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Title: THE EFFECTS OF HYDROSTATIC PRESSURE AND TEMPERATURE ON THE UPTAKE AND UTILIZATION OF AMINO ACIDS BY A FACULTATIVELY PSYCHROPHILIC MARINE BACTERIUM

Abstract approved: Redacted for Privacy
Dr. Richard Y. Morita

The transport response of four marine psychrophilic bacteria to a variety of substrates was determined and related to environmental conditions. Studies on pressure and temperature effects on glutamic acid transport and utilization indicated that high pressures and low temperatures inhibit glutamate transport, while permitting glutamate respiration. Similar results were obtained for glycine, phenylalanine, and proline. The respiration patterns for all four amino acids differed at 5 C. The differences in pressure effects on respiration is thought to be due to differences in the pressure response of the enzymes of the respiration pathways and not those of the biosynthetic pathways. Pressure effects on the transport systems of all four amino acids were reversible to some degree. Both

proline and glutamic acid were able to protect their transport proteins against pressure damage. The significance of these results for pressure studies is discussed.

The Effects of Hydrostatic Pressure and Temperature on the Uptake and Utilization of Amino Acids by a Facultatively Psychrophilic Marine Bacterium

by

Kala Lapidus Paul

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Professor of Microbiology and Oceanography
in charge of major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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Typed by Cheryl E. Curb for Kala Lapidus Paul

Dedication:

To those

whom I love.

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With sincere thanks to:

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All my parents;

and

Jim, who knows what it all means.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF FIGURES (Cont.)

LIST OF FIGURES (Cont.)

LIST OF TABLES

THE EFFECTS OF HYDROSTATIC PRESSURE AND TEMPERATURE ON THE UPTAKE AND UTILIZATION OF AMINO ACIDS BY A FACULTATIVELY PSYCHROPHILIC MARINE BACTERIUM

INTRODUCTION

Microorganisms living in the open ocean must be able to transport and metabolize nutrients for cell maintenance and growth at various salinities, low temperatures, and under varying hydrostatic pressure. The salinity of seawater ranges from 30 to 40°/oo (parts per thousand) and averages 35°/oo (Sverdrup et al., 1942). Temperatures in the marine environment vary from -1.9 C to 30 C (Sverdrup et al., 1942); however, the greater part (90%) of the world ocean is at temperatures below 5 C (ZoBell, 1961). Hydrostatic pressure increases with increasing depth of the water column by approximately 1 atm per 10 m, the average pressure being about 380 atm (ZoBell, 1961).

There has been ample documentation of microorganisms able to survive under these conditions. MacLeod (1965) described marine organisms requiring seawater or its equivalent for growth; Kates and Hagen (1964), Morita and Haight (1964), and Evison and Rose (1965) reported on psychrophilic bacteria with low optimum and maximum growth temperatures and ZoBell and Morita (1957-59) confirmed the existence of barophilic marine forms.

In a nutrient spare environment with less than 1 mg of utilizable, dissolved organic carbon per liter (Duursma, 1965), efficient nutrient uptake and utilization has definite survival value, and the effect of changes in the physical environment on the nutrient sequestering system of a marine microorganism would have definite survival repercussions.

This investigation is concerned with the effect of pressure and temperature on amino acid transport and utilization in a bacterium designated as MP-38, a facultatively psychrophilic marine bacterium.

LITERATURE REVIEW

Information about the nature of bacterial transport systems is necessary for an understanding of pressure-temperature effects on such a system. Pardee (1968) and Kay and Gronlund (1969) present a comprehensive picture of active transport which suggests that uptake is an enzymatic process. The substrate specificity of a system, its dependence on energy metabolism, temperature sensitivity, adherence to Michaelis-Menten kinetics, and the loss of uptake ability through mutation all point to permease or protein mediated transport.

Application of hydrostatic pressure has a variety of consequences for proteins, including degree of hydration, ionization, electroconductivity, and dipolar interaction (Morita, 1967). These factors, as well as the degree of ionization of the buffer, solvent structure changes, pH variation and weakening of hydrogen bonds, all affect the stability and reactivity of proteins under pressure (Johnson, Eyering, and Polissar, 1954).

