilic bacteria were isolated having the ability to grow and accumulate ^{14}C-TMG only at temperatures below 20 C. It was shown that galactose rather than lactose was the primary inducer and utilizable substrate for the permease system. Further studies with a Vibrio species (Ant-12), have indicated that there was only one inducible galactose uptake system which differs in inducer and substrate specificity from those found in Escherichia coli. The initial rate of ^{14}C-TMG uptake exhibited saturation kinetics when measured as a function of increasing external ^{14}C-TMG concentrations. A reciprocal plot of these data yielded an apparent Km value of 4.8×10^{-6} M. Phosphorylation was not found to be a prerequisite for uptake and accumulation of ^{14}C-TMG. An exogenous source of energy was required for accumulation

of ^{14}C-TMG, while an exogenous precursor supply of an amino acid, in addition to the inducer, was essential for induction. Electron transport and general metabolic inhibitor studies gave further support that uptake was energy dependent.

It was found that a specific Na^+ requirement existed for uptake and that this requirement varied quantitatively with the substrate being transported. A relatively higher specific Na^+ requirement was also evident for induction. Furthermore, Ant-12 was shown to have a specific K^+ requirement and that the levels of K^+ required for uptake, growth and induction were the same.

At suboptimal salinities, uptake, growth and induction were inhibited more by the generally low nonspecific solute concentration than by a specific ion deficiency. The effect of the nonspecific solute may not be totally osmotic since the solute requirement varied both quantitatively and qualitatively with cellular function. A higher nonspecific solute requirement was found for induction than for growth or uptake, the one least requiring the additional solute. In accordance, increasing salinities supported in the following order: induction > growth > uptake.

Salinity-temperature and salinity-nutrient interaction studies have shown salinity to be the primary factor in controlling the pattern of cellular function. Only at near maximum growth temperatures and suboptimal growth salinities was the maximum temperature for growth and induction found to increase with increasing salinities.

Ion and Salinity Effects on Induction and Function of Galactose Permease in an Antarctic Psychrophilic Marine Bacterium

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INTRODUCTION

The inducibility of enzyme systems in the marine environment is of paramount importance since the sea is inundated with various substrates from freshwater runoff from land. In order to more definitively understand the effect of salinity and temperature on an inducible system, it was suggested that a psychrophilic marine bacterium having an inducible lactose permease be studied. Literature concerning the lactose operon in Escherichia coli would then serve as the necessary background data.

An inducible permease system would be of interest since salinity and temperature effects on induction could be examined with respect to alteration in transport activity. It has been hypothesized that the increased thermal resistance of bacteria at higher salinities was due to the stabilization of proteins by added salts (37, 78). This study could help clarify whether membrane or intracellular proteins are being primarily stabilized.

In addition to salt concentration effects, specific ion requirements have been demonstrated in marine organisms (57). Studies were undertaken to determine both the type and amount of salt required for an inducible uptake system. This investigation should prove

useful in interpreting the effect of salinity on induction at various temperatures.

REVIEW OF LITERATURE

Transport Systems for Galactose and Galactosides in Escherichia coli

The bacterium Escherichia coli has been the organism of choice in nearly all basic transport studies. The elucidation of the galactose transport systems and their mechanism of operation has proved no exception. Genetic and biochemical studies have shown E. coli K12 to have five transport systems for galactose and galactosides (15, 30, 64, 69). These systems may be distinguished by various substrate and inducer specificities, although they have many in common (69). One system, where lactose provides a specific assay, has proven to be identical to the lactose operon (7, 51, 64). Another system is induced specifically by galactinol, transports methyl-1-thio-β-D-galactoside but not lactose, and is not active in cells grown at 37 C (61, 74). The third system transports and is induced specifically by fucose, does not accumulate methyl-1-thio-β-D-galactoside, and possesses binding proteins having a dissociation constant of 10^{-6} M for galactose (1, 3, 4, 68). Another transport system specifically accumulates D-galactose (6, 22), while the fifth system is mediated by the phosphoenolpyruvate-dependent phospho-transferase system (35, 36, 67).

Mechanisms of Active Transport

Early investigations have suggested that active transport in bacteria was obligately coupled to phosphate bond energy (70, 71). A number of findings seem to be consistent with this view, including Kepes (29) finding of a 1:1 stoichiometric relationship between the amount of oxygen required to transport a molecule of galactose into E. coli to that required to synthesize one ATP molecule. Furthermore, it is well established that a number of active transport systems are sensitive to inhibitors of oxidative phosphorylation (33, 64). The importance of Mg^{+2}, Ca^{+2}-ATPase in the use of ATP for anaerobic proline accumulation has also been implicated in ATPase inhibitor (32) and ATPase deficient mutant studies (72).

More recent evidence suggests that active transport by bacteria may likewise be driven oxidatively without being directly influenced by phosphorylation. Kaback and coworkers (26, 31, 62) have shown that oxidative phosphorylation was not required for active transport of a variety of amino acids and sugars by membrane vesicles of E. coli. Transport by the membrane vesicles was coupled primarily to a flavin-linked membrane-bound D-lactate dehydrogenase. Intact cell studies using E. coli have further suggested that aerobic accumulation of amino acids and galactosides are not prevented by low intracellular levels of ATP (32) or mutants deficient in Mg^{+2}, Ca^{+2}-ATPase (72, 76).

It seems clear that energy for active transport can be derived independently from either respiration or ATP hydrolysis. The observation that the same transport system can be driven by both energy pathways further suggests a common coupling point to transport (2, 32). An energized membrane state has been proposed to be coupled to transport processes since uncoupling of oxidative phosphorylation can inhibit uptake by either pathway (32, 52). The hypothesis that emerges is that respiration or ATP hydrolysis can activate the membrane, and this high energy membrane is the immediate donor for bacterial transport (28, 32, 52, 72, 76). Mitchell (49) has proposed that the energized membrane is an electrochemical potential created by the extrusion of protons during respiration or ATP hydrolysis. This concept is supported by the finding that the rate of lactose inflow correlates with the simultaneous influx of protons (86, 87).

Salt Requirement for Maintenance of Cellular Integrity

A requirement for the isolation of marine bacteria is the use of seawater or various salts in the medium. This is not surprising in light of the fact that marine bacteria are generally found to lyse in distilled water or in solutions hypotonic to seawater (10, 11, 41, 59). In the past, it had been assumed that the protection afforded marine bacteria by sea salts could be attributed to the maintenance of the

correct osmolarity. However, evidence now indicates that the effect of salts in preventing lysis may be more ionic in nature than osmotic (45, 46, 79). The effectiveness of the salt is dependent on both the organism and ions present. In general, divalent cations have been found to be more effective in preventing lysis of marine bacteria than monovalent cations. Mg^{+2} is several times more effective in preventing lysis of marine bacteria than Na^{+} or K^{+}, the least effective (10, 34, 41, 83). In some cases, a monovalent cation can not maintain cell integrity without the addition of a divalent cation (34). On the other hand, divalent cations alone, some in very low concentrations, are able to maintain cell structure. The effectiveness is in the order $Cu^{+2} > Zn^{+2} > Ni^{+2} > Ca^{+2} > Mn^{+2} > Mg^{+2}$ (34).

Much of the influence of Mg^{+2} in preventing cell lysis has been attributed to its ability to maintain the stability of the cell envelope (10), particularly the cytoplasmic membrane (9, 73). It has been suggested that Mg^{+2} bridges occur in the cell membrane between anionic groups as phosphoryl head groups of phospholipids and carboxyl groups associated with amino acids. The effectiveness of any given ion is attributed to its polarizing power to adequately shield the mutual repulsion between these free negative charges (10, 11).

Salt Requirement for Transport of Substrates into Bacteria

Although Mg^{+2} is important for maintaining cell stability, other ions are required for cellular activity. A requirement for both Na^{+} and K^{+} for active transport has been found for a marine (40) and terrestrial bacterium (18). MacLeod and coworkers (12, 13, 14, 40, 82, 88) have shown a specific Na^{+} and K^{+} requirement for active transport of α-aminoisobutyric acid-C^{14} (^{14}C-AIB) in their marine pseudomonad. Na^{+} and K^{+} participate in active transport at the cytoplasmic membrane rather than the cell wall as determined from comparative studies of whole cells, mureinoplasts and protoplasts (12). Thompson and MacLeod (82), proposed that active transport of ^{14}C-AIB is apparently mediated by two distinct cation dependent steps. The initial step involves the entry and equilibration of the substrate into the cells by a Na^{+}-dependent facilitated diffusion mechanism. The second stage, which is dependent on intracellular K^{+}, brings about the accumulation of ^{14}C-AIB against a concentration gradient. Evidence that Na^{+}, rather than K^{+}, may play the more direct role in transport is indicated by the observation that only the quantitative Na^{+} requirement for uptake varies with the substrate being transported. Three to four times more Na^{+} was found to be required for the transport and oxidation of dicarboxylic acids than for aliphatic monocarboxylic acids or sugars (13, 14, 40). These

authors concluded that the difference in the Na^+ requirement could be accounted for by assuming that a ternary sodium-sugar or amino acid-carrier complex was being formed (40). Formation of such a complex could account for the difference in Na^+ requirement for transport of different substrates, since the amount of Na^+ required for the formation of the ternary complex would vary with the molecular composition of the substrate and carrier.

A second function of Na^+ that indirectly relates to its role in Na^+ dependent transport is the capacity to control the porosity of the cytoplasmic membrane (12, 81). In this role, Na^+, which can be replaced at least partially by other ions, prevents the release of compounds accumulated by active transport (88).

Ionic Requirement for Growth of Marine Bacteria

The requirement for seawater upon initial isolation has been used to differentiate marine from terrestrial bacteria (90). Zobell (89) studied the salt dependency of the bacterial flora from seawater and showed that the maximum viable counts were obtained at salt concentrations corresponding to seawater. In contrast, the highest bacterial number from terrestrial polluted bay sediment occurred in media containing seawater diluted 10-20% with distilled water (89). Hidaka and coworkers (19, 20, 21), correspondingly have reported that a majority of their marine bacteria had salinity optima in the

5-7% range whereas most terrestrial strains had an optima near 0.5%. These percentages are somewhat in error since these authors failed to account for the salt present in media constituents. Nevertheless, their results do suggest that marine bacteria may be differentiated from terrestrial or halophilic bacteria according to salinity requirements (19).

The need for seawater in a chemically defined medium has been shown to be due to the ability of seawater to supply the kinds and amounts of inorganic ions required for growth by organisms (42). Most of the requirements for ions by marine bacteria are similar to that required by all bacteria. However, there are two unusual features of the qualitative mineral requirement of marine bacteria which need mention. First, the need for Na^+ by these forms appears to be almost unique among bacteria so far investigated (42, 43, 44). Secondly a few marine bacteria have been found to require Cl^- for growth or optimum rate of growth (42). It would seem that true marine bacteria may be distinguished from land forms not by having a seawater requirement but rather that they have a readily detectable NaCl need for growth.

The requirement for Na^+ appears to be highly specific, although in some organisms it can be partially replaced by other ions (43, 56, 57, 58, 60, 80). Pratt (56) has indicated that KCl or sucrose replaced a substantial portion of the Na^+ growth requirement in

approximately one-half of his isolates. Variations in the concentration of Na^+ required to satisfy the specific requirement was said to account for the differences in sparing effect of the nonspecific solute (58, 60, 80).

Ion Requirements for Induction

Substrate induction is known to be affected by salt. The concentration of NaCl was found to directly affect the rate of induction of glutamic dehydrogenase in a marine vibrio (77). Furthermore, both NaCl and KCl were found by Payne (53, 54) to be indispensable to Vibrio natriegens for induction and activity of enzymes responsible for the oxidation of glucuronate and galacturonic acid. It was further indicated that Na^+ was needed for the induction of the substrate transport and oxidative mechanisms, while the action of K^+ was restricted to oxidative activity (53, 54). Induction of resting cells of V. natriegens to oxidize L-arabinose and mannitol was similarly found to be dependent on the presence of Na^+ with normal seawater levels of Mg^{+2} and K^+ in the suspending medium (63). Extension of this study has indicated that both the synthesis of mannitol binding protein and net synthesis of cellular protein require Na^+ (85). MacLeod (39), in contrast, has suggested that induction of the permease system may depend on transport of the substrate by basal level permease which would require Na^+. Thus, induction would only seem to depend on

Na^+, but in reality only transport would be Na^+ dependent. Nevertheless, Payne's argument is strengthened by the observation that a high concentration of K^+ (no Mg^{+2} or Na^+) can facilitate entry of mannitol but not the synthesis of protein (63, 85). More labeled mannitol was found to enter the cells when incubated with 0.3 M K^+ (no Mg^{+2} or Na^+) than cells suspended with Na^+ (no Mg^{+2} or K^+). Only under the latter condition, however, did induction of the penetration and oxidation mechanism occur since such cells took up and oxidized mannitol linearly when they were incubated with labeled mannitol, Na^+, K^+, and chloramphenicol.

Interaction of Salinity and Temperature on Growth and Cellular Function

The growth behavior of bacteria has been shown to be affected by the interaction of the salt concentration and the environmental temperature. Generally, the maximum and/or optimum growth temperature of bacteria have been found to be significantly increased with increasing NaCl concentration. A number of coliforms (37), lactic acid bacteria (17), halophiles (16, 23, 24), fungi (65, 66), and marine bacteria (25, 78) all have demonstrated this phenomenon. The effect of salt on the maximal growth temperature of a marine vibrio was shown to be due to the presence of cations (78). The order of effectiveness of cations on restoring the maximal growth temperature when

added to dilute seawater was $Na^{+} > Li^{+} > Mg^{+2} > K^{+} > Rb^{+} > NH_4^{+}$. Only SO_4^{-2}, of the various anions tested, showed any effect on the growth temperature response. It has been hypothesized that the increased thermal resistance of bacteria was due to the stabilization of proteins by added NaCl (37, 78).

To help clarify such variations in the maximum growth temperature of microorganisms, other cellular functions have been investigated. Cooper and Morita (8) have presented data that definitely establishes a relationship between temperature and salinity in protein and RNA synthesis. They found that protein synthesis in a marine vibrio was significantly inhibited at 22 C at a salinity of 25‰ but not at a salinity of 35‰ until the cells were incubated for 20 minutes at 24 C. Loss of viability was shown to occur simultaneously with the thermal inhibition of protein synthesis. In contrast, under these same circumstances, total RNA synthesis and proline uptake continued unabated after protein synthesis ceased. It was concluded that although the critical temperature of the lesion of protein synthesis increased with increasing salinity, loss of cell viability was due to the thermal inhibition of protein synthesis at the translational level.

In another study, Ishida and coworkers (25) have suggested that the influence of temperature on the minimum and optimum salinities for growth could be best explained in terms of alteration in transport activities. It was found that the temperature at which the cells

were grown controlled the minimum salinity for substrate uptake, rather than the temperature at which uptake was observed.

MATERIALS AND METHODS

Isolation of the Bacterium

Samples for lactose enrichment cultures were obtained by either filtering two liters of seawater sample through a Millipore filter (0.45 μ) or obtaining approximately 25-50 g (wet weight) of sediment. Filters were placed into 125 ml sterilized flasks containing 100 ml of seawater, while sediment samples were added to 500 ml sterilized flasks containing 200 ml of seawater. Filter sterilized solutions of lactose and Bacto-yeast extract were added to these flasks to give a final concentration of 5×10^{-3} g/ml and 1×10^{-5} g/ml respectively. After 2-3 weeks of incubation at 3 C, samples were streaked out onto lactose medium plates containing the following: 2 g Bacto-peptone, 1 g Bacto-yeast extract, 5 g lactose 2×10^{-2} g brom thymol blue, 32 g Rila Salt Mix (Rila Products Co., Teaneck, N. J.), 15 g agar, and distilled water (1000 ml). The medium was adjusted to pH 8.0 with 5.0 N HCl and sterilized by autoclaving for 15 minutes at 2 atmospheres. Colonies which changed the color of the surrounding medium from blue to yellow were picked after 1-2 weeks incubation at 3 C and restreaked twice for purification. Cultures were further selected for their ability to grow only at temperatures below 20 C and to accumulate β-methyl-D-thiogalactoside [β-methyl-^{14}C] (^{14}C-TMG).

Organism and Maintenance Conditions

A psychrophilic marine bacterium, isolated from Antarctic waters (USNS Eltanin, Cruise 51) was used in this work. This organism which is referred to as Ant-12 was obtained from a core sample at a depth of 497 m (Station 7, Lat: 71° 34.68' S, Long: 171° 44.98' E).

Ant-12 was subcultured monthly and maintained on 3.2% glycerol artificial seawater (GAS) slants at 5 C. A 3.2% GAS medium was prepared by mixing 750 ml of solution A containing 20.8 g NaCl, 0.56 g KCl, 4.8 g $MgSO_4$, 4.0 g $MgCl_2 \cdot 6H_2O$, 0.008 g K_2HPO_4, 0.0008 g $FeSO_4 \cdot 7H_2O$; 240 ml of solution B containing 2.0 g Bacto-peptone, 1.0 g Bacto-yeast extract, 2 ml glycerol, 2.0 g Rila Salt Mix; and 10 ml of solution C containing 0.484 g Tris. Agar was added when required at a concentration of 15 g per liter. The medium was adjusted to pH 8.0 with 5.0 N HCl and sterilized by autoclaving. Various salt concentrations of GAS were prepared by proportionally increasing or decreasing the salt concentration in solution A.

Growth and Preparation of Cells

The inoculum, cells grown in 3.2% GAS medium for 72 hours, was added at 1% (v/v) to 500 ml Erylenmeyer flasks containing 3.2% GAS medium or 3.2% GAS + 0.5% galactose (GGAS) in order to obtain

noninduced or induced cells respectively. All flasks were shaken at 5 C in a Psychrotherm Incubator Shaker (New Brunswick) for 36 hours.

Cells were harvested by centrifugation at 9150 x g for 5 minutes at 0 C in a RC2-B Sorvall refrigerated centrifuge. The cells were then washed three times in either 3.2% ASW or magnesium buffer. ASW consisted of the following: 750 ml of solution A, 10 ml of solution C, and 240 ml of distilled water. Various concentrations of ASW were prepared by proportionally increasing or decreasing the salt concentration in solution A. Magnesium buffer contained 6.0 g $MgSO_4$, 5.0 g $MgCl_2 \cdot 6H_2O$, 0.484 g Tris, and distilled water (1000 ml). Both ASW and magnesium buffer solutions were adjusted to pH 8.0 with 5 N HCl. The cell suspension had an $OD_{600} = 0.1$ using 3.2% ASW as a reference on a Bausch and Lomb Spectronic 20 Colorimeter. All subsequent OD_{600} measurements are made with reference to 3.2% ASW.

Chemicals

The following inorganic (analytical or reagent grades) and organic compounds were used: β-methyl-D-thiogalactoside [β-methyl-^{14}C] and U-^{14}C-L-glutamic acid (^{14}C-Glut) (New England Nuclear Corp., Boston, Mass.); D-glucose, L-glutamic acid, D-lactose, D-mannose, iodoacetic acid, and NaCN (J. T. Baker

Chemical Co., Phillipsburg, N. J.); D-fucose, isopropyl-β-D-thiogalactoside (IPTG), β-methyl-D-galactoside (MG), methyl-β-D-thiogalactoside (TMG), and Ouabain (Sigma Chemical Co., St. Louis, Mo.); D-galactose and chloramphenicol (CAP) (Mann Research Lab., N. Y., N. Y.); NaF (Baker and Adamson, N. Y., N. Y.); p-chloromercuribenzoate (PCMB)(Calbiochem, L. A., Calif.); 2, 4-dinitrophenol (DNP) (Eastman Kodak Co., Rochester, N. Y.); NaN_3 (Matheson Coleman and Bell, Cinn., Ohio).

^{14}C-TMG Uptake

Ten ml of induced cells having an OD_{600} = 0. 1 were centrifuged and resuspended in 0. 9 ml of 3. 2% ASW containing 3. 42 x 10^{-2} M glycerol. The experiment was initiated by adding 0. 1 ml of 27. 8 μg/ml ^{14}C-TMG (2 μCi/ml). With the exception of the temperature and salinity interaction studies on uptake, all ^{14}C-TMG uptake assays were performed at 0 C. Tenth ml portions were filtered after 5 minutes through a Millipore filter apparatus fitted with a 0. 45 μ Millipore membrane filter. A negative pressure of 103 mm of Hg were used to facilitate filtration. Membrane filters were rinsed with two 5 ml portions of 3. 2% ASW, dried in an oven at 70 C for 10 minutes and placed in scintillation vials for counting.