Hydrostatic pressure may cause macromolecules to undergo a molecular volume decrease or prevent a molecular volume increase (Morita, 1957). Laidler (1951) proposed that pressure inhibits enzyme activity by preventing the unfolding of a protein necessary for the normal molecular volume increase during formation of the enzyme-substrate complex. Haight and Morita (1962) demonstrated

that the aspartase of Escherichia coli was inhibited at pressures which prevented a molecular volume increase. Hill and Morita (1964) and Morita (1957) showed that the activity of the dehydrogenases of Allomyces macrogynus and E. coli, respectively, decreased with increasing pressure applied at the same temperature.

High temperature effects on proteins are also partially due to molecular volume change. Since thermal denaturation of proteins is accompanied by an increase in molecular volume (Johnson et al., 1954), the negative molecular volume change effected by pressure should counterbalance high temperature effects. This was shown to be the case for a malic dehydrogenase (Morita and Haight, 1962) and an inorganic pyrophosphatase from bacteria (Morita and Mathemeier, 1964) where hydrostatic pressure permitted enzyme function above 100 C, a normally restrictive temperature. Haight and Morita (1962) used pressure to stimulate deamination of aspartic acid by an aspartase from E. coli at temperatures above the optimum temperature at 1 atm. At 37 C and 45 C, increasing pressure decreased total aspartase activity, but increasing pressure at 56 C increased enzyme activity. Pressure also protected the enzyme from thermal denaturation in the presence of the substrate.

Landau (1966, 1970) has published data on the effect of pressure on protein and nucleic acid synthesis. His conclusion, based on comparison of amino acids and nucleotides, is that the order of pressure

inhibition of macromolecular synthesis is protein, DNA, and RNA. These findings agree with those of Pollard and Webber (1966) and Albright and Morita (1968).

METHODS AND MATERIALS

Organisms

A facultatively psychrophilic marine bacterium, designated as MP-38, isolated from station NH-4 (Department of Oceanography, Oregon State University, R/V Acona, Cruise 6406) by R. D. Haight, was used for experiments on pressure and temperature effects on transport and studies on nutrient specificity. Although not given a genus and species name, the taxonomic characteristics are listed in the appendix. Vibrio marinus, MP-1 (ATCC 15381) an obligately psychrophilic marine bacterium, MP-37, and MP-39, unidentified marine psychrophiles were also used for nutrition studies. Stock cultures of these organisms were kept on agar slants at 5 C and transferred bi-monthly.

Media

Cells were grown in a dilute nutrient saline medium (NSM) consisting of (g/L) Rila Marine Salts (Rila Products Co., Teaneck, N.J.) 35; succinic acid, 0.02; polypeptone (BBL), 0.5; yeast extract (Difco), 0.3. The nutrients were kept in a sterile concentrated solution, pH 7.4, and diluted 1:10 with 35 $^{o}/_{oo}$ (parts per thousand) artificial seawater (ASW) before being dispensed. The ASW was adjusted to pH 7.4 with NaOH. The dispensed medium was autoclaved at 15 psi for

10-20 min., depending on volume, and cooled to 15 C prior to inoculation. ASW, in all cases, refers to a solution of Rila salts of 35 g/l, pH 7.4. When a solid medium was needed, NSM was solidified with 1.5% agar (Difco).

Growth Curve

A growth curve was determined for MP-38 in NSM at 15 C with shaking in an incubator-shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The inoculum was prepared from a 12 hr incubation of a transfer from a 12 hr culture. The optical density of the second transfer was read at 600 nm on a colorimeter (Bausch and Lomb, Spectronic 20) in 12 X 112 mm tubes against a medium blank. Dilution of the inoculum was made in NSM in a 500 ml Erlenmeyer flask equipped with a side arm so that the initial O.D. 600 nm would be 0.01. Readings were made at 2 hr intervals for 26 hr.