The ^{14}C-TMG uptake system was characterized by kinetic, competitive inhibitor and metabolic inhibitor studies. Unless

otherwise stated, cells grown in GGAS medium were utilized. Assay for ^{14}C-TMG uptake was performed as already described with the exception that various metabolic inhibitors, competitive inhibitors or energy sources were added to the assay solution.

^{14}C-Glutamic Acid Uptake

Assay of ^{14}C-Glut uptake followed the same procedure as the ^{14}C-TMG uptake assay except that 0.1 ml of 3.96 μg/ml ^{14}C-Glut (2 μCi/ml) was used.

Radioactive Determination

Five ml portions of Omnifluor (New England Nuclear) was added to the scintillation vials containing the dried filters. Radioactivity was measured in a liquid scintillation spectrometer. The counts were made for 4-10 minutes giving total counts in the range 10^3-10^4 cpm. The average background counts were always less than 20 cpm. The optimum counting setting was used to give the maximum counts on the main channel. The channel-ratio method was utilized to correct the counting rate to the disintegration rate of a given sample. A quench correction curve was prepared using a toluene and chloroform technique as described by Wang and Willis (84).

Radiochromatography of Accumulated ^{14}C-TMG

Radiochromatography studies were performed with galactose induced cells which were washed and resuspended in 3.2% ASW. The ^{14}C-TMG assay was scaled up to 6.0 ml in order that four 0.2 ml samples could be filtered at intervals. All four filters were placed in separate scintillation vials. Two of these samples were immediately prepared for scintillation counting. The other two samples were treated with 0.5 ml of boiling water and then frozen. Frozen samples were later thawed and spotted on Whatman #1 chromatography paper using a 10 µl pipette. Chromatograms were run in the ascending direction for 10 cm in a 2-propanol:water (3:1 v/v) solvent system (27). One centimeter strips were cut and placed in fluor for counting. The solvent system gave an Rf value of approximately 0.5 and 0.1 for ^{14}C-TMG and phosphorylated forms of galactoside respectively. Possible phosphorylation of ^{14}C-TMG was further determined by mixing 0.1 ml of thawed sample with 0.1 ml of either acid or alkaline phosphatase. Stock solutions of acid phosphatase (Calbiochem) contained 1.0 mg in 0.15 M sodium acetate, pH 5.0 (48); while alkaline phosphatase (Mann Research Laboratories) contained 1.0 mg in 0.2 M Tris-HCl buffer + 0.014 M $MgCl_2 \cdot 6H_2O$, pH 8.0 (48). Samples were then rechromatographed and compared with the nonphosphatase treated sample.

Induction of Resting Cells

Cells grown in 3.2% GAS medium were centrifuged and washed three times in 3.2% ASW or magnesium buffer. The cells were resuspended in various salt solutions at an OD_{600} = 0.1. After one hour of incubation at the experimental temperature, peptone or glutamic acid (adjusted with Tris to pH 8.0) was added at a concentration of 1.5×10^{-4} g/ml and 9×10^{-4} M respectively. This peptone and glutamic acid concentration gave a maximum rate of induction without growth within the time period of the experiment. Induction was then initiated by the addition of 1×10^{-3} M fucose. Two 10 ml samples were removed with time after further incubation at experimental conditions. One sample was centrifuged, washed once, and assayed for ^{14}C-TMG uptake using 3.2% ASW as previously described. The other sample was centrifuged and washed three times in 3.2% ASW before resuspending in 2 ml of 1×10^{-2} M potassium buffer for protein determination.

Other inducers and energy sources were substituted for fucose and glutamic acid respectively in order that the induction system could be further characterized. Resuspension and cell washes were done in 3.2% ASW.

Protein Determination

Cell-free extracts were held on ice and treated with an ultrasonic cell disrupter (Bronwill Scientific, Rochester, N. Y.). The extract was centrifuged for 5 minutes at 13, 200 x g and decanted for protein determination. Soluble protein was estimated by the Lowry method (38) employing bovine serum albumin as a standard.

Ionic Requirement Studies for ^{14}C-TMG Uptake

Na^+ and K^+ ion requirements were investigated with GGAS medium grown cells centrifuged and washed three times with magnesium buffer. The magnesium ion requirement was determined with cells washed three times in NaCl buffer consisting of 26. 0 g NaCl, 0. 484 g Tris-HCl (pH 8. 0), and distilled water (1000 ml). Twenty ml of cell suspension having an OD_{600} = 0. 2 were then centrifuged and resuspended in 40 ml of basal ion solution (BIS) varying in Na^+, K^+, or Mg^{+2} concentration. A complete BIS solution contains the following: 26. 0 g NaCl, 6. 0 g $MgSO_4$, 5. 0 g $MgCl_2 \cdot 6H_2O$, 0. 7 g KCl, 0. 484 g Tris-HCl (pH 8. 0), and distilled water (1000 ml). After incubation at 5 C for 2. 5 hours, 10 ml samples were removed, centrifuged and resuspended in 0. 9 ml of the same ionic solution as previously incubated, with the addition of 3. 42 x 10^{-2} M glycerol. Assay of ^{14}C-TMG uptake was initiated by adding 0. 1 ml of 27. 8 μg/ml ^{14}C-TMG (2 μCi/ml).

Ionic Requirement Studies for Growth

Determination of the quantitative Na^+ and K^+ requirement for growth of the bacterium was made by inoculating the organism in minimal medium of varying Na^+ or K^+ concentrations. Minimal medium was composed of the following: 3.0 g casein, 2 ml glycerol, 6.0 g $MgSO_4$, 5.0 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$, 0.01 g K_2HPO_4 (for the Na^+ requirement experiment) or 0.008 g Na_2HPO_4 (for the K^+ requirement experiment) 0.001 g $FeSO_4 \cdot 7H_2O$, 0.484 g Tris-HCl (pH 8.0), 26.0 g NaCl, 0.7 g KCl, and distilled water (1000 ml). Forty ml portions of minimal medium in 250 ml side-arm flasks were inoculated with 0.1 ml of inoculum. All flasks were shaken at 5 C on a reciprocating shaker at 100 strokes/minute. Growth was estimated by following the change in optical density at 600 nm in a Spectronic 20 colorimeter.

Ionic Requirement Studies for Induction of ^{14}C-TMG Uptake

Induction requirements for Na^+ or K^+ and Mg^{+2} were ascertained with GAS medium grown cells centrifuged and washed three times with magnesium buffer or NaCl buffer respectively. Twenty ml of cell suspension having an $OD_{600} = 0.2$ was resuspended in 40 ml of BIS varying in Na^+, K^+, or Mg^{+2} concentrations. After incubation at 5 C for one hour, fucose and glutamic acid (adjusted with

Tris to pH 8.0) were added at a concentration of 1×10^{-3} M and 9×10^{-4} M respectively. Two 10 ml samples were removed with time and prepared for ^{14}C-TMG uptake assay and protein determination as already described.

Temperature-Salinity and Nutrient-Salinity Interaction on Uptake of ^{14}C-TMG

Cells grown in GGAS medium were centrifuged and washed three times with magnesium buffer. Ten ml samples having an $OD_{600} = 0.2$ were centrifuged and resuspended in 1.8 ml of various dilutions of ASW equilibrated at the experimental temperature. The cells were further incubated at the appropriate temperature for various times. Assay of ^{14}C-TMG uptake was performed by the addition of 0.1 ml of 0.342 M diluted glycerol and then 0.1 ml of 55.6 μg/ml ^{14}C-TMG (4 μCi/ml). Stock solutions of glycerol were prepared and equilibrated at the same salinity and temperature as the experiment. Samples of 0.1 ml cell suspension were removed at intervals and processed for scintillation counting as previously described.

For nutrient-salinity interaction studies, GGAS medium grown cells were centrifuged and washed three times with 3.2% ASW. The washed pellet was suspended in 3.2% ASW to an $OD_{600} = 0.2$ prior to incubation for 24 hours at 5 C. The cell suspension was then treated as in the above temperature-salinity study with the exception that

^{14}C-TMG assays were performed at 0 C using various concentrations of glycerol.

Temperature-Salinity Interaction on Growth

The determination of the effect of temperature and salinity on the growth of Ant-12 was made by inoculating the organism into various dilutions of GAS medium on a Temperature Gradient Incubator (Scientific Industries Inc., Mineola, N. Y.). Ten ml portions of GAS medium in tubes were inoculated with 0.05 ml of inoculum. All tubes were shaken at 60 strokes/minute. Optical density readings were taken at various intervals using a Spectronic 20 colorimeter (600 nm).

Temperature-Salinity and Nutrient-Salinity Interaction on Induction of ^{14}C-TMG Uptake

Cells grown in GAS medium were centrifuged and washed three times with magnesium buffer before resuspending to an $OD_{600} = 0.1$ in 40 ml of various concentrations of ASW. Cells were equilibrated at the experimental temperature and salinity for one hour before peptone and fucose were added to give final concentrations of 1.5×10^{-4} g/ml and 1×10^{-3} M respectively. At various intervals, two 10 ml samples were removed, centrifuged and washed for ^{14}C-TMG uptake assay and protein determination.

Nutrient-Salinity interaction experiments were treated as

indicated above with the exception that induction was performed at 5 C using various concentrations of peptone.

RESULTS

Lactose Enrichment Culture

Several Antarctic marine bacteria, including Ant-12, were isolated with the ability to grow only at temperatures below 20 C and utilize lactose. Induction studies on these organisms showed that galactose rather than lactose was the primary inducer for ^{14}C-TMG uptake. The natural substrate for this transport system in lactose induced cells also appeared to be galactose since the carrier possessed its greatest affinity for galactose and almost no affinity for lactose.

Characterization of Ant-12

The growth temperature range of Ant-12 was found to be from below 0 C to 16 C with a maximum growth rate at 8.5 C. A number of salts or seawater was also found to be essential for growth of this organism. Based on a classification scheme by Shewan (75) the isolate has been tentatively identified as being a member of the genus Vibrio.

Characterization of the Inducible ^{14}C-TMG Uptake System

Studies were carried out to characterize the ^{14}C-TMG uptake system of Ant-12. Induction studies (Table 1) demonstrated that

galactose and fucose were the primary inducers. Lactose produced a comparable induction only at higher concentrations, whereas glucose did not cause induction.

Table 1. Induction of ^{14}C-TMG uptake employing various inducers.

Inducers[a]	Concentration (M)	10^3 x DPM/mg protein
None	--	0.8
Fucose	1×10^{-3}	82.5
Galactose	1×10^{-3}	66.4
IPTG	1×10^{-3}	24.2
TMG	1×10^{-3}	18.8
Lactose	1×10^{-3}	0.9
	1.4×10^{-2}	56.5
Glucose	1×10^{-3}	1.0
	1×10^{-2}	0.7

[a]Inducers were added to give a final concentration of 10^{-2} M or 10^{-3} M to 12 hour 3.2% GAS medium grown cells. Cells were induced for 12 hours.

Competitive inhibition studies of ^{14}C-TMG uptake (Table 2) have also shown that galactose was the primary utilizable substrate. This was true for cells induced with one of a number of galactose derivatives (Table 2). Furthermore, these differently induced cells

showed the same pattern of competitive inhibition of ^{14}C-TMG uptake. Galactose, glucose, MG, and TMG all showed relatively higher percent inhibition for any given induced cell than did D-fucose, IPTG and lactose. Specificity for galactose and galactoside uptake was shown by the lack of competitive inhibition by glutamic acid and mannose.

Table 2. Competitive inhibition of ^{14}C-TMG uptake with non-radioactive compounds.

Substrate[a] Inhibitor	Galactose[b] Induced	Fucose[b] Induced	TMG[b] Induced	IPTG[b] Induced	Lactose[b] Induced
Control	0	0	0	0	0
D-Fucose	89	89	87	89	91
D-Galactose	98	98	97	97	97
D-Glucose	98	98	98	97	97
IPTG	93	92	93	88	88
Lactose	11	11	11	12	6
MG	98	98	97	94	96
TMG	98	97	96	93	96
Mannose	10	10	--	--	5
Glutamic Acid	0	0	--	--	0

[a]Substrate inhibitors and ^{14}C-TMG were used at concentrations of 13.85×10^{-4} M and 13.85×10^{-6} M respectively.

[b]Cells were induced at a concentration of 10^{-3} M.

The uptake system did not require phosphorylation as a prerequisite for uptake and accumulation. No phosphorylation was detected during the accumulation process, although free ^{14}C-TMG was found to be phosphorylated after accumulation by the cells was nearly completed (Figure 1). ^{14}C-TMG, however, was not found to be respired even after 6 hours of incubation.

For galactose induced cells, uptake of ^{14}C-TMG was linear for approximately 15 minutes followed by saturation after 30 minutes (Figure 2). The initial rate of ^{14}C-TMG uptake exhibited saturation kinetics when measured as a function of increasing external ^{14}C-TMG concentration (Figure 3). A reciprocal plot of this data yielded an apparent Km value of 4.8×10^{-6} M.

Cells in the exponential phase of growth were found to be initially capable of accumulating ^{14}C-TMG. Cells starved for 3 hours required an additional metabolizable substrate (Figure 4). Glycerol, casein, or glutamic acid permitted starved cells to regain their ability to accumulate ^{14}C-TMG against a concentration gradient.

Further support that uptake of ^{14}C-TMG is energy dependent has been shown by the use of certain electron and general metabolic inhibitors (Table 3). The best inhibitor was DNP, followed by NaN_3 and NaCN. Of the two mercaptide forming agents tested, PCMB prevented uptake whereas iodoacetate had no effect. NaF, a known inhibitor of the phosphotransferase system, did not inhibit uptake.

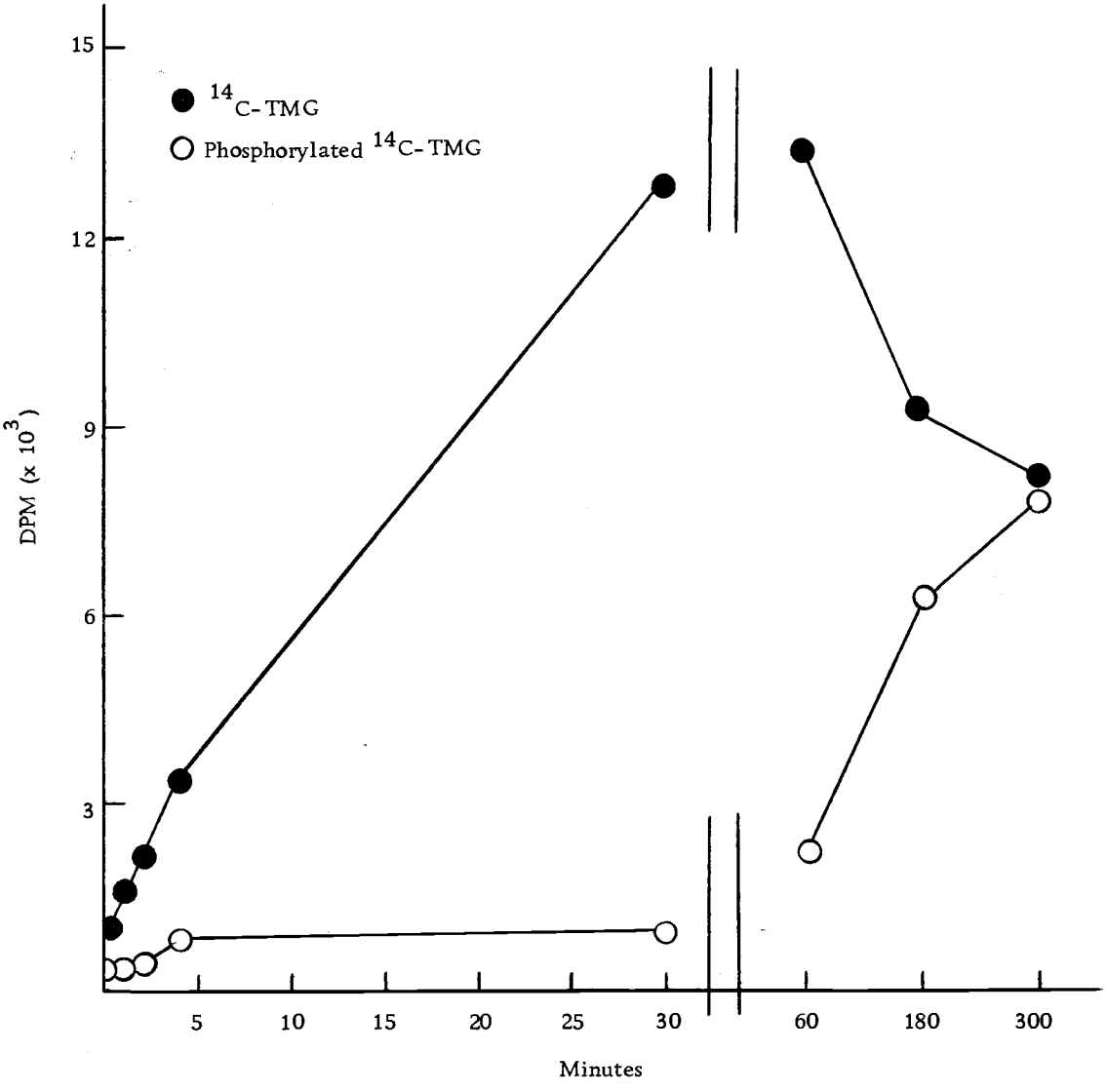

Figure 1. Time course of ^{14}C-TMG phosphorylation.

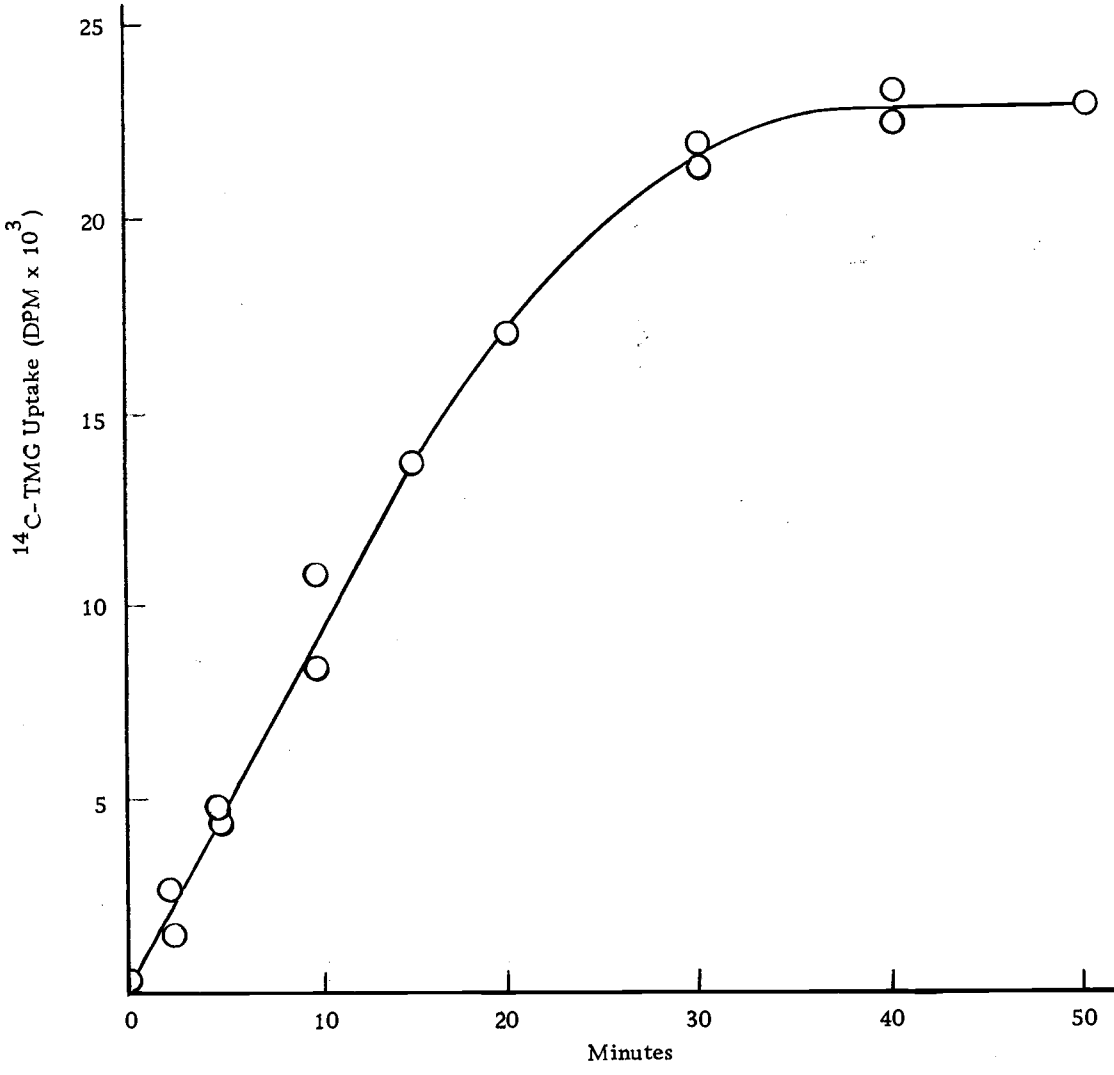

Figure 2. Uptake of ^{14}C-TMG with time.

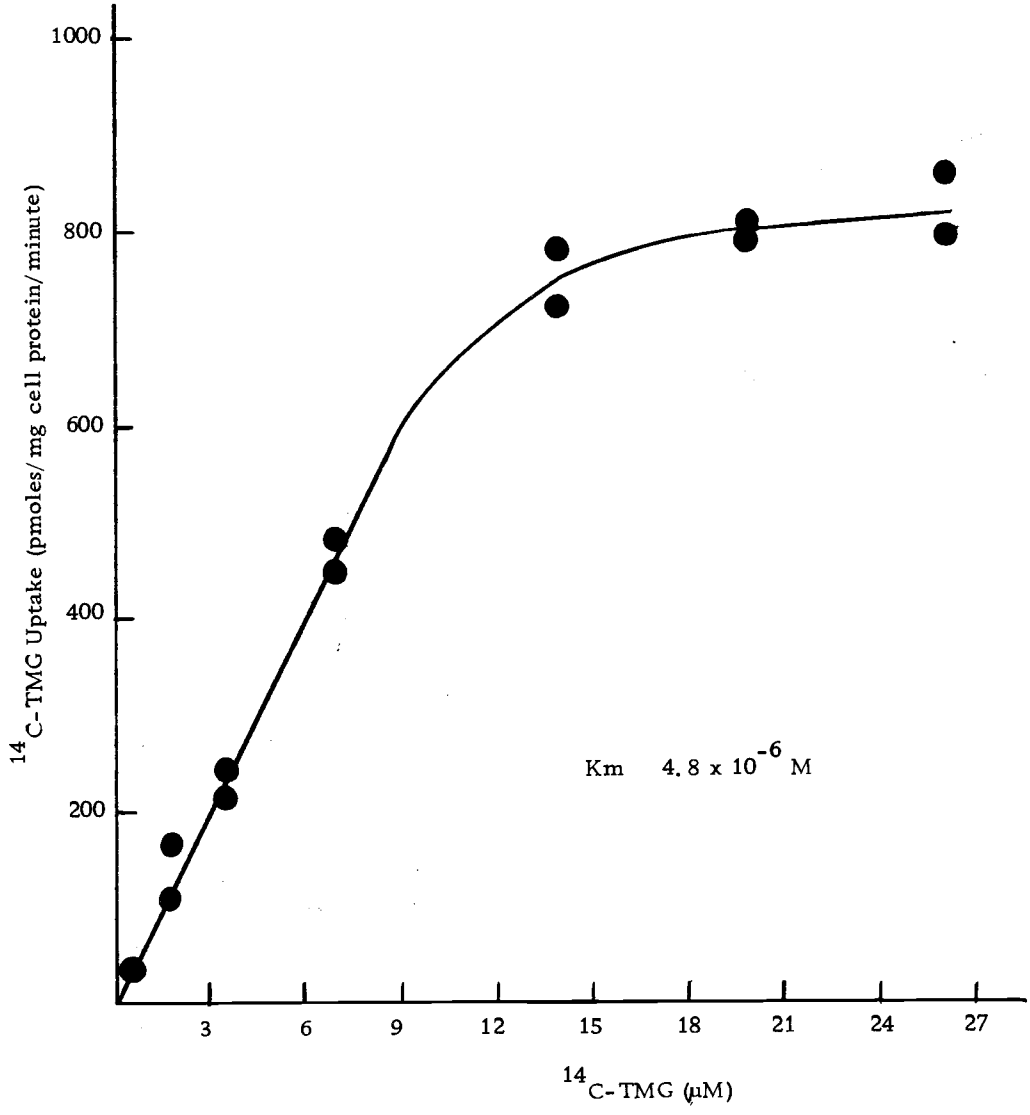

Figure 3. Kinetics of ^{14}C-TMG uptake with various concentrations of ^{14}C-TMG.

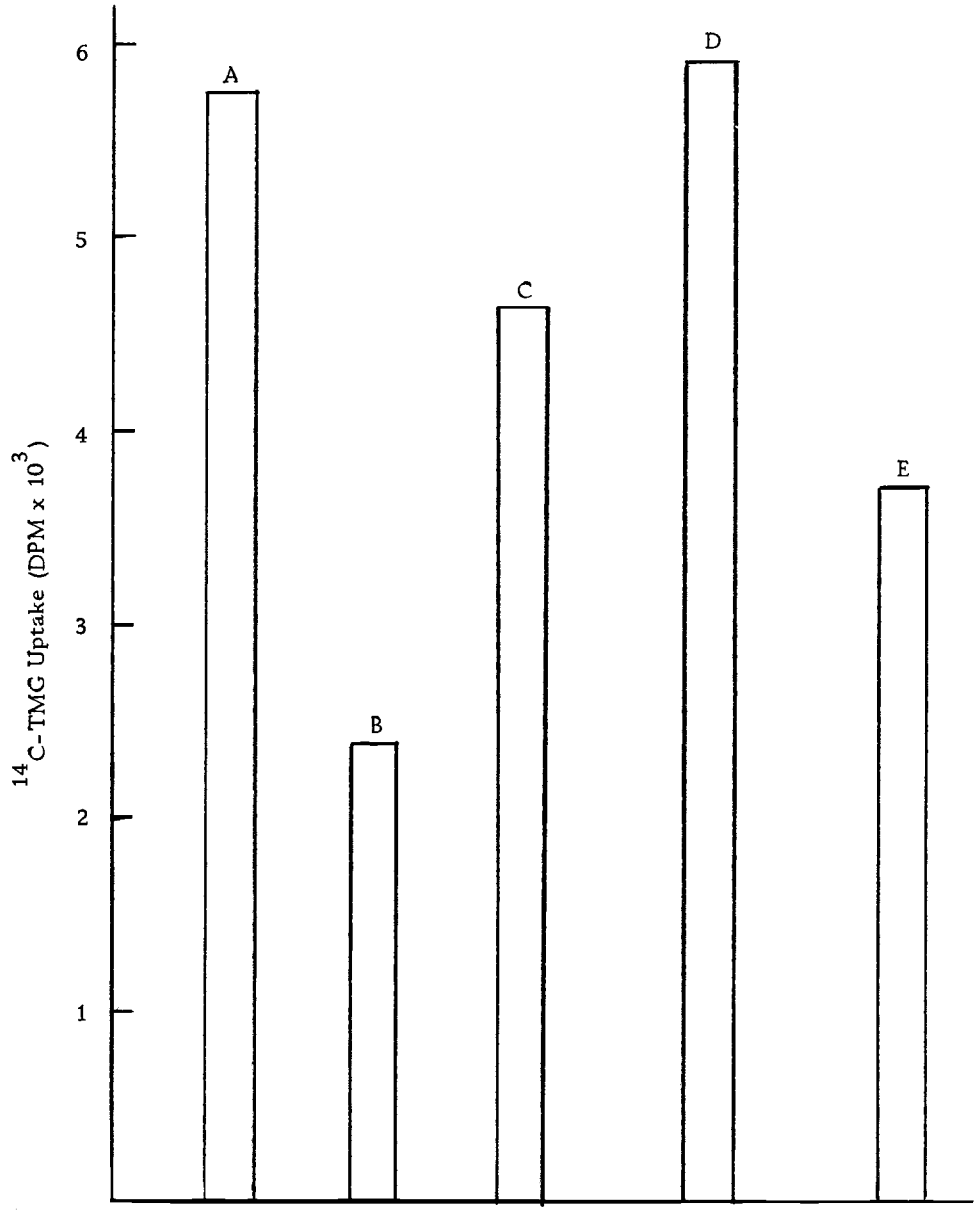

Figure 4. The requirement for an energy source for uptake of ^{14}C-TMG. Cells were assayed for ^{14}C-TMG uptake with or without an energy source after 0 hour and 3 hours of starvation. A = 0 hr (no energy source), B = 3 hrs (no energy source), C = 3 hrs (3.4 x 10^{-2} M glycerol), D = 3 hrs(1.5 x 10^{-3} g/ml Bacto-casein), E = 3 hrs (9 x 10^{-3} M glutamic acid).

This is in agreement with the TMG phosphorylation study. Ouabain, an inhibitor of Na^+ dependent transport in animal cells, similarly had no inhibitory effect on this bacterial uptake system.

Table 3. Effects of metabolic inhibitors on the uptake of ^{14}C-TMG.

Inhibitor	Concentration (M)	% Inhibition
NaCN	10^{-3}	42
	10^{-2}	69
NaN_3	10^{-3}	78
Iodoacetic Acid	10^{-3}	10
	10^{-2}	15
NaF	10^{-3}	0
	10^{-2}	10
DNP	10^{-3}	98
	10^{-4}	95
PCMB	10^{-3}	52
Ouabain	10^{-3}	12

An amino acid, in addition to the inducer, was required for induction of the ^{14}C-TMG uptake system (Figure 5). Glutamic acid alone was found to induce cells to the same extent as peptone or casamino acid, although the induction rate was one-half as fast (Figures 6, 7). Neither glycerol nor galactose was able to replace the amino acid requirement suggesting a possible precursor role for the amino acid.

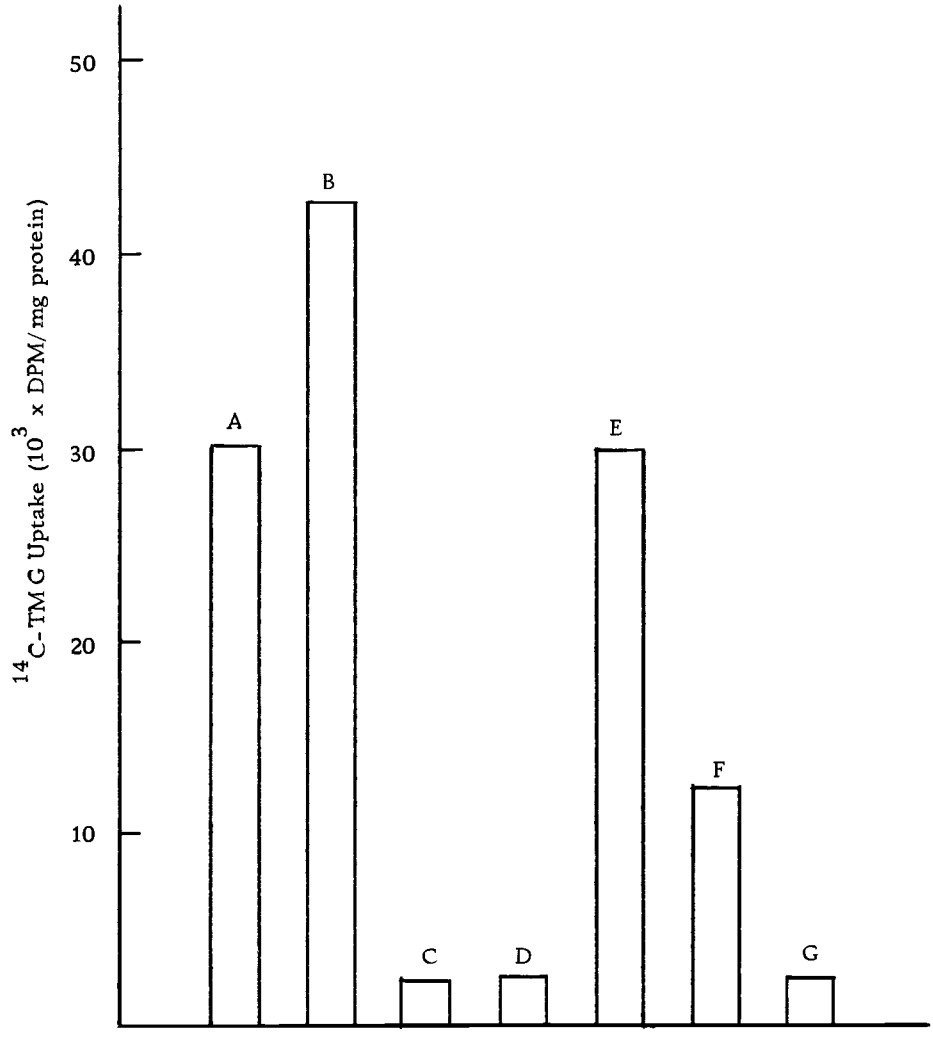

Figure 5. The effect of various substrates on induction of ^{14}C-TMG uptake. Cells were induced for 2 hours with fucose (10^{-3} M) and the following substrates: A = 1.5 x 10^{-4} g/ml peptone, B = 2.5 x 10^{-4} g/ml casamino acid, C = 2.7 x 10^{-2} M glycerol, D = 2.7 x 10^{-2} M galactose, E = 1.5 x 10^{-4} g/ml peptone + 2.7 x 10^{-2} M glycerol, F = 1.0 x 10^{-3} M glutamic acid, G = none.

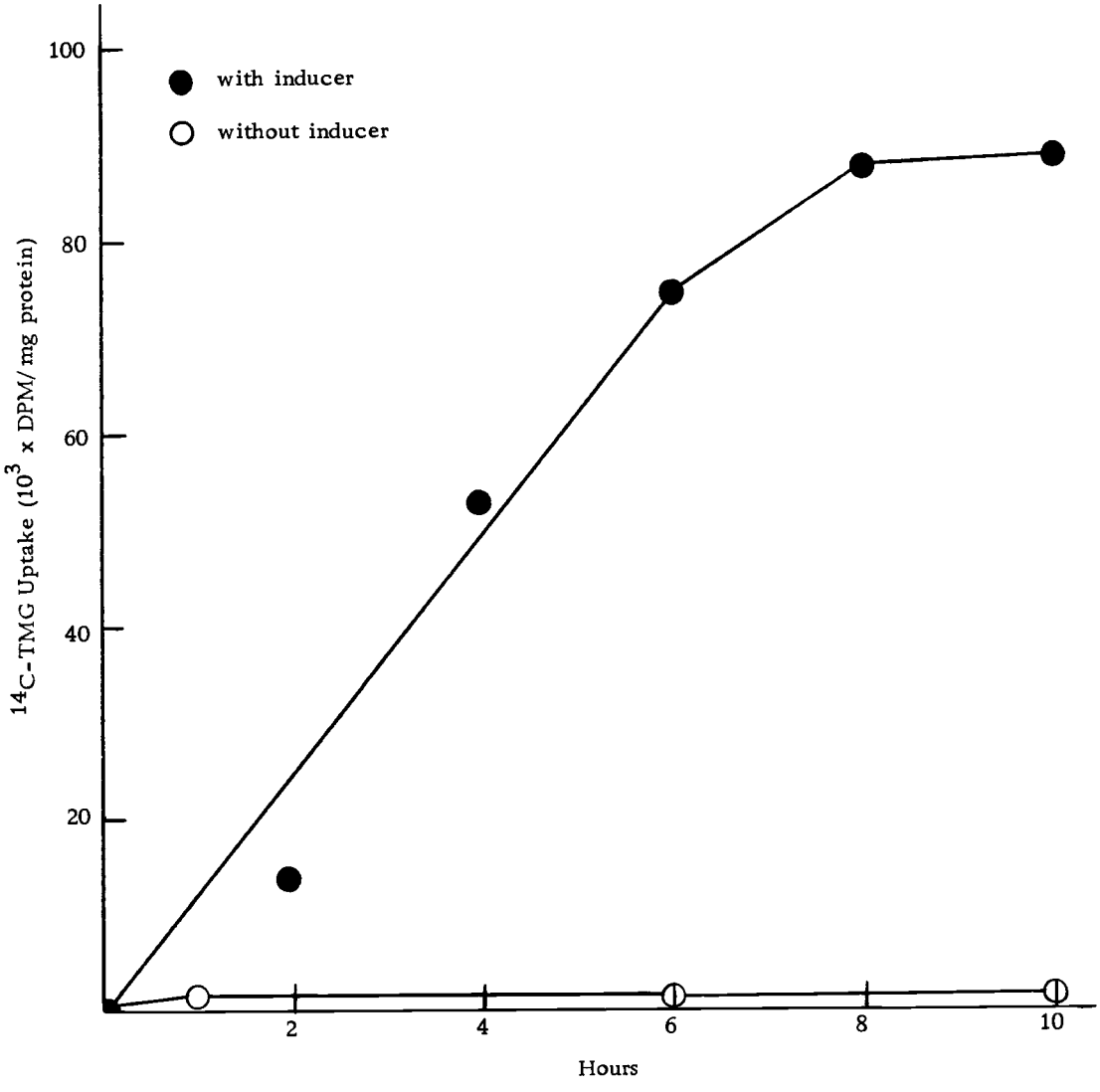

Figure 6. Induction of ^{14}C-TMG uptake using fucose (10^{-3} M) and glutamic acid (9×10^{-4} M) as the inducer and precursor source respectively.

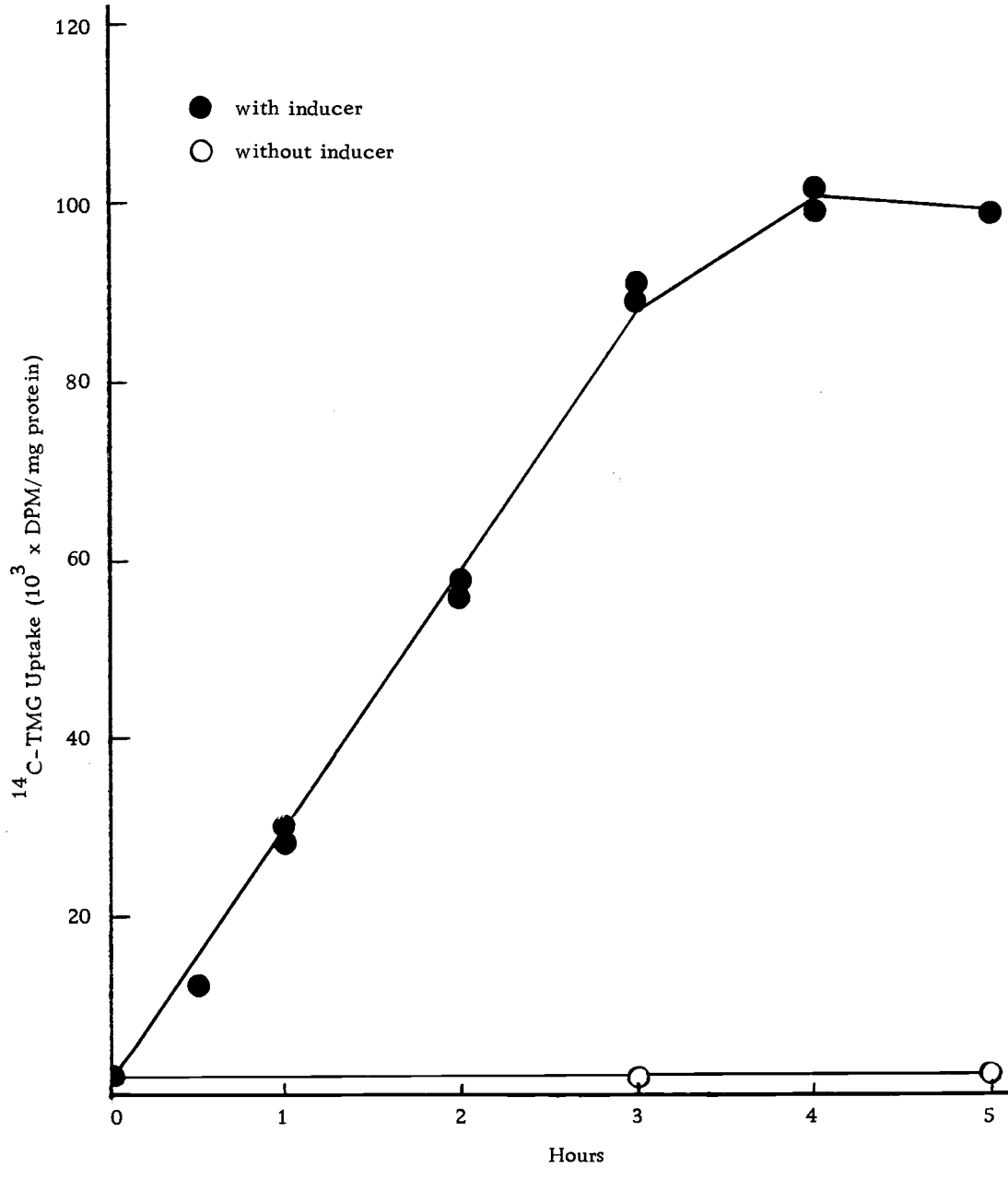

Figure 7. Induction of ^{14}C-TMG uptake using fucose (10^{-3} M) and Bacto-peptone (1.5×10^{-4} g/ml) as the inducer and precursor source respectively.