Optimum Growth Temperature

Optimum growth temperature for MP-38 was determined using a polythermostat constructed by Morita and Haight (1966) after that by Oppenheimer and Drost-Hansen (1960). Tubes containing 15 ml NSM were equilibrated in the polythermostat for 4 hr prior to inoculation. An inoculum (0.1 ml) of a 12 hr culture was placed in each tube and the contents mixed. Readings were made after 8.5 hr on a Beckman

DB spectrophotometer at 600 nm in a cuvette with a 1 cm lightpath.

Nutrient Studies

Cells for nutrient studies were grown for 12 hr in NSM using a 1% (v/v) inoculum from a 12 hr culture. The cells were harvested by centrifugation for 5 min at 10,400 X g in a Sorvall RC2-B refrigerated centrifuge, washed two times with Millipore filtered (MF) ASW and suspended to an $OD_{600\ nm}$ corresponding to approximately 6.0×10^8 cells/ml. Viable count vs. OD was established by plate count using the Bausch and Lomb Spectronic 20 for OD readings. Cell suspensions were diluted $1{:}10^3$ with MF ASW and 15 ml dispensed into 50 ml serum bottles. The ^{14}C- labeled compounds, proline, glycine, phenylalanine, glutamate, glucose, glycolic acid, and acetic acid (Amersham/Searle) were added to a concentration of approximately 20 mg/l, and the bottles capped. Carbon dioxide was not collected.

Viability vs. Pressure

A time course of viability vs. pressure with increasing temperature was done for MP-38 at 5, 20, and 25 C. Portions of the cell suspensions exposed to 100, 300, and 500 atm for 1, 2.5, and 5 hr were plated on NSM agar, incubated at 15 C, and counted after 18 hr. A similar study was done for MP-1 at 5 C.

Pressure Studies

Cells to be used for pressure studies were grown and harvested as in the nutrient studies. Washed cells were suspended to an OD_{600nm} of 0.25, approximately 6.0 X 10^8 cells/ml. The suspension was diluted at 1:10^3 with MF ASW and the ^{14}C-amino acids were added to a concentration of 200 μg/L and 0.02 μc/ml. The radioactive amino acids, L-glycine-^{14}C(u) (Amersham/Searle), L-phenylalanine -^{14}C(u) L-methionine-^{14}C(u), L-proline-^{14}C(u) and L-glutamic acid-^{14}C(u) (International Chemical and Nuclear Corp.) were made up to a concentration of 2 μg/ml (0.2 μc/ml) with cold DL-amino acid (Nutritional Biochemicals Co.) and stored frozen at -10 C. The specific activity of each solution was 100 μc/mg. After addition of the substrate, 10 ml portions of the iced cell suspension were drawn into plastic syringes, tightly capped and kept on ice until pressurized. The syringes were placed in pressure cylinders, described by ZoBell and Oppenheimer (1950), and pumped up to the desired pressure. The blank was treated as a 500 atm sample, only up to temperature equilibration time, after which the cylinder was depressurized. The syringe contents were then emptied into 50 ml serum bottles containing 0.6 ml 0.1 N H_2SO_4 to fix the cells and release CO_2 (Wright and Hobbie, 1965). The bottles were quickly stoppered with rubber serum caps equipped with a plastic bucket containing a 25 X 57 mm piece of

fluted Whatman #1 filter paper. At least ten min after fixing, 0.2 ml phenylethylamine was introduced with a syringe on to the filter paper through the serum cap for $^{14}CO_2$ adsorption. All serum bottles were left sealed at room temperature at least one hour before uncapping (Hobbie and Crawford, 1969).

The fluted filter papers were placed in scintillation vials and counted in 5 ml scintillation fluor containing 5 g/L 2,5-diphenyloxazole (PPO) and 0.3 g/L triphenyldioxazole (POPOP) in toluene. Twelve hr incubation of the filter papers in fluor at 15 C was necessary to permit clearing of the fluor and stabilization of counts. Cells were collected by filtration through 0.45 μ, 25 mm diameter membrane filters, air dried at 60 C for two hr, and counted in 2 ml fluor in a Nuclear Chicago Mark I liquid scintillation counter. The counting efficiency was determined from channels ratio using toluene-^{14}C(u) standard (International Chemical and Nuclear Corp.) quenched with chloroform. Experiments on the effect of substrate concentration or exposure time were set up in the manner described above, with these parameters varied. Exposure time for each temperature in the pressure series was determined from the viability study.

Substrate Utilization Under Pressure

Samples were prepared as above, incubated with ^{14}C-glutamate or ^{14}C-proline for three hr at 5 C, 1 atm, and pressurized to 500 atm.

Cells were depressurized and fixed at three hr and seven hr after pressurization.

Reversibility of Pressure Effect

Samples in syringes were incubated at 5 C under 500 atm pressure for five hr. The pressure was then released and the samples further allowed to incubate at 1 atm for six hr. Sampling was done on release of pressure and during atmospheric incubation.

RESULTS AND DISCUSSION

The effect of temperature on the growth of MP-38 is shown in Figure 1. Optimum growth temperature, defined as the temperature at which the greatest $OD_{600_{nm}}$ is reached in a given amount of time, is about 22 C. MP-38 exhibits a wide temperature tolerance range, being able to grow below 0 C and above 30 C. A growth curve for MP-38 in NSM at 15 C was determined indicating that this organism takes about 24 hr to reach the maximum stationary phase of growth with no major inflection points. The midpoint of the log phase of growth was chosen for harvesting the cells, and a 12 hr incubation period was routinely used for culturing all organisms.

Table 1 represents the uptake response of MP-37, 38, and 39 and V. marinus, MP-1 to a variety of labeled substrates. Uptake was scored positive if 0.5% or more of the available radioactivity was detected in the cells. These counts were not corrected for respiration. All organisms were pre-incubated with the substrates to permit synthesis of inducible transport systems. All four organisms took up the amino acids and not the organic acids. Only MP-1 took up glucose. Figures 3 and 4 indicate that any problem with glucose transport for the three other organisms were not a function of substrate concentration or exposure time. MP-1 actively transported glucose and glutamate under conditions where the other organisms only took up

Table 1. Response of four marine bacteria to a variety of substrates.

Organism	Substrate						
V. marinus	Acetate	Glucose	L-Glutamate	Glycine	Glycollate	L-Phenylalanine	L-Proline
MP-1	-	+	+	+	-	+	+
MP-37	-	-	+	+	-	+	+
MP-38	-	-	+	+	-	+	+
MP-39	-	-	+	+	-	+	+

Uptake response of four marine psychrophiles to several naturally occurring substrates. Cell number was 5.0-7.5 X 10^5 cells/ml in 15 ml samples in ASW. Substrate concentration was about 20 µg/L. Uptake was scored positive if more than 0.5% of the available radioactivity was detected in the cell. No CO_2 was collected.