Induction of ^{14}C-TMG uptake was found to be inhibited by CAP (50 μg/ml). CAP, however, was not used further in induction studies since washing the cells once in ASW was sufficient to remove the inducer and thus prevent further induction.

Salinity Effects on Uptake, Growth, and Induction

Salinity studies have shown that Ant-12 has a salinity range for growth extending from approximately 1.5% to over 7.5% (w/v) ASW with an optimum growth rate at 4.0% ASW (Figure 8). In order to determine if variations in growth at different salinities were due to a specific or nonspecific ion effect, it was necessary to ascertain if any specific ion was deficient. To suboptimal dilutions of ASW, where growth, uptake and induction appreciably decreased, additional salts were added singularly in an attempt to restore optimum growth and cell function. Optimum uptake was obtained with additional NaCl, $NaNO_3$, $MgSO_4$-$MgCl_2$, KCl, or LiCl (Figure 9). Surprisingly, $MnCl_2$ was found to inhibit uptake. All sodium salts tested were similarly able to support optimum growth and induction rates when added to suboptimal salinities (Tables 4, 5). In addition, $MgCl_2$ or KCl supported near optimal growth rates but did not restore optimal induction rates. A reverse order of effectiveness is seen for $MgCl_2$ and KCl in their ability to increase growth and induction rates. It is also interesting to note that while LiCl supported optimal uptake

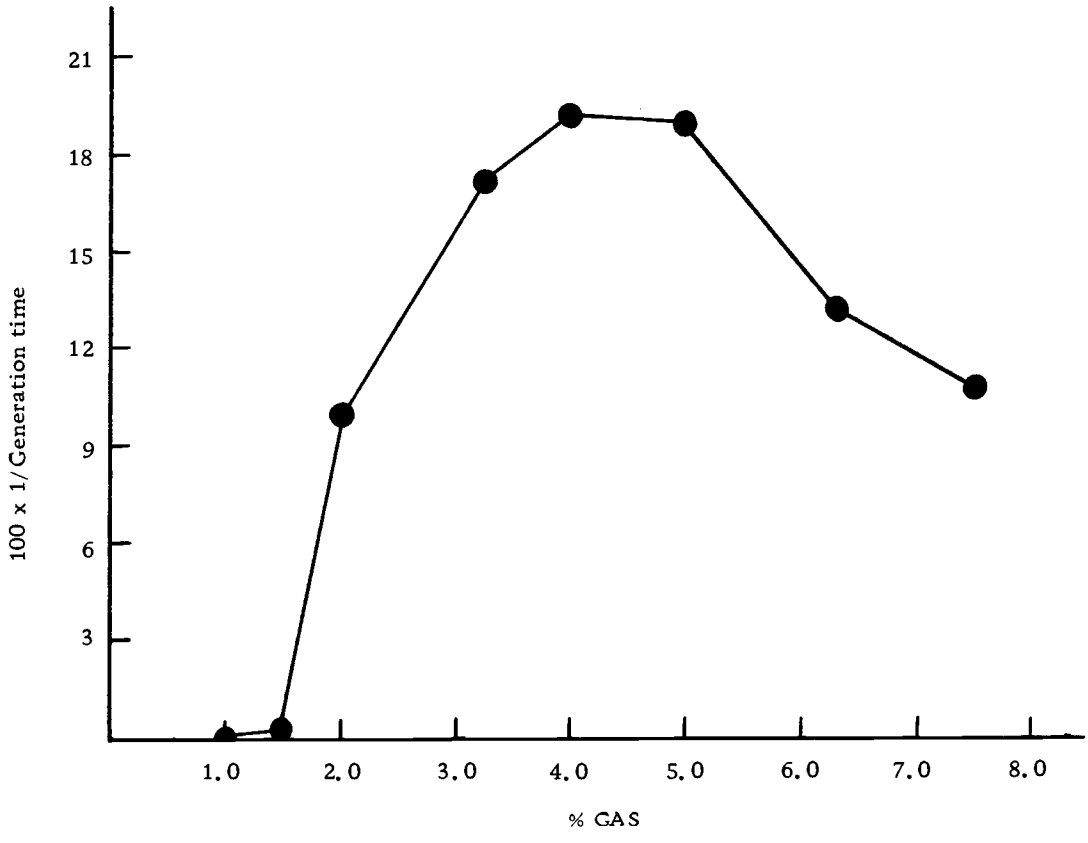

Figure 8. The growth rate of Ant-12 at various % GAS (5 C).

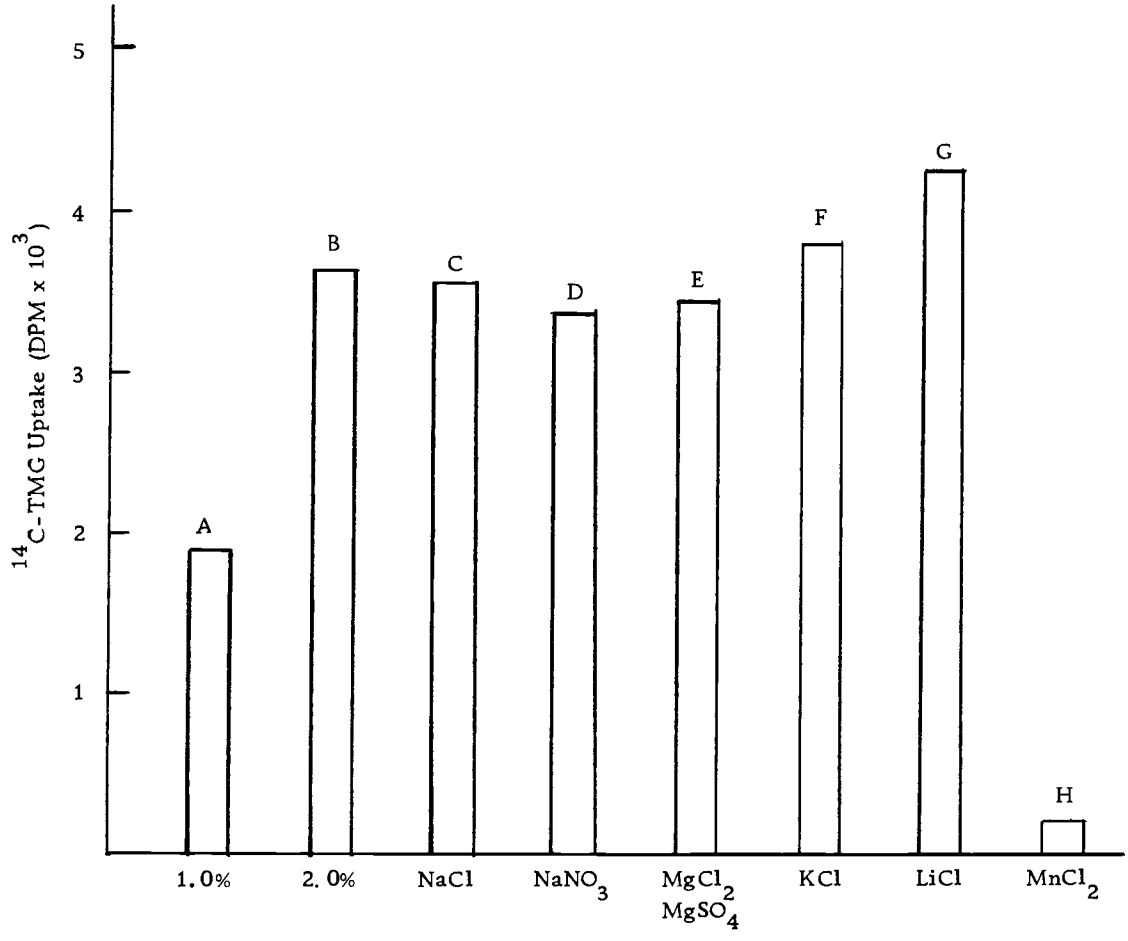

Figure 9. Nonspecific solute requirement for ^{14}C-TMG accumulation. Cells were incubated and assayed for ^{14}C-TMG uptake in the following solutions: A = 1.0% ASW, B = 2.0% ASW, C-H = 1.0% ASW + 0.11 M additional salt.

Table 4. Nonspecific solute requirement for growth.

Added Salt[a]	Growth Log (OD_{600} x 100) 36 hrs	60 hrs
NaCl	1.04	1.71
$NaNO_3$	0.95	1.79
Na_2SO_4	0.84	1.62
KCl	0.90	1.40
MgCl	1.04	1.86
LiCl	0	0.30
RbCl	0	0
None	0.30	0.60
2.5% GAS	1.00	1.70

[a]Cells were grown in 1.5% GAS medium with 0.282 M additional salt.

Table 5. Nonspecific solute requirement for induction of ^{14}C-TMG uptake.

Added Salt[a]	Induction (10^3 x DPM/mg cell protein) 2 hrs	5 hrs
NaCl	12.8	55.2
$NaNO_3$	11.0	55.1
Na_2SO_4	14.8	72.1
KCl	9.5	38.4
$MgCl_2$	4.3	23.2
LiCl	2.1	5.6
RbCl	3.4	6.1
None	0.9	1.6
3.0% ASW	13.1	57.0

[a]Cells were prepared for induction in 1.0% ASW with 0.33 M additional salt.

it did not promote growth and induction.

Ion Effects on Uptake, Growth, and Induction

A BIS solution containing Na^+, K^+, and Mg^{+2} ions was shown to be able to support both uptake and induction (Tables 6, 7). Moreover, both Na^+ and K^+ are specifically required for these processes since neither ion could be replaced (Tables 8, 9, 10, 11). Ion substitutes for Na^+ were not inhibitory to permease action since ^{14}C-TMG uptake occurred on further addition of NaCl (Table 8). In contrast, deletion of Mg^{+2} did not seem to affect either uptake or induction (Tables 6, 7), although it was required for growth along with other ions.

Table 6. Evidence for a Na^+ and K^+ requirement for accumulation of ^{14}C-TMG.

Ion Requirements	Uptake (DPM) 5 minutes	30 minutes
BIS (Complete)	5020	19600
BIS (-Na^+)	820	1800
BIS (-K^+)	1126	3055
BIS (-Mg^{+2})	4386	18323

Table 7. Evidence for a Na^+ and K^+ requirement for induction of ^{14}C-TMG uptake.

Ion Requirements	Induction (10^3 x DPM/mg cell protein) 2 hrs	5 hrs
BIS (Complete)	13.5	64.3
BIS ($-Na^+$)	0.7	1.1
BIS ($-K^+$)	1.4	3.8
BIS ($-Mg^{+2}$)	13.7	66.1

Table 8. Specific Na^+ requirement for the accumulation of ^{14}C-TMG.

Salt Added[a]	Uptake (DPM) No NaCl Added	0.1 M NaCl Added
None	1554	7423
NH_4Cl	1257	5120
LiCl	1724	7521
RbCl	1524	6911
$NaNO_3$	7591	
Na_2SO_4	5321	

[a]Cells were suspended in BIS solution containing a substitute salt for NaCl. Salts were tested at 0.1 M, except Na_2SO_4 which was added at 0.05 M.

Table 9. Specific K^+ requirement for the accumulation of ^{14}C-TMG.

Salt Added[a]	Uptake (DPM)
None	3231
NH_4Cl	2926
LiCl	3628
RbCl	3162
KCl	8132
KNO_3	7941
K_2SO_4	6313

[a]Cells were suspended in BIS solution containing a substitute salt for KCl. Salts were tested at 0.009 M, except K_2SO_4 which was added at 0.0045 M.

Table 10. Specific Na^+ requirement for induction of ^{14}C-TMG uptake.

Salt Added[a]	Induction (10^3 x DPM/mg cell protein) 4 hrs
LiCl	0.7
RbCl	1.0
KCl	0.6
$MgCl_2$	0.5
NaCl	57.9
$NaNO_3$	43.8
Na_2SO_4	50.3

[a]Cells were prepared for induction in BIS solution containing a substitute salt for NaCl. Salts were tested at 0.45 M, except for Na_2SO_4 which was added at 0.23 M.

Table 11. Specific K^+ requirement for induction of ^{14}C-TMG uptake.

Salt Added[a]	Induction (10^3 x DPM/mg cell protein) 4 hrs
LiCl	2.4
RbCl	4.0
NaCl	2.8
$MgCl_2$	3.7
KCl	60.7
KNO_3	51.1
K_2SO_4	49.8

[a]Cells were prepared for induction in BIS solution containing a substitute salt for KCl. Salts were tested at 8.4×10^{-3} M, except K_2SO_4 which was added at 4.2×10^{-3} M.

Quantitative Na^+ and K^+ Requirement for Uptake, Growth, and Induction

The foregoing results have demonstrated that both a specific ion and nonspecific solute requirement exist for uptake, growth, and induction. For determination of the optimal specific quantitative Na^+ requirement for these processes, the nonspecific solute requirement was satisfied by the addition of an appropriate solute to BIS or Minimal medium lacking Na^+. Having satisfied this requirement, it was found that 0.05-0.11 M NaCl was required for optimal ^{14}C-TMG uptake (Figure 10), 0.11-0.22 M NaCl for optimal growth (Figure 11)

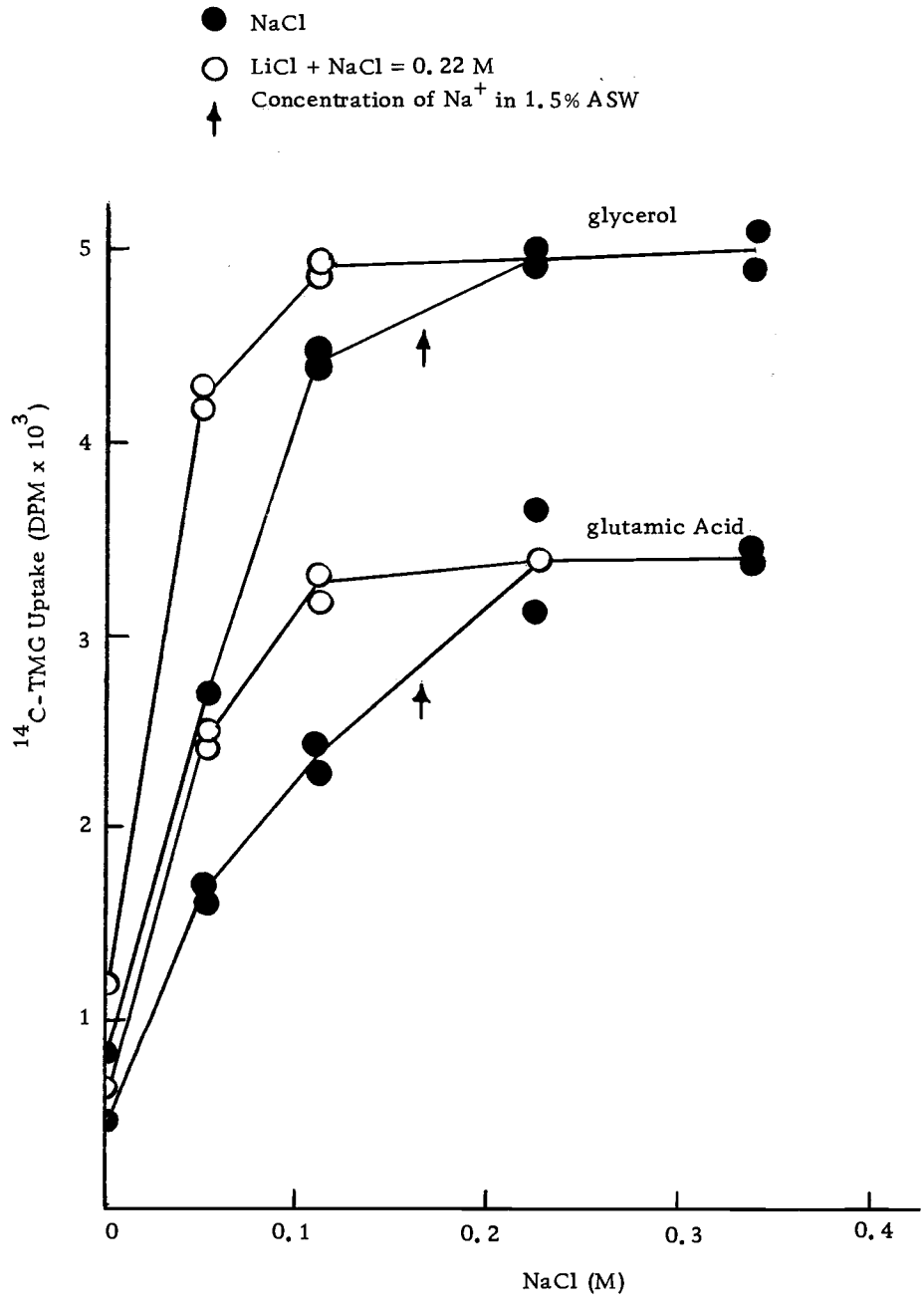

Figure 10. Quantitative Na^+ requirement for uptake of ^{14}C-TMG using glycerol or glutamic acid as the energy source.

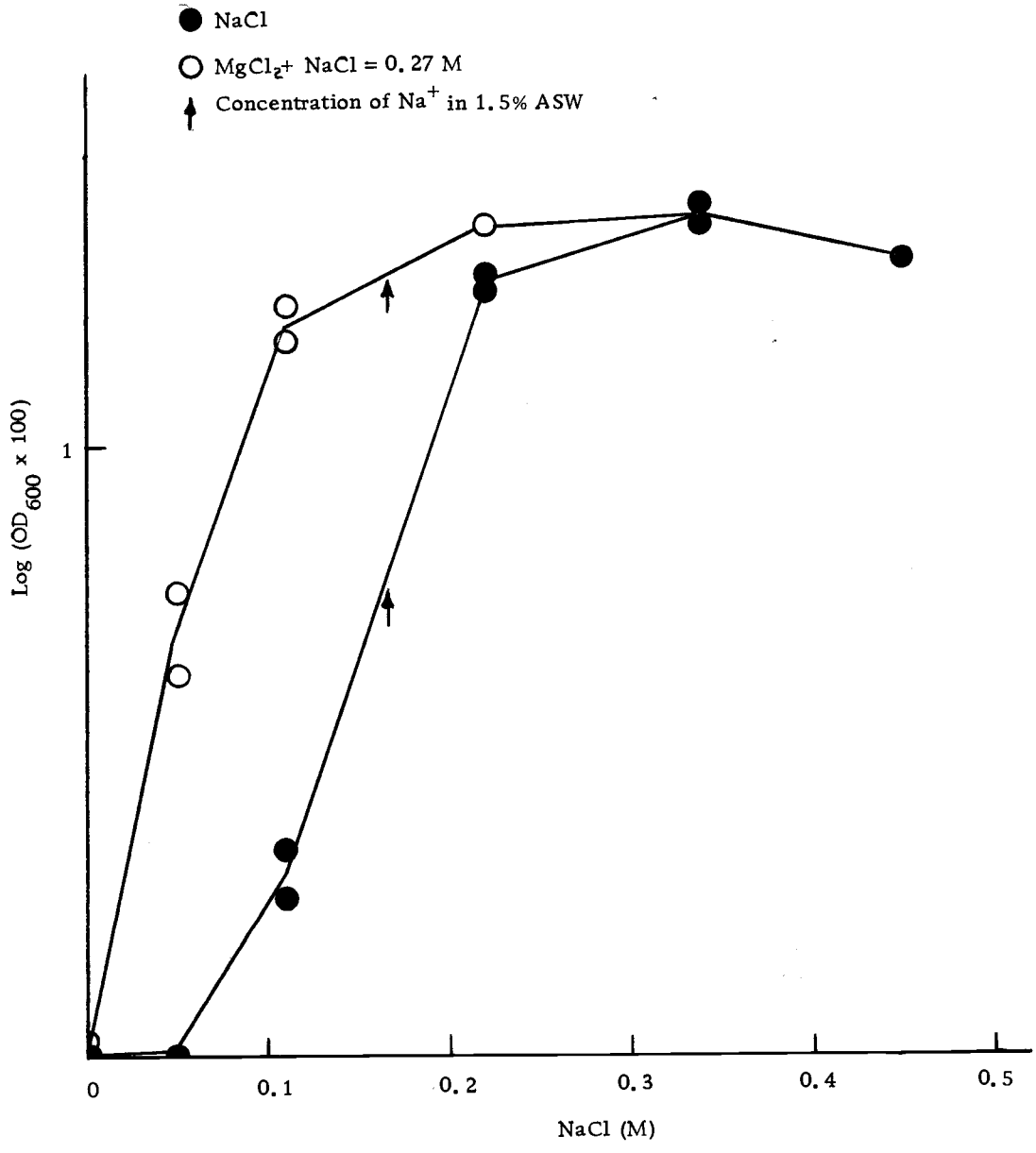

Figure 11. Quantitative Na^+ requirement for growth (30 hours of growth).

and ^{14}C-Glut uptake (Figure 12), and 0.22-0.33 M NaCl for optimal induction (Figure 13).