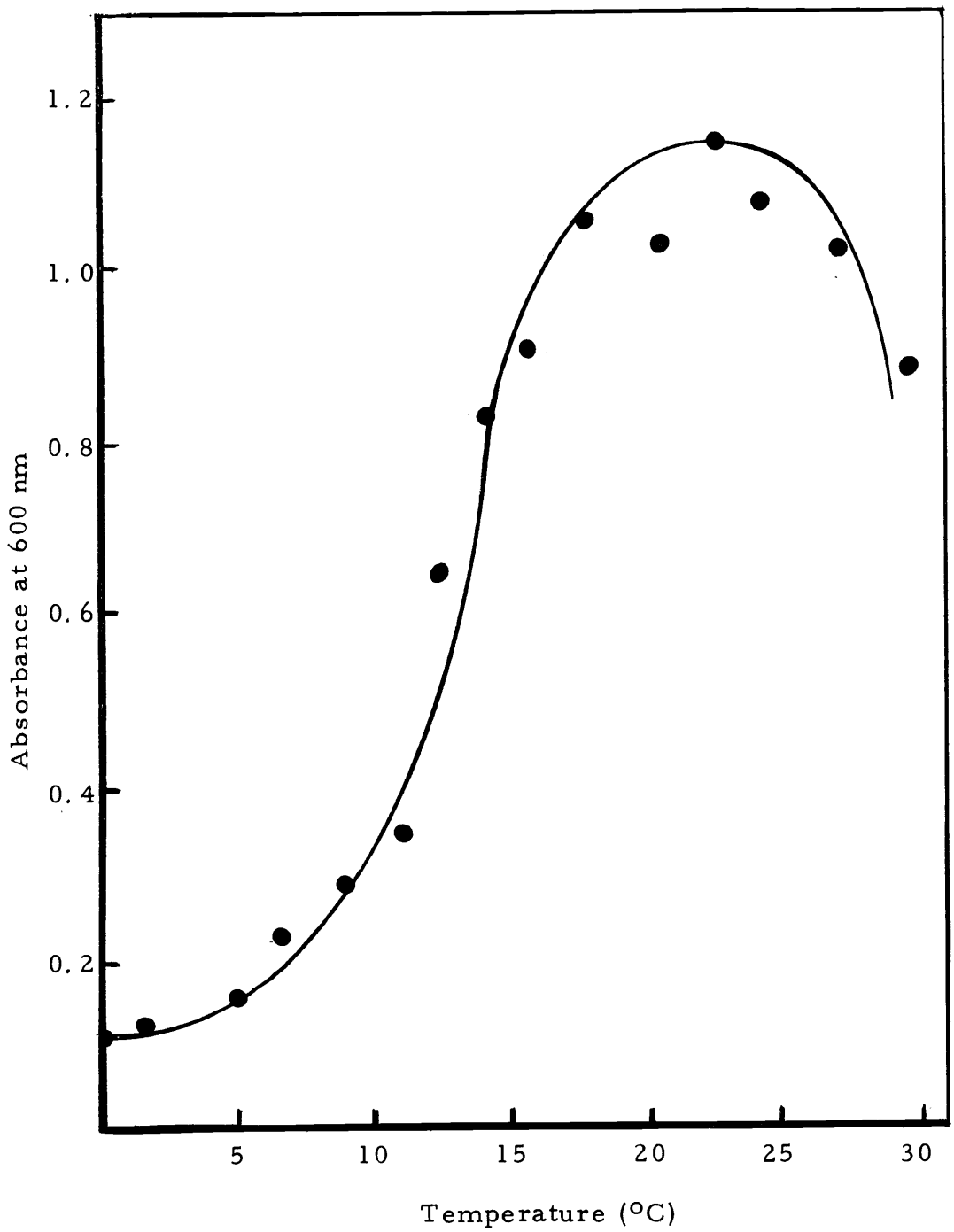

Figure 1. Effect of temperature on growth of MP-38, measured after eight hr incubation in a polythermostat in nutrient seawater medium, using 0.1 ml of a 12 hr culture as an inoculum.