Previous studies by MacLeod and coworkers (13, 14, 40) have shown that the quantitative Na^+ requirement for uptake was not fixed but varied with the substrate. In our experiments, amino acids were required for induction and growth while glycerol was used as an energy source for ^{14}C-TMG uptake. The possibility therefore exists that the differences in Na^+ requirement could be attributed to uptake of different precursors and/or energy sources rather than any real difference in the Na^+ requirement for uptake, growth, and induction. This possibility was evident for growth and ^{14}C-TMG uptake studies as was shown from the higher Na^+ requirement for ^{14}C-Glut uptake than for ^{14}C-TMG uptake (Figures 10, 11, 12). This difference, however, does not account for the total difference in Na^+ requirement between uptake and induction (Figures 12, 13). Furthermore, the use of glycerol or glutamic acid as an energy source did not change the optimum Na^+ requirement for ^{14}C-TMG uptake (Figure 10).

The participation of the nonspecific solute is seen from the difference in cell function with and without additional nonspecific solute. It is noteworthy that induction seems to have a higher nonspecific solute requirement than growth or uptake, the one least requiring the additional solute (Figures 10, 11, 12, 13).

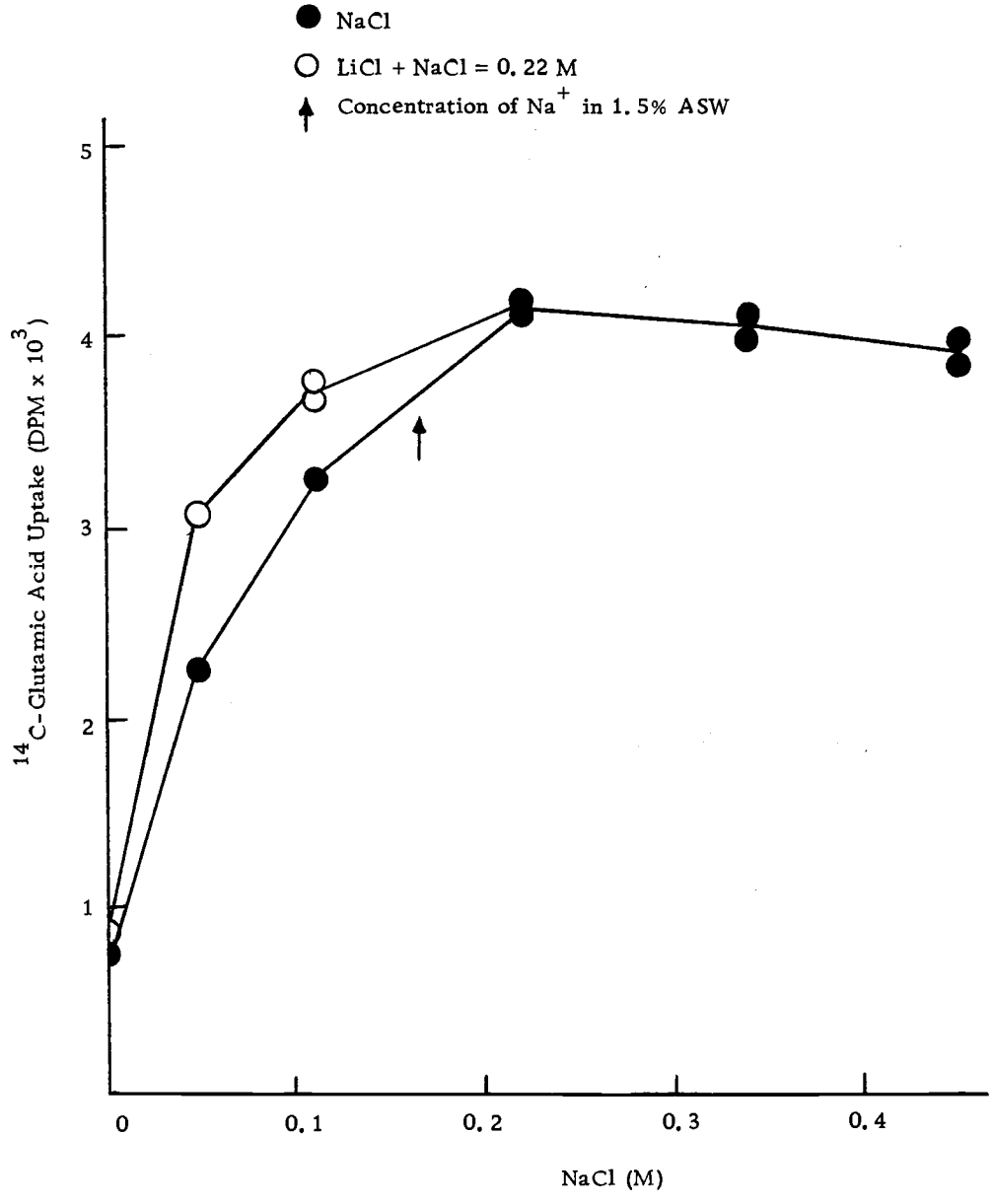

Figure 12. Quantitative Na^+ requirement for uptake of ^{14}C-glutamic acid.

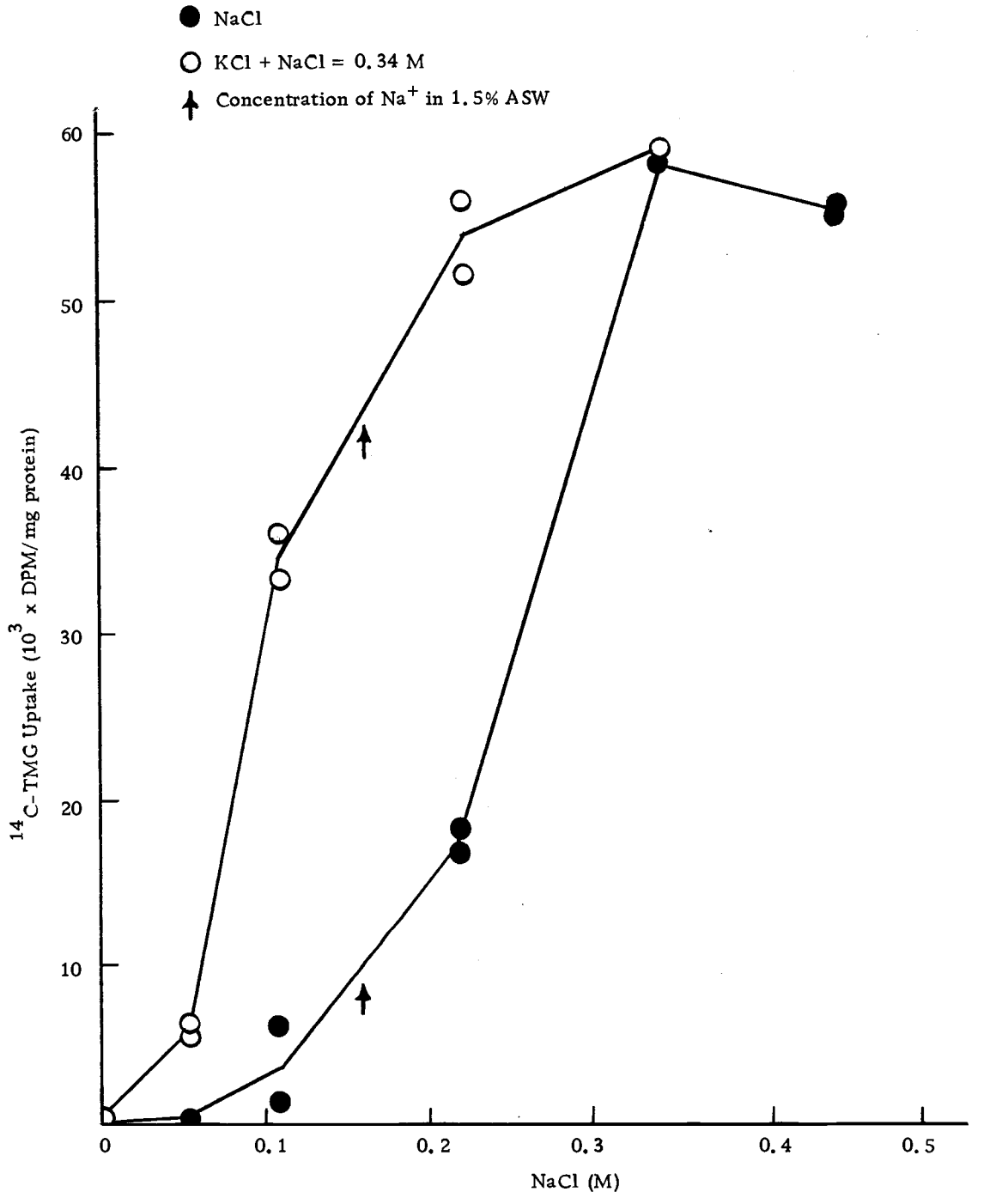

Figure 13. Quantitative Na^+ requirement for induction of ^{14}C-TMG uptake (4 hour induction period).

The optimum quantitative K^+ requirement was found to be the same for uptake, growth, and induction. This optimal K^+ requirement was approximately 12×10^{-4} M (Figures 14, 15, 16).

Temperature-Salinity Interaction on Uptake, Growth, and Induction

Salinity and temperature interactions, within the growth range of this organism, had little synergistic effect on uptake, growth, and induction. Only at near maximum growth temperature was there any indication of an interaction. Here the maximum growth and induction temperature was found to increase from 14.5 C to 16.0 C as the salinity increased above 1.5% ASW (Figures 17, 18). The situation was less clearly observed for uptake (Figure 19).

Uptake studies of ^{14}C-TMG have shown that 2.0%-3.2% ASW gave optimal uptake at all growth temperatures, and induction was optimal at 4.0%-5.0% ASW (Figures 17, 18, 19). It is interesting that at 6.5% ASW, despite an appreciable decrease in uptake, growth, and induction were greater than at 2.0% ASW. Increasing salinities tend to support in the following order: induction > growth > uptake.

Nutrient-Salinity Interaction on Uptake and Induction

The pattern of induction and uptake at various salinities was not affected by the nutrient concentration (Figures 20, 21). Synergistic

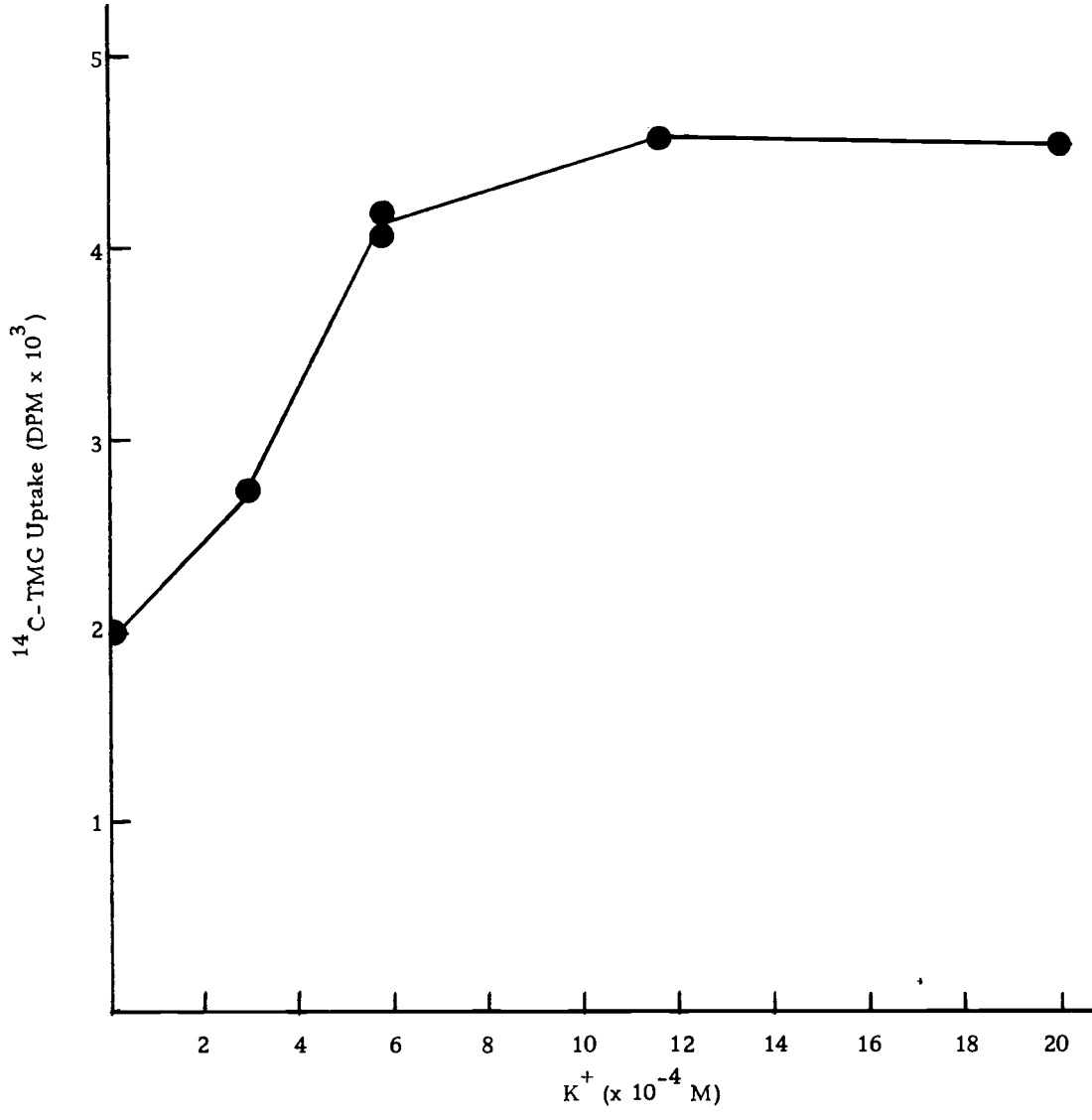

Figure 14. Quantitative K^+ requirement for ^{14}C-TMG uptake.

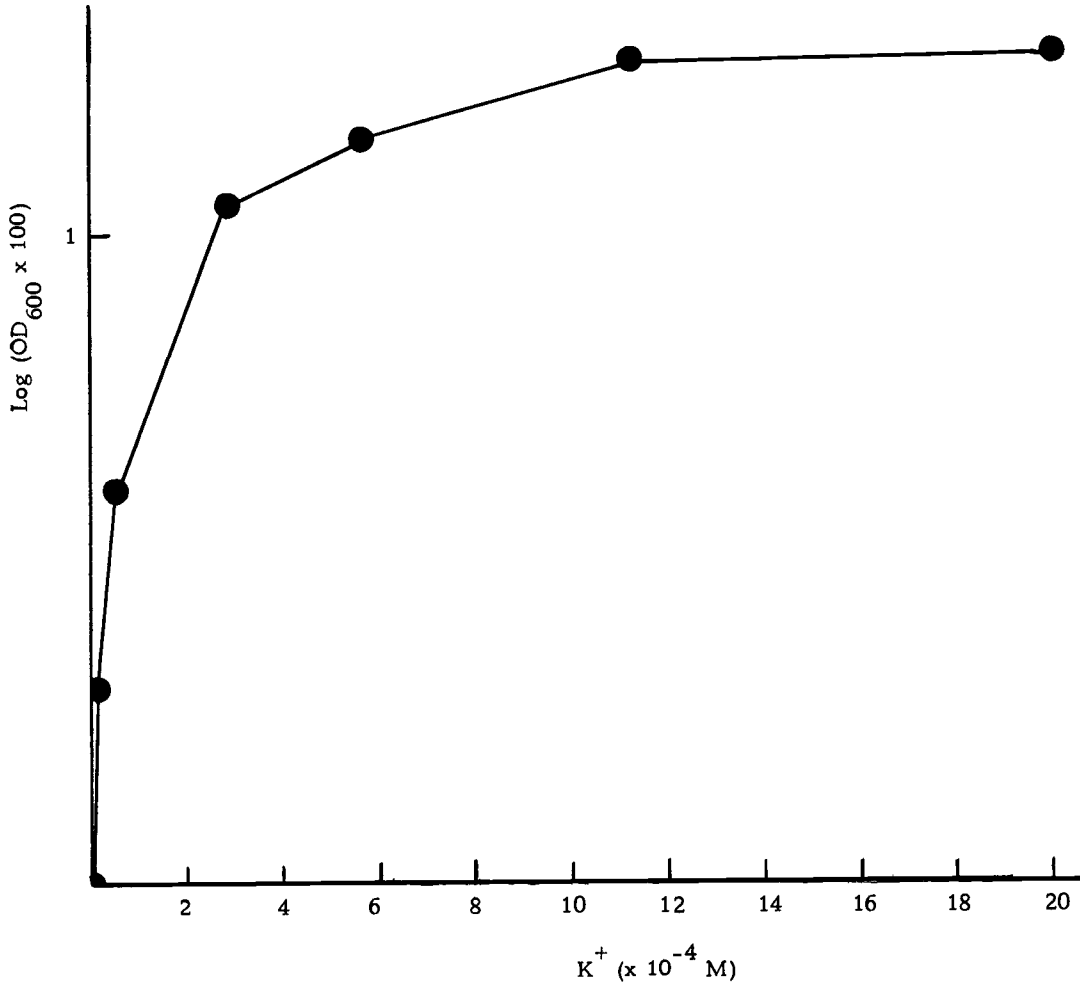

Figure 15. Quantitative K^+ requirement for growth (30 hours of growth).

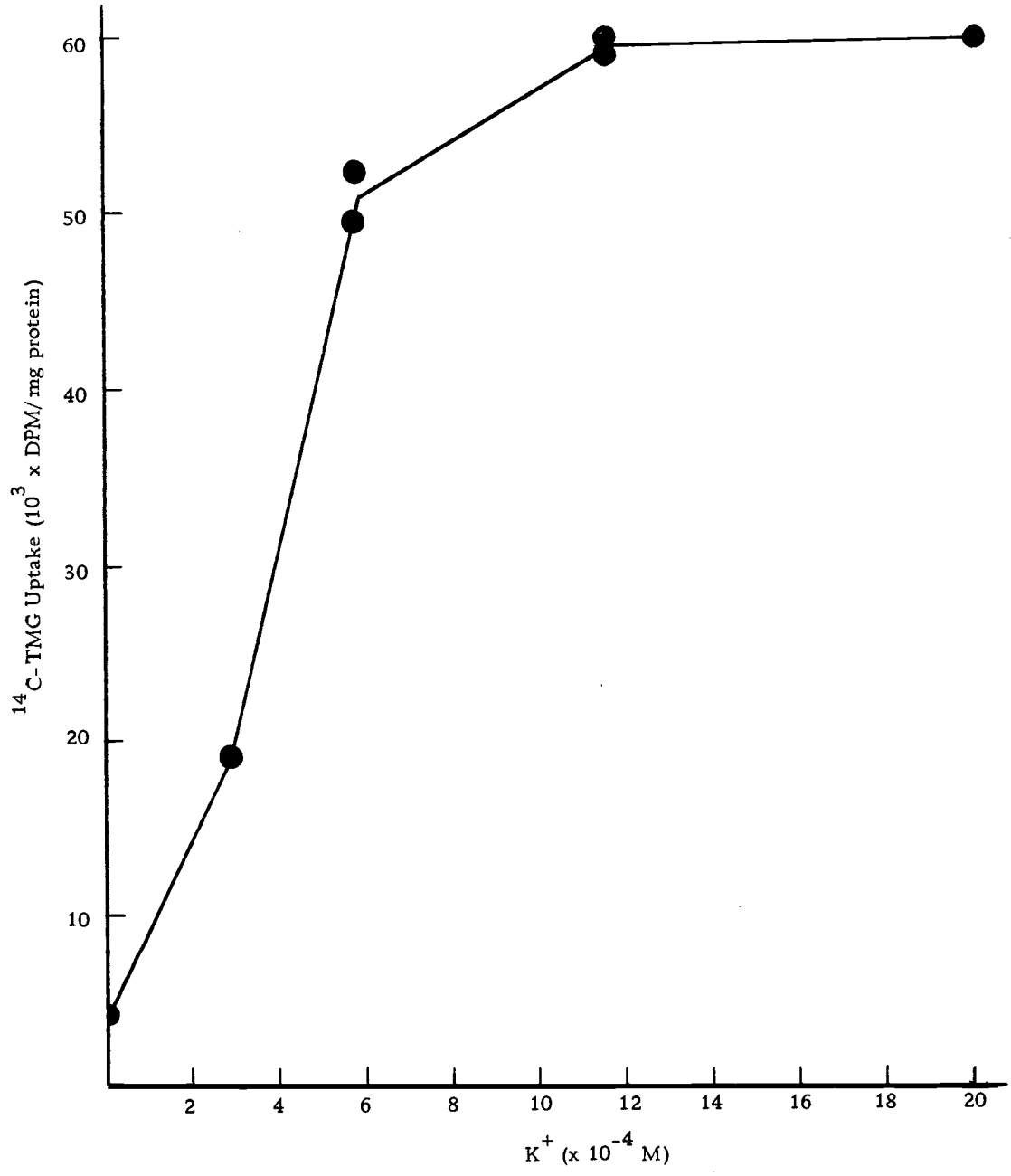

Figure 16. Quantitative K^+ requirement for induction of ^{14}C-TMG uptake (4 hour induction period).

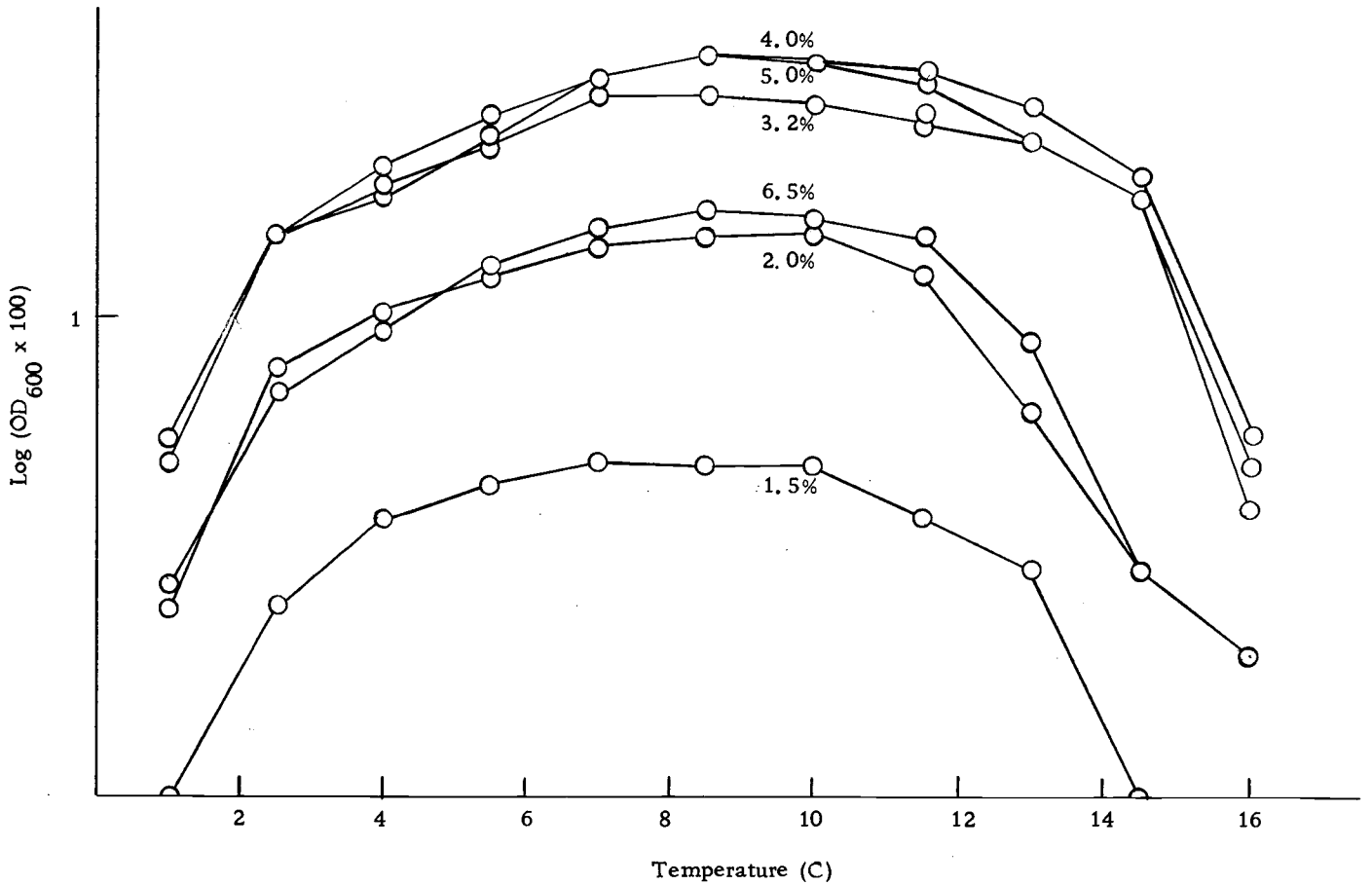

Figure 17a. Salinity-temperature effects on growth (30 hours of growth).

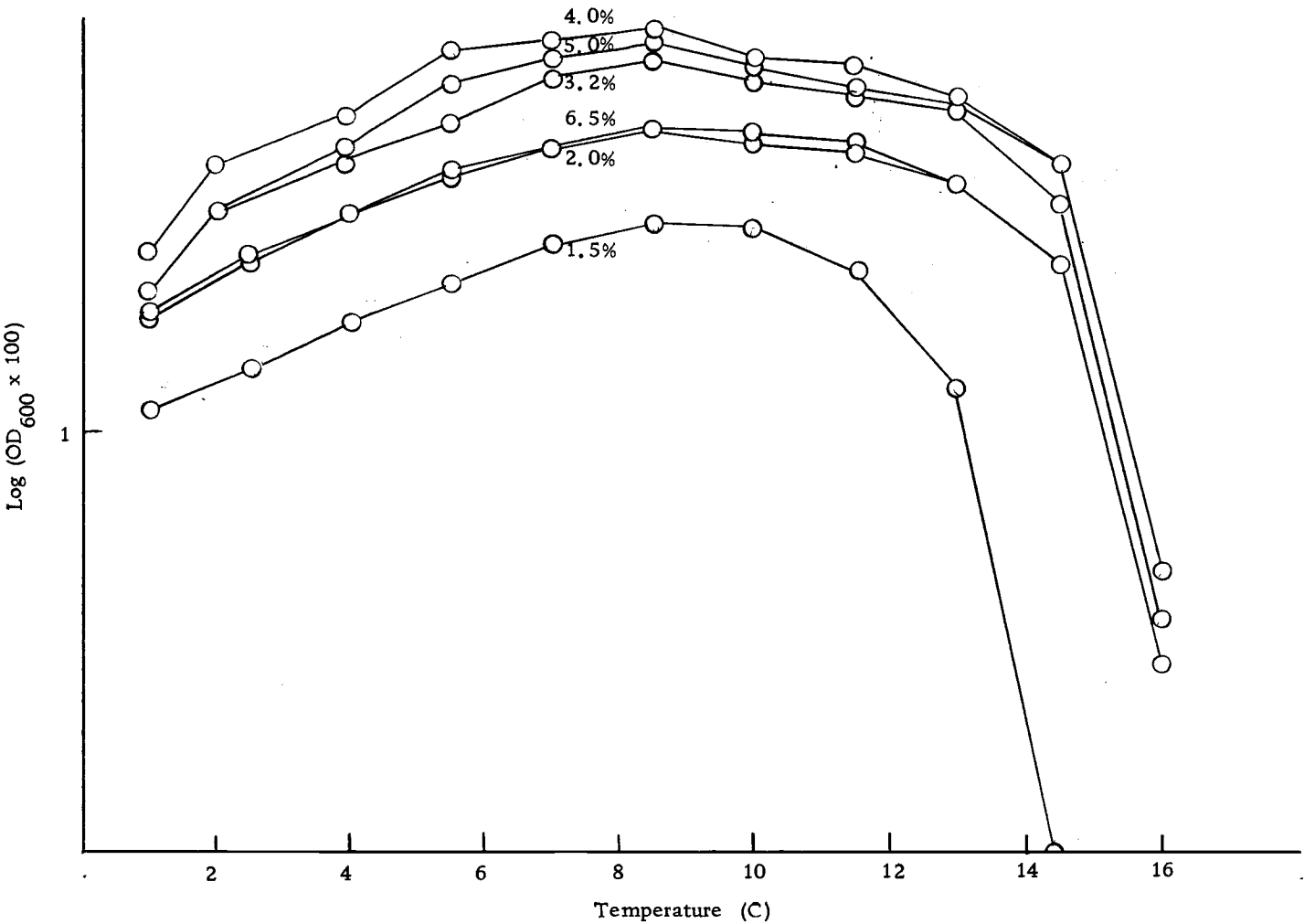

Figure 17b. Salinity-temperature effects on growth (48 hours of growth).

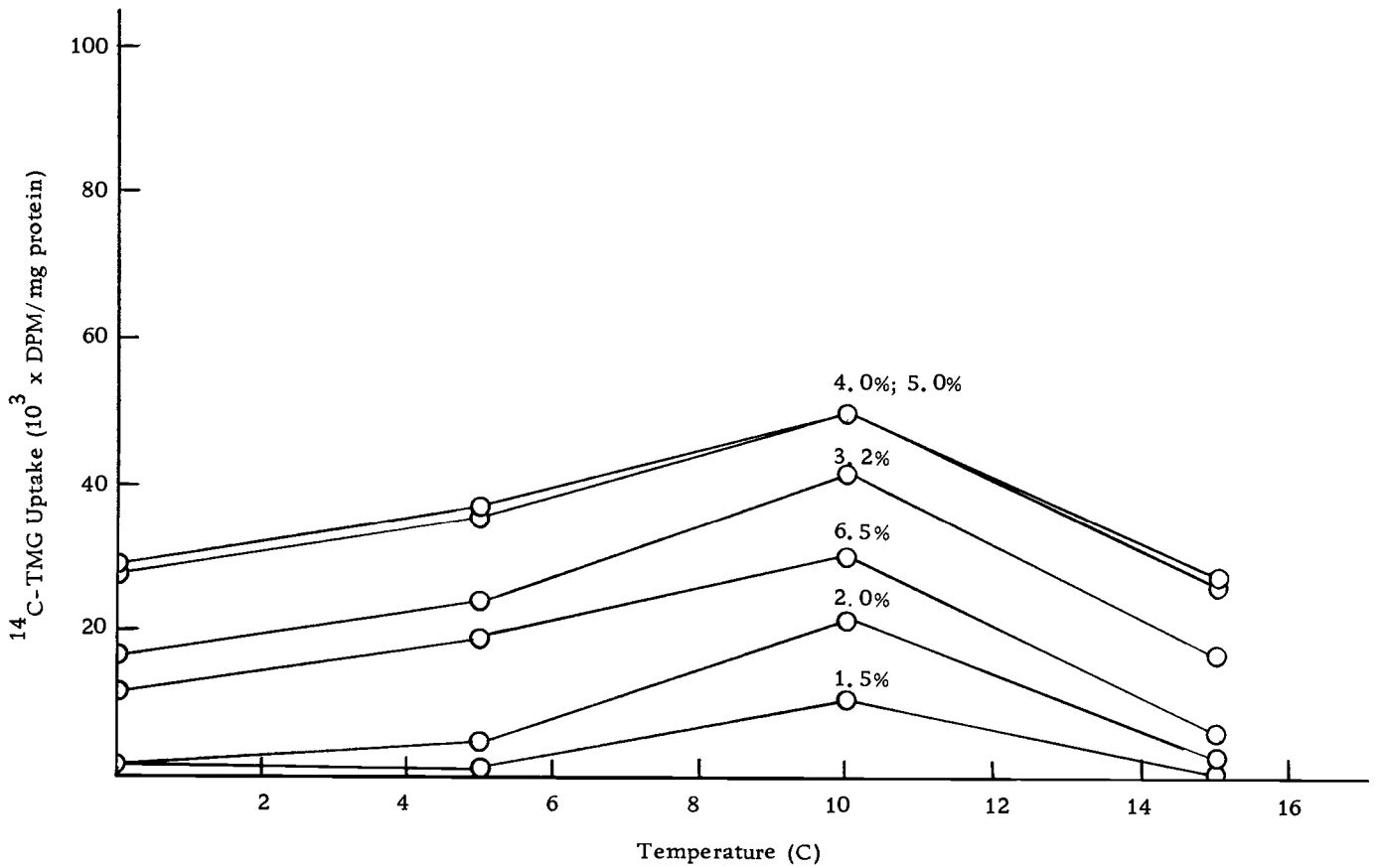

Figure 18a. Salinity-temperature effects on induction of ^{14}C-TMG uptake (2 hour induction period).

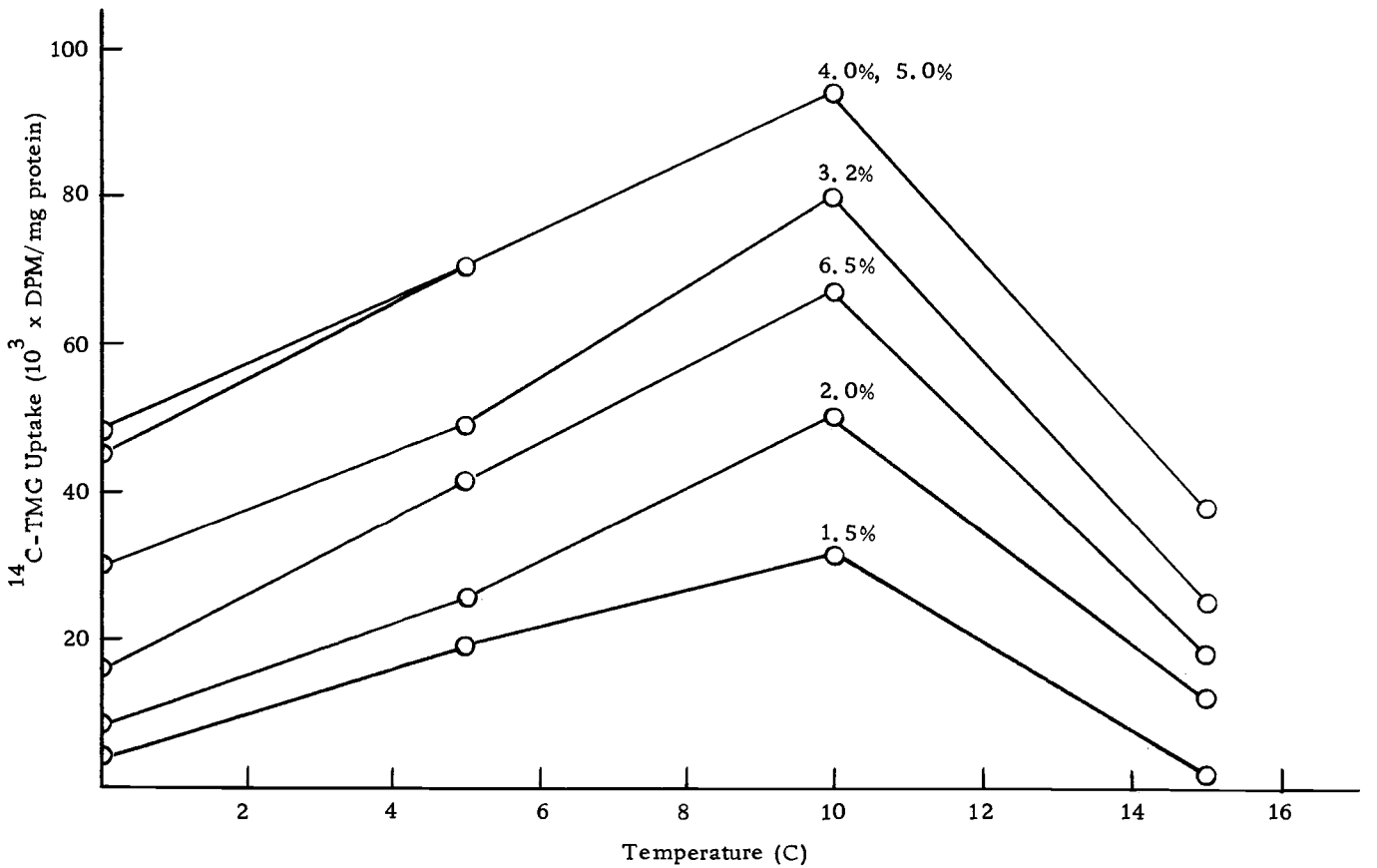

Figure 18b. Salinity-temperature effects on induction of ^{14}C-TMG uptake (6 hour induction period).

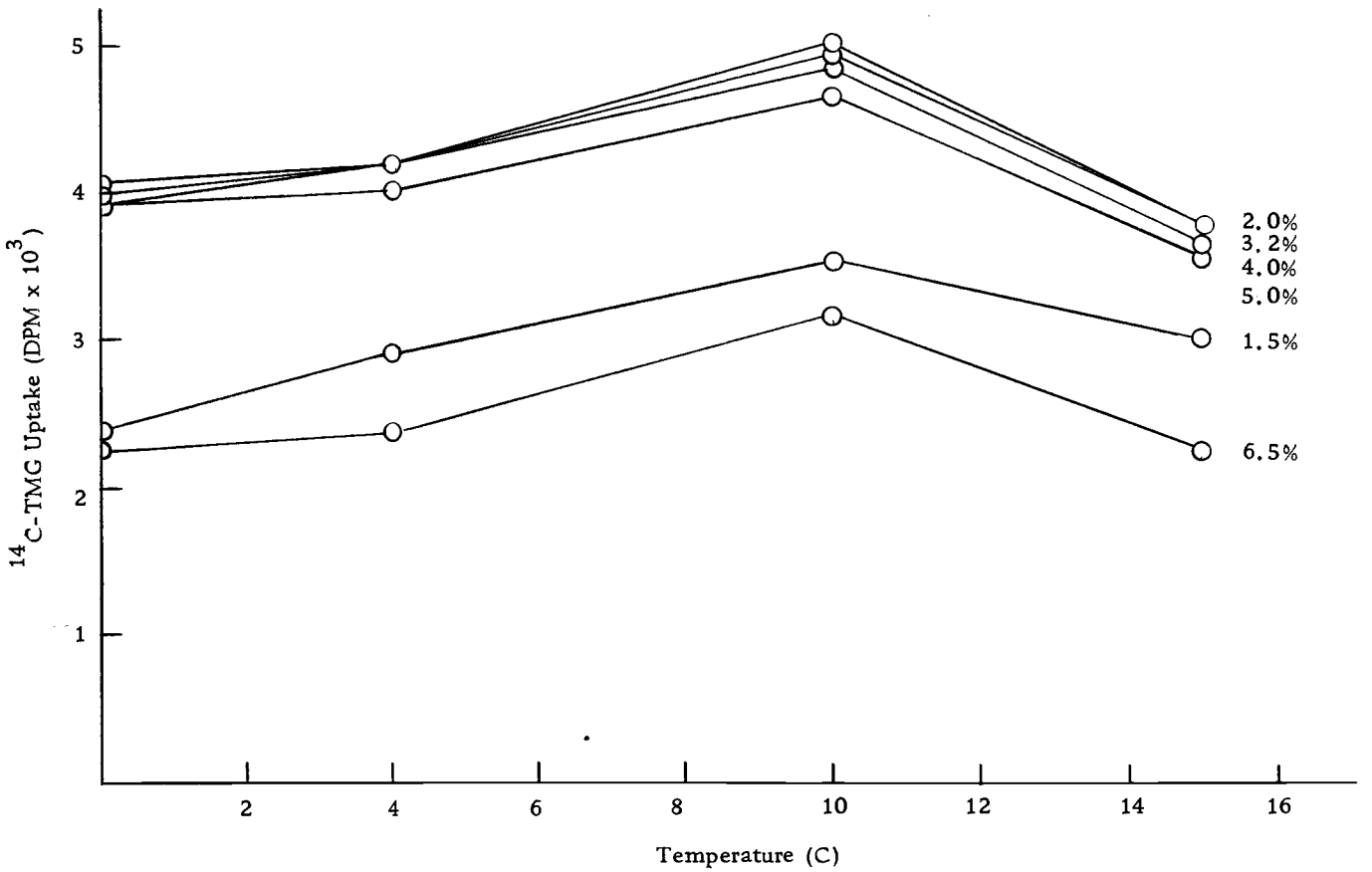

Figure 19a. Salinity-temperature effects on uptake of ^{14}C-TMG (2 hour incubation).