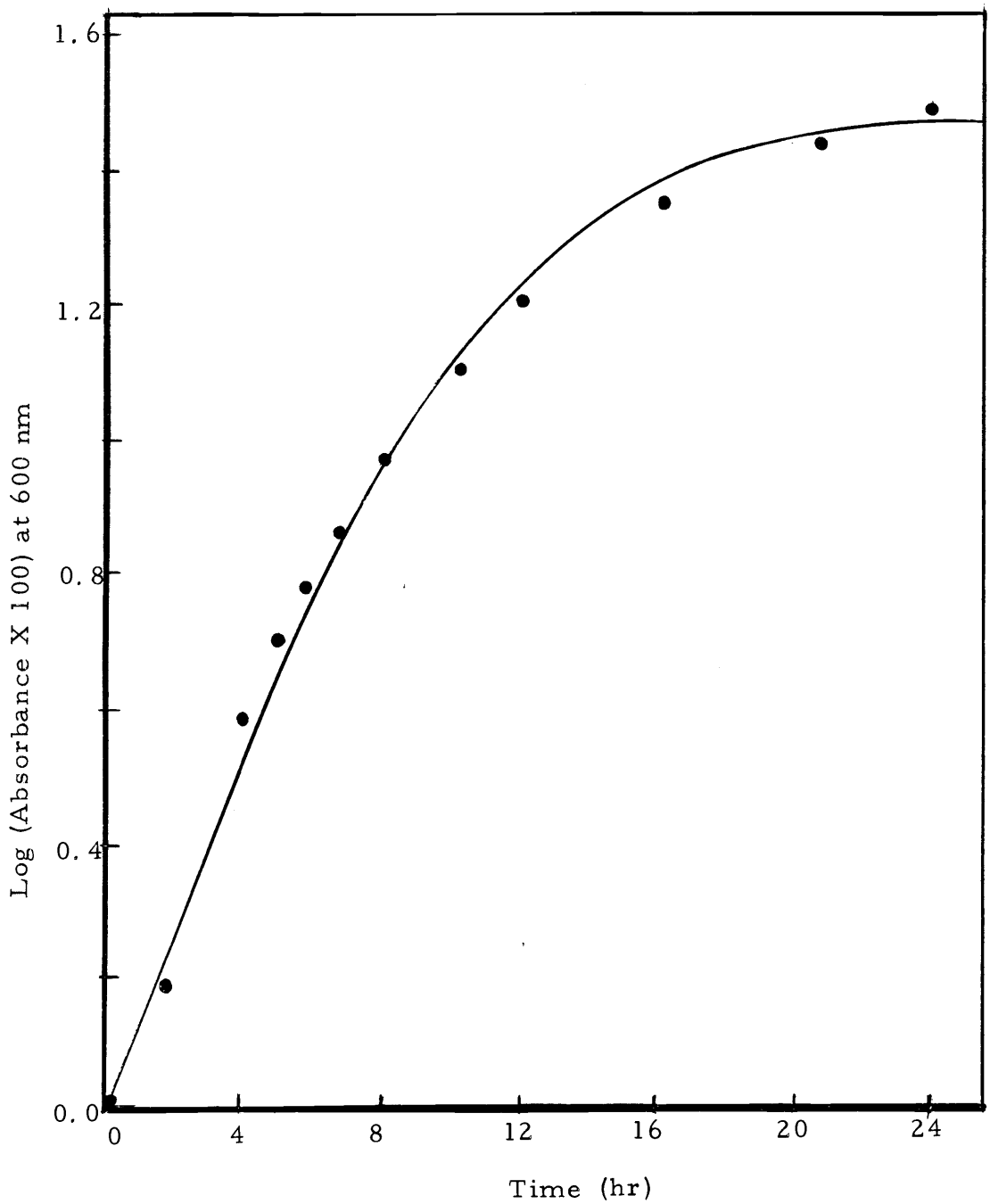

Figure 2. Growth curve for MP-38 incubation with shaking in nutrient seawater medium at 15 C. The inoculation was diluted to give an initial OD of 0.01.