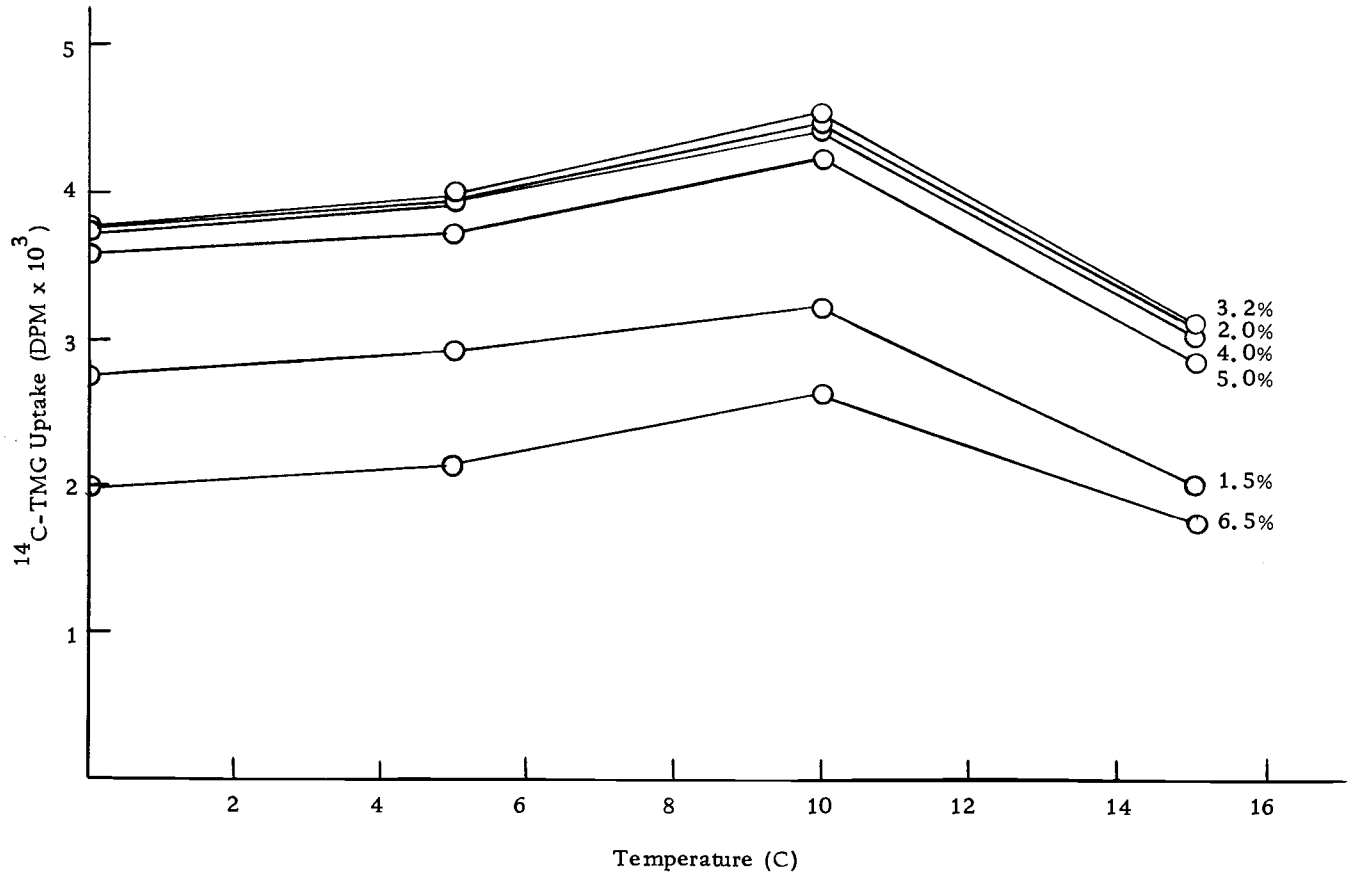

Figure 19b. Salinity-temperature effects on uptake of ^{14}C-TMG (6 hour incubation).

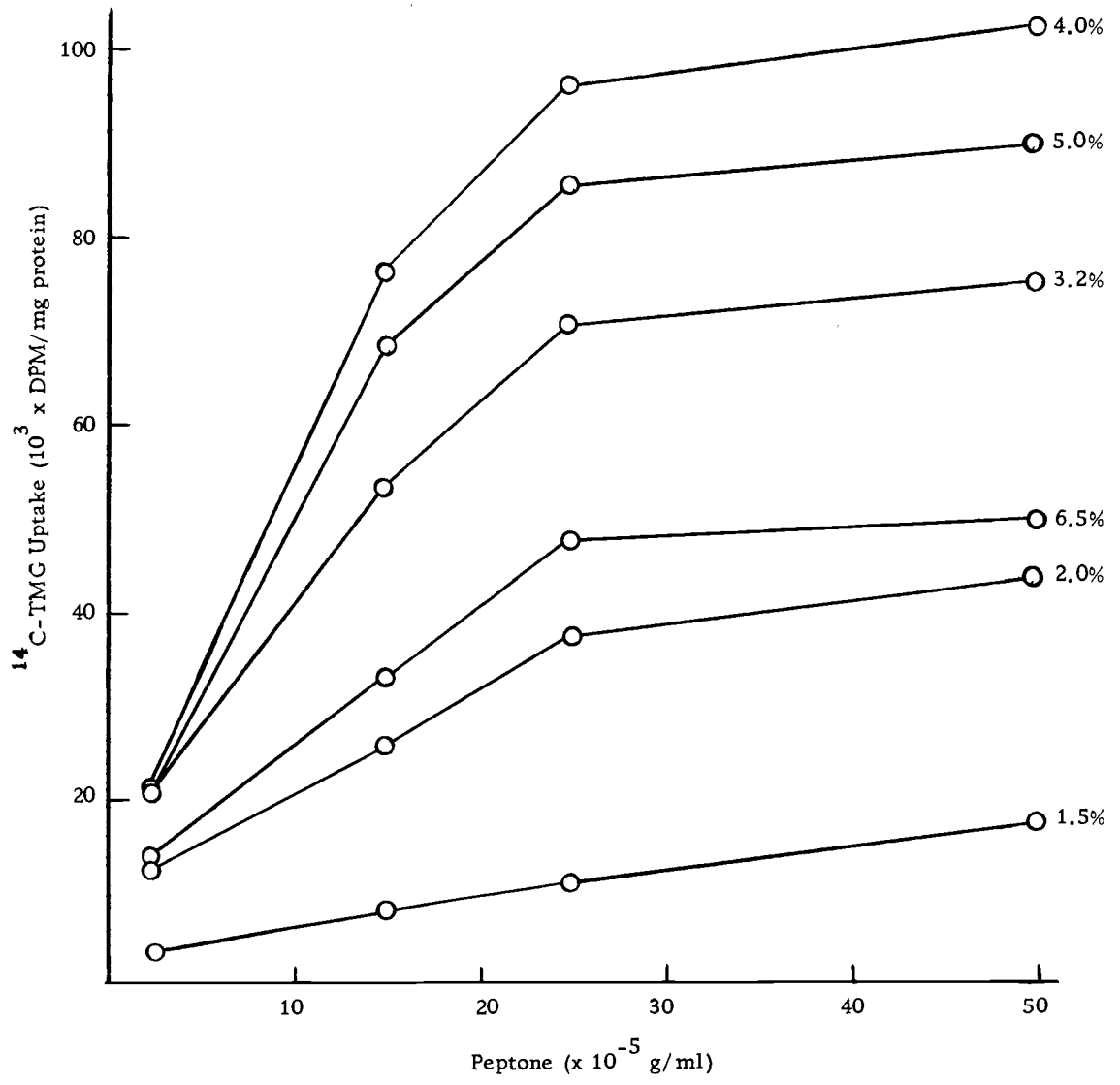

Figure 20. Salinity-nutrient effects on induction of ^{14}C-TMG uptake.

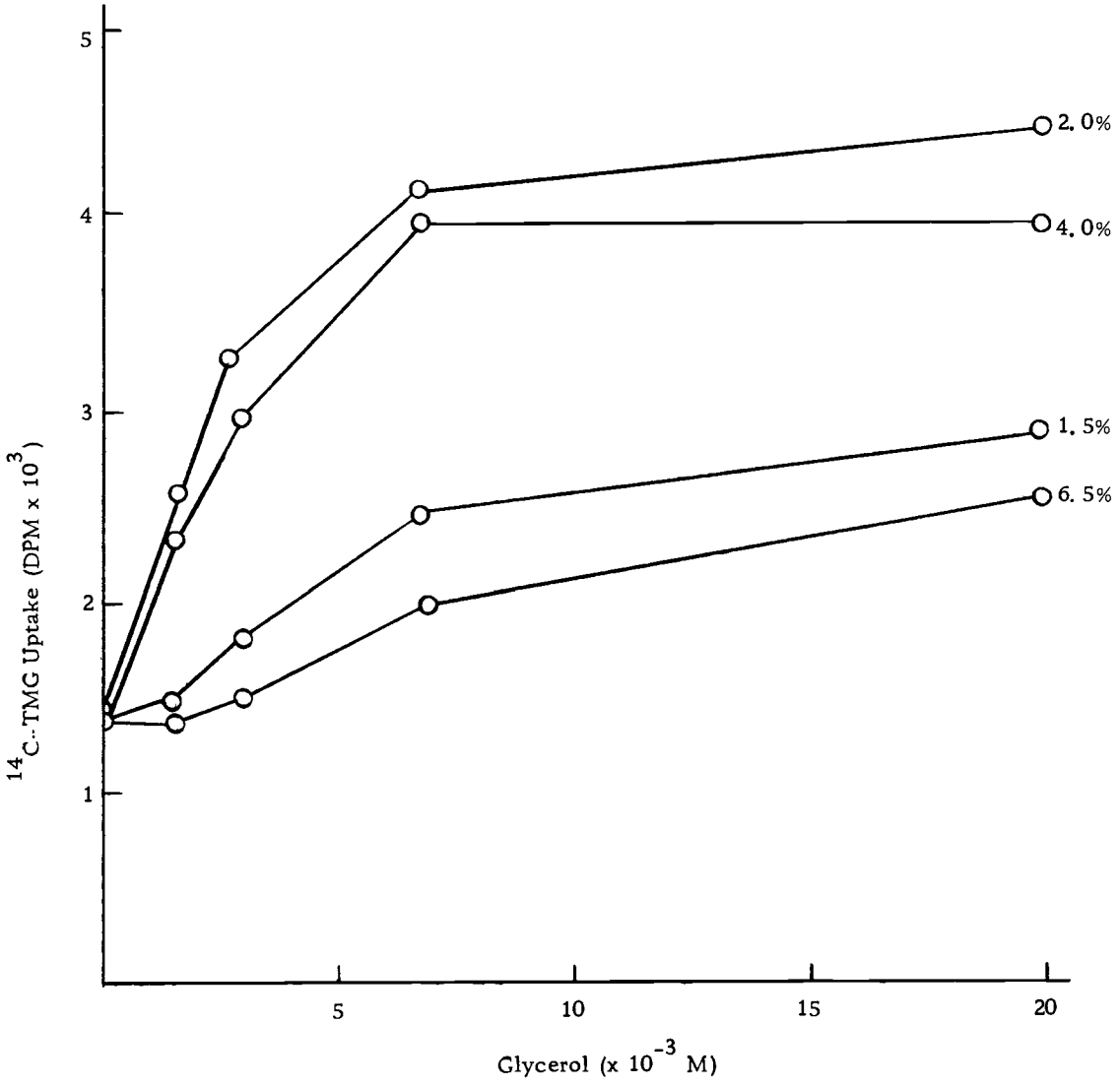

Figure 21. Salinity-nutrient effects on uptake of ^{14}C-TMG.

interactions between these parameters were not observed for induction and uptake.

Relative Percent Cellular Function at Various Temperature-Salinity or Nutrient-Salinity Combinations

Figures 17-21 were replotted to show relative percent cellular function at various temperature-salinity or nutrient-salinity combinations (Figures 22-26). The replotted graphs are useful in predicting the relative amount of cellular function which results from the interaction of these oceanic parameters.

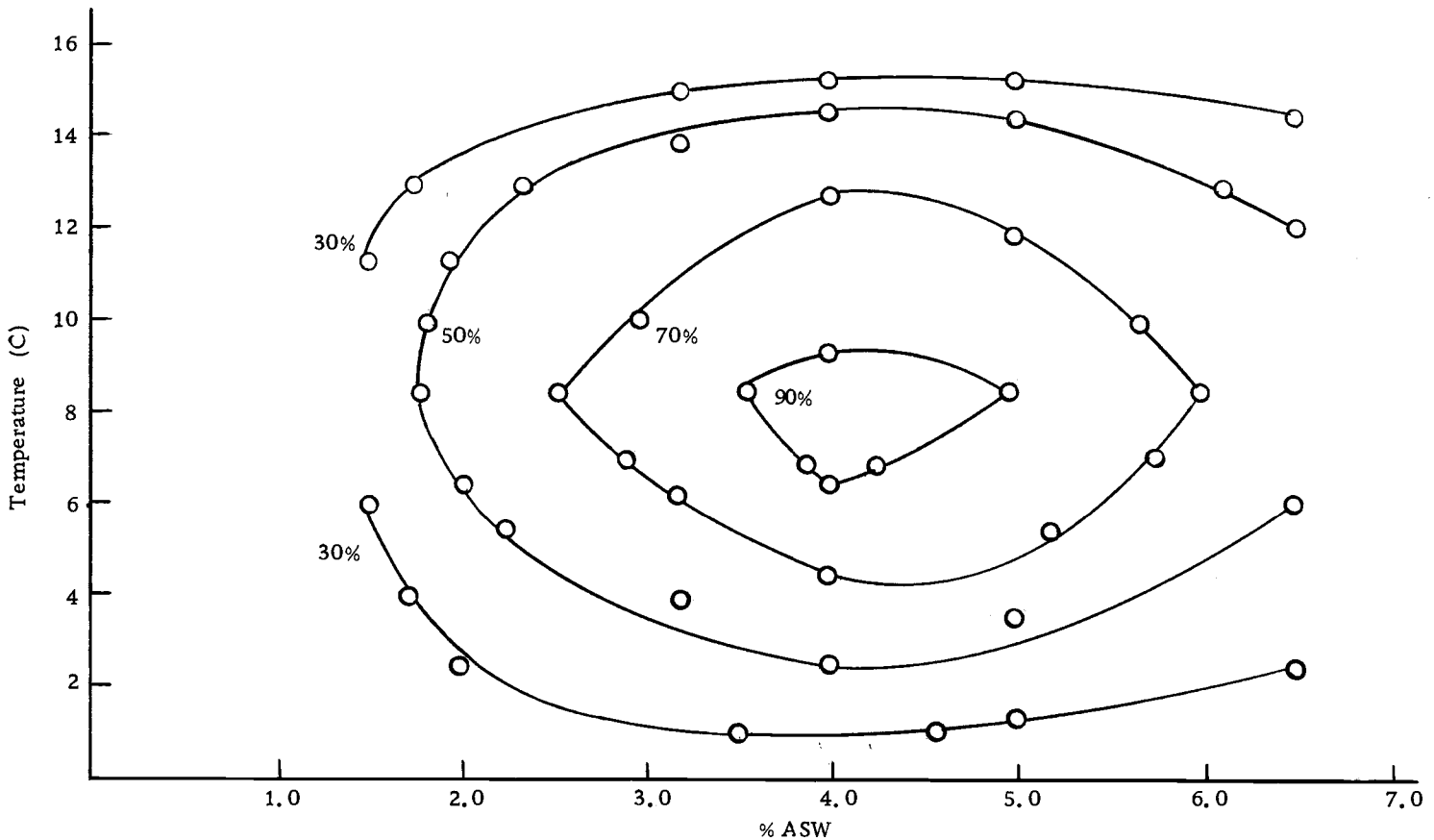

Figure 22. Relative percent growth of Ant-12 at various salinities and temperatures (replot of Figure 17b).

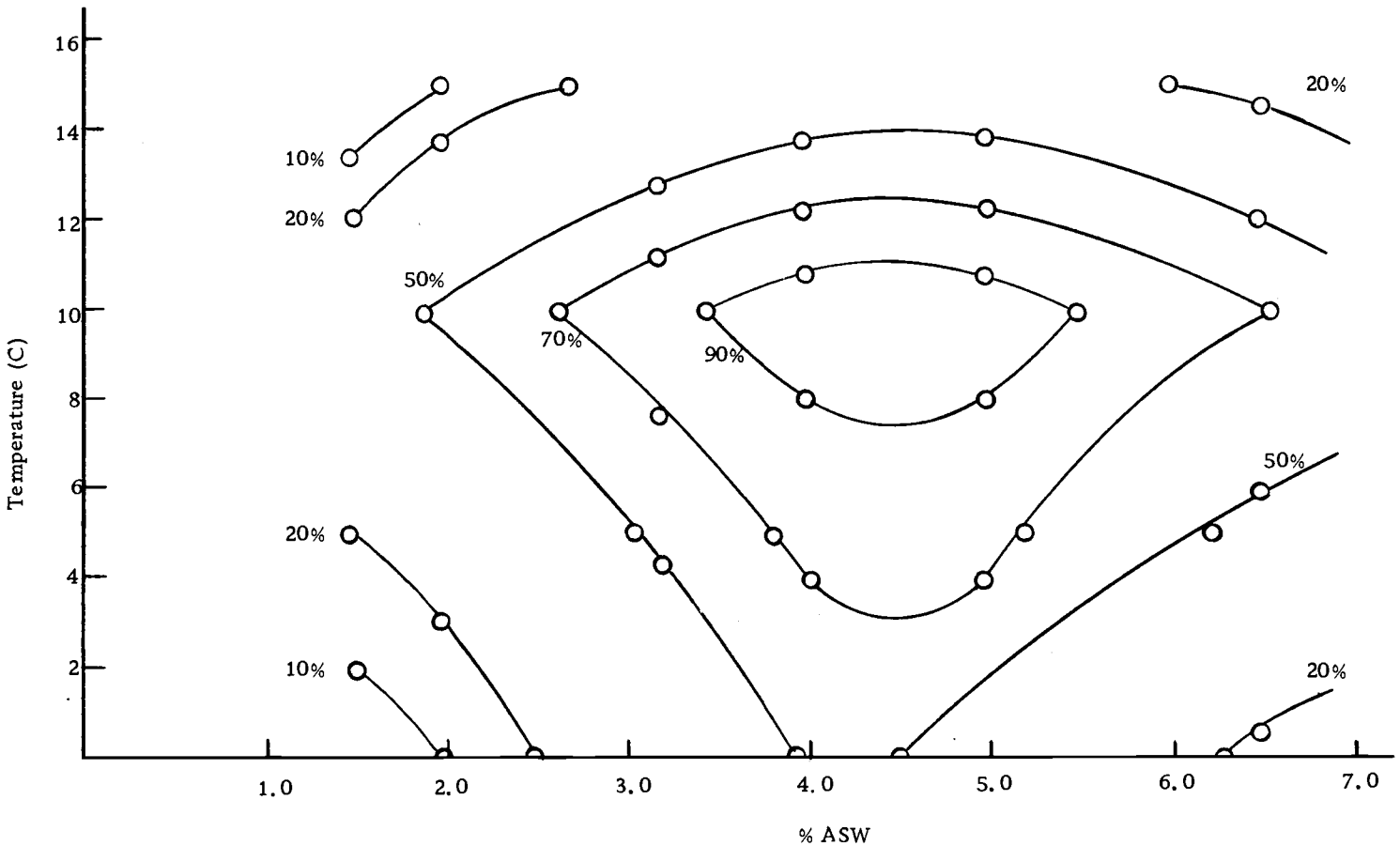

Figure 23. Relative percent induction of ^{14}C-TMG uptake at various salinities and temperatures (replot of Figure 18b).