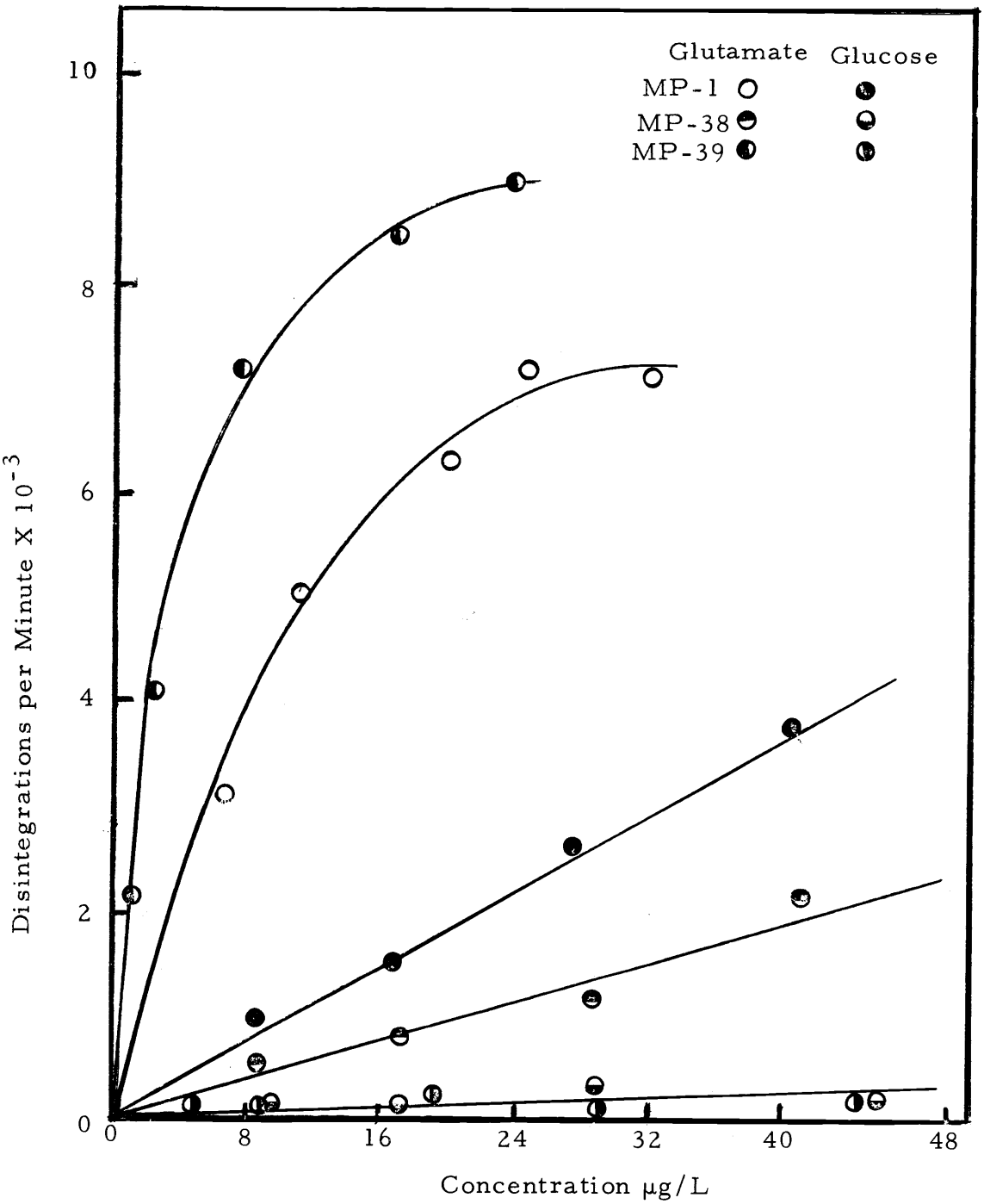

Figure 3. Uptake of ^{14}C-glucose and ^{14}C-glutamate by MP-1, MP-38, and MP-39 at 15 C in 20 min at 1 atm. Cell concentration was approximately 5.0-7.5 X 10^5/ml. No correction was made for respiration.