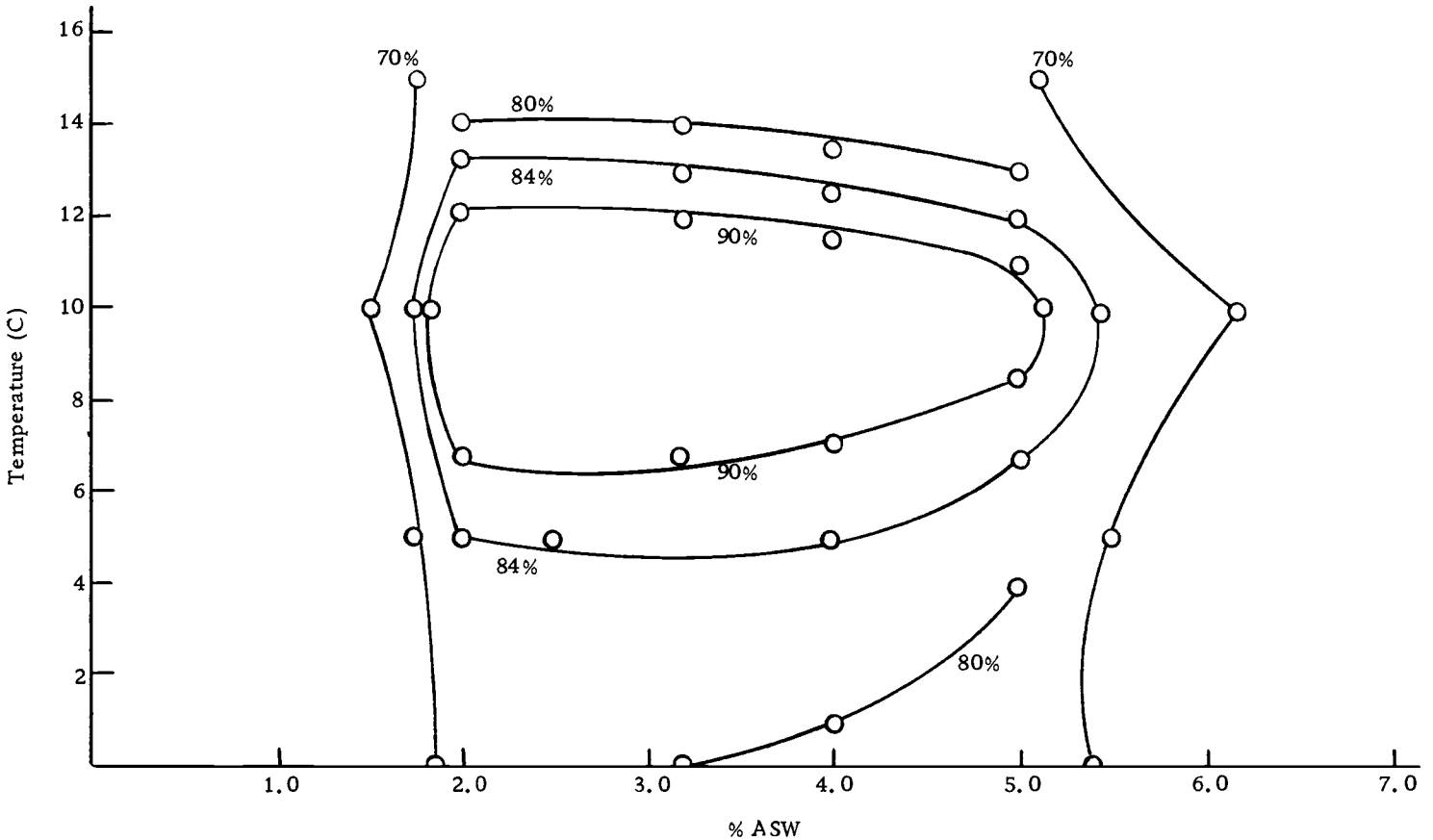

Figure 24. Relative percent uptake of ^{14}C-TMG at various salinities and temperatures (replot of Figure 19a).

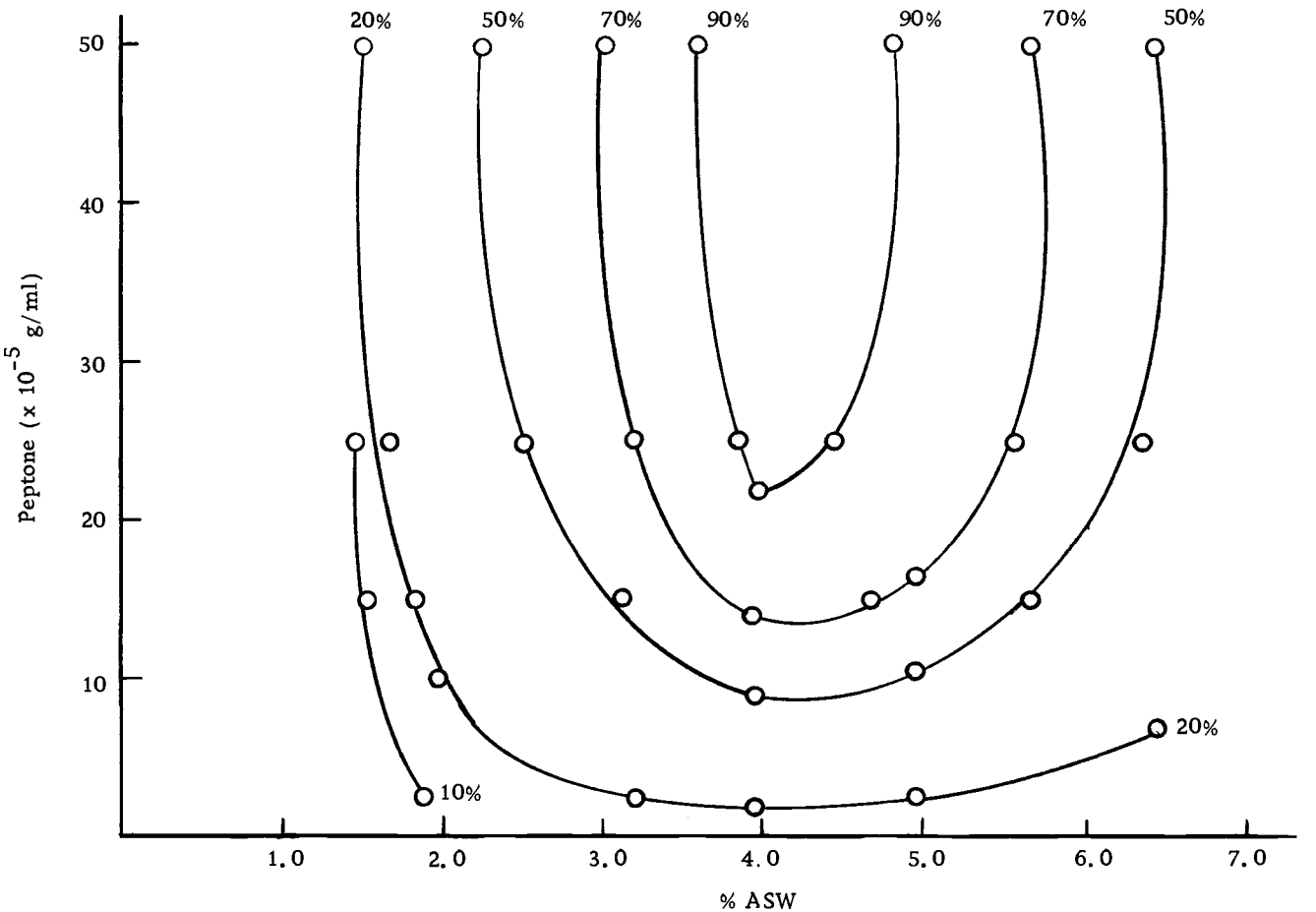

Figure 25. Relative percent induction of ^{14}C-TMG uptake at various salinities and nutrient concentrations (replot of Figure 20).

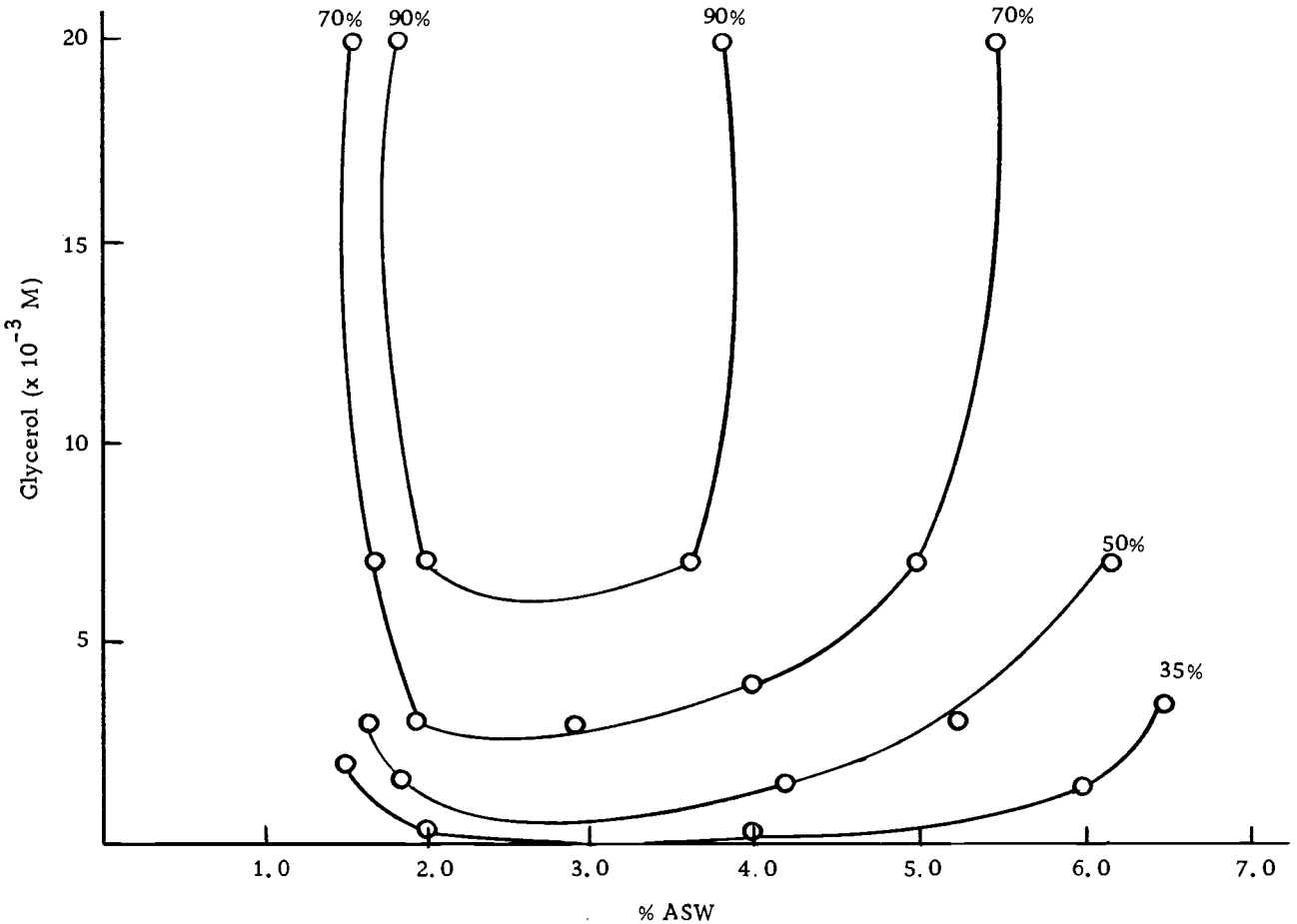

Figure 26. Relative percent uptake of ^{14}C-TMG at various salinities and nutrient concentrations (replot of Figure 21).

DISCUSSION

A number of Antarctic marine psychrophilic bacteria were isolated with the ability to ferment lactose. However, none of the organisms could accumulate ^{14}C-TMG via a lactose permease. The results suggest that a lactose permease, similar to that found for E. coli, is not present in these isolates.

Substrate and inducer specificities reveal that the galactose system in Ant-12 is also different from that found in E. coli. Although induction and competitive inhibition studies clearly demonstrate galactose as being the primary utilizable substrate, the substrate specificity of the system does not coincide with any of the five galactose uptake systems of E. coli (15, 30, 64, 69). The possibility that two or more uptake systems may be functioning in Ant-12, however, does not appear to be the case. Preferential inducers and substrate specificities of the E. coli systems failed to distinguish in Ant-12 more than one galactose system. Furthermore, kinetic studies of ^{14}C-TMG uptake did not show a diphasic pattern which would be expected if two uptake systems of different affinities were present (31). Unless similar affinities exist or different inducers are required, Ant-12 would seem to possess but one uptake system for galactose. Genetic evidence would be needed to positively clarify this question.

A number of inherent difficulties in using this isolate for induction and uptake studies became apparent on defining this inducible

uptake system. Unlike E. coli, which has an appreciable quantity of endogenous substrate, our isolate could only accumulate ^{14}C-TMG for a short time after the energy source was removed. Whether Ant-12 did not possess a significant store of utilizable endogenous substrate or could shut down its metabolism under starvation conditions is a matter of speculation. In any case, it became apparent that an exogenous energy source would be needed for accumulation of the non-metabolizable ^{14}C-TMG. Results involving ^{14}C-TMG uptake might, therefore, be influenced by the uptake of the energy source. This problem is somewhat alleviated by the use of glycerol as the energy source. Glycerol is known to be able to penetrate the osmotic barrier of bacteria freely (50). Furthermore, assay of ^{14}C-TMG uptake done before endogenous reserves were depleted gave results similar to those where glycerol was added. The results differing with respect to the quantity of ^{14}C-TMG accumulated rather than any change in the general pattern of uptake at different salinities and temperatures.

Galactose induction was similarly found to require an exogenous precursor supply of an amino acid in addition to the inducer. This would seem to be in accordance with the amino acid requirement for growth of this isolate. It is perhaps an advantage to the organism that both induction and growth require amino acids since induction would only occur under conditions where growth was possible. The phenomenon of substrate accelerated death would, otherwise, be a

distinct possibility (55). An ideal experimental induction system, however, would be one where endogenous reserves could promote induction. Such a system would be relieved of the possibility that uptake of exogenous substrates would be the rate limiting step in induction. In this respect, it is significant that our results suggest that salinity effects on induction are not due to uptake of either the inducer or precursor substrate.

Much of the research on specific Na^+ requirements for cellular functions in bacteria has been done on marine pseudomonads. Since a Vibrio species was used in this study, a comparison of the Na^+ requirement for uptake, growth, and induction between these genera is in order. A number of similarities were found between Ant-12 and the pseudomonad studied by MacLeod and associates (13, 14, 82). These include a specific Na^+ requirement for uptake, variations in the quantitative specific Na^+ requirement with the substrate being transported, and a parallel quantitative specific Na^+ requirement for uptake and growth. In addition, Ant-12 was found to have a specific Na^+ requirement for induction. This was demonstrated by a higher optimum specific Na^+ requirement for induction than for growth or uptake. Synthesis of luciferase by Vibrio fisheri has also been found to require a higher NaCl concentration than growth (47). Both the type and concentration of salt used was said to be important for its synthesis. Rhodes and Payne (63) have similarly noted that their

Vibrio species required substantially more Na^+ for glucuronate induction than did MacLeod's pseudomonad species for uptake of substrates. Moreover, Webb and Payne (85) have demonstrated that a specific Na^+ requirement exists for both induction of mannitol binding protein and general net synthesis of cellular protein.

The K^+ has been similarly shown to affect transport, growth, and induction. Our study has indicated that the K^+ requirement for these processes was not only specific but quantitatively the same. This parallelism in requirement suggests that K^+ may either be specific for only one cellular function or a number of functions, one of which is rate limiting. Thompson and MacLeod (82) have given evidence that K^+ was specifically required for accumulation of substrate into the cell. On the other hand, Payne (53, 54) has pointed out the influence of K^+ on the energy-generating mechanism in his bacterium. These suggestions could in fact be interrelated since K^+ may possibly be involved in the coupling of a high energy intermediate to the transport processes.

Perhaps the most important ion requirement of Ant-12 with respect to the marine environment is the nonspecific solute requirement. This requirement may be defined as the additional solute required to promote optimal growth and cell function after all specific ion deficiencies are satisfied. The term nonspecific may be misleading since our results indicate that only a restricted number

of ions can satisfy this requirement and that these ions may differ both quantitatively and qualitatively with cellular function. Considering the differing size, polarizing power, and hydrophobic effect of various ions this is not surprising. Furthermore, these results lend credence to the idea that the nonspecific solute effect is not totally osmotic. The importance of nonspecific solute to our isolate is evident at suboptimal growth salinities. Growth and uptake are limited not by a specific ion deficiency but by the generally low solute concentration. Thus it would appear that at various salinities within the growth range of the organism, the nonspecific solute requirement rather than any specific ion deficiency controls growth and uptake. Induction similarly would follow this pattern, although at suboptimal salinities for growth, a specific Na^+ deficiency is evident. The higher nonspecific solute requirement for induction and growth is further emphasized by the observation that high salinities are relatively more inhibitory to uptake than induction or growth.

The influence of salinity on controlling cellular function is also evident from both salinity-temperature and salinity-nutrient interaction studies. Within the growth range of this organism, salinity was shown to be the primary factor in controlling the pattern of cellular function. Only at near maximum growth temperatures and suboptimal growth salinities were synergistic interactions observed for induction and growth. This interaction was an increase in maximal induction or

growth temperature with increased salinity. Other studies have indicated that the optimal temperature for growth similarly displayed this salinity-temperature relationship (17, 23), although this was not observed here. It has been hypothesized that the increased thermal resistance of bacteria was due to the stabilization of proteins by added salt (37, 78). Whether the stabilization effect is associated with membrane and/or intracellular proteins is unknown. Our results suggest that uptake would probably not be the primary location for this stabilization, since it is less sensitive to low salinities than growth and induction. Of significance, therefore, is the finding by Cooper and Morita (8) that synergistic temperature-salinity interactions occur for net protein and RNA synthesis.

This study shows that the induction and function of galactose permease in a marine psychrophilic Vibrio is controlled by the inducer, exogenous substrate and ionic environment, all of which can be postulated to influence induction in nature. Further work should be undertaken to extend this study to natural environments, hopefully to allow a better understanding of the conditions under which organisms are induced in natural systems.

SUMMARY

Lactose enrichment cultures of Antarctic water failed to isolate a psychrophilic marine bacterium with an inducible lactose permease. Instead, a marine psychrophilic Vibrio (Ant-12) which could accumulate nonmetabolizable ^{14}C-TMG through a galactose permease system was isolated. Substrate and inducer specificities revealed that Ant-12 possessed only one galactose uptake system which was different from those found in E. coli. Uptake and accumulation of ^{14}C-TMG did not require phosphorylation and had a Km value of 4.8×10^{-6} M. Accumulation of ^{14}C-TMG was energy dependent, as was shown by electron and metabolic inhibitor studies and the requirement for an exogenous energy source. The addition of a precursor supply of an amino acid, in addition to the inducer, was required for induction.

Studies on specific ion requirements for cellular function indicated that a specific Na^+ requirement exists for uptake and that this requirement varied quantitatively with the substrate being transported. Similarly, induction had a specific Na^+ requirement which was higher than that required for growth and uptake. A specific K^+ requirement was found to be quantitatively the same for uptake, growth, and induction.

Salinity studies have demonstrated that the specific ion requirements for cellular function were satisfied at suboptimal growth

salinities. A nonspecific solute requirement controlled cellular activities within the growth range of the organism. Evidence was given that the effect of the nonspecific solute was not completely osmotic in nature. Both salinity and the nonspecific solute requirement tended to support in the following order: induction > growth > uptake.

Salinity rather than temperature or nutrient concentration was shown to be the primary factor in controlling the pattern of cellular function. At near maximum growth temperatures and suboptimal salinities, the maximum temperature for growth and induction was found to increase with increasing salinities.

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