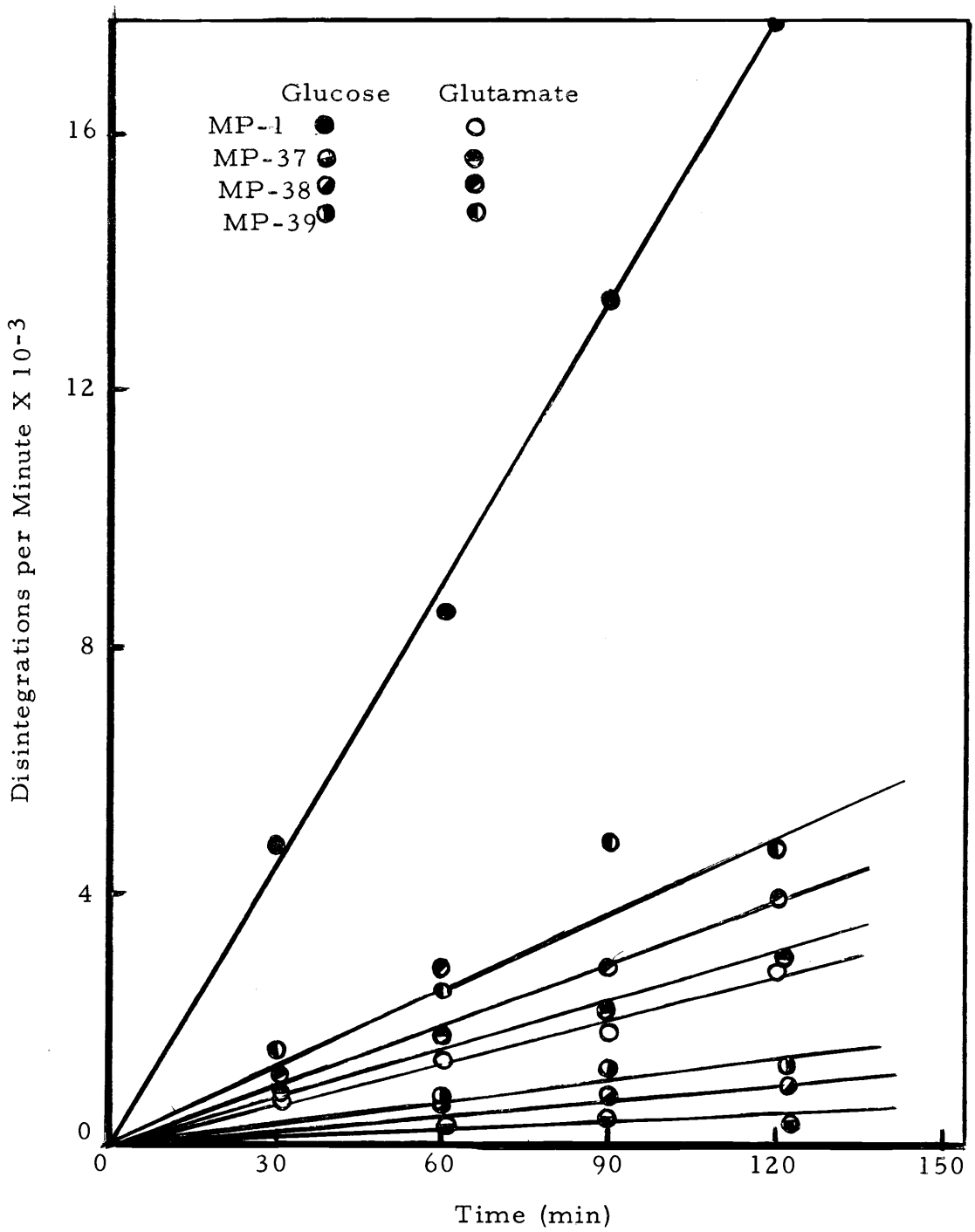

Figure 4. Uptake of ^{14}C-glutamate and ^{14}C-glucose by MP-1, MP-37, MP-38, and MP-39 at 15 C, 1 atm. Cell concentration was 5.0-7.5 x 10^5 cells/ml. in 10 ml. Substrate concentration was 20 μg/L; 0.02 μc/μg ^{14}C-glutamate; 0.03 μc/μg ^{14}C-glucose. No correction was made for respiration.

glutamate. It must be noted that MP-1 is an organism which has been in constant use over the last seven yr, during which time it acquired the ability to use glucose as a sole carbon source (unpublished data, Stanley and Morita, 1964). The other organisms were maintained over a similar period of time in a complex medium which contained a variety of non-carbohydrate sources of organic carbon.

Several investigators (Vaccaro, 1969; Vaccaro and Jannasch, 1966, 1967; Vaccaro et al., 1968; Lewin and Lewin, 1960) have used glucose in studying natural populations in seawater. Although glucose is present in seawater (Menzel, 1964), studies on glucose assimilation or uptake kinetics for a given population may have a built-in error in that they select for glucose utilizers which may be a small portion of the hetertrophic population. In the Antarctic, the heterotrophic microbial population does not appear to utilize glucose but other substrates are taken up readily (unpublished date, Gillespie, Jones and Morita, 1971). Therefore, ^{14}C-glucose, although used by many investigators for the determination of hetertrophic uptake, may not be the correct substrate to employ in the marine environment.

Figure 5 presents the uptake of a variety of amino acids by MP-38 indicating both the specificity of transport proteins and their differing substrate affinities. These data include a CO_2 correction for uptake. The picture of uptake for MP-38 presented by these figures shows a marked preference for amino acids over the other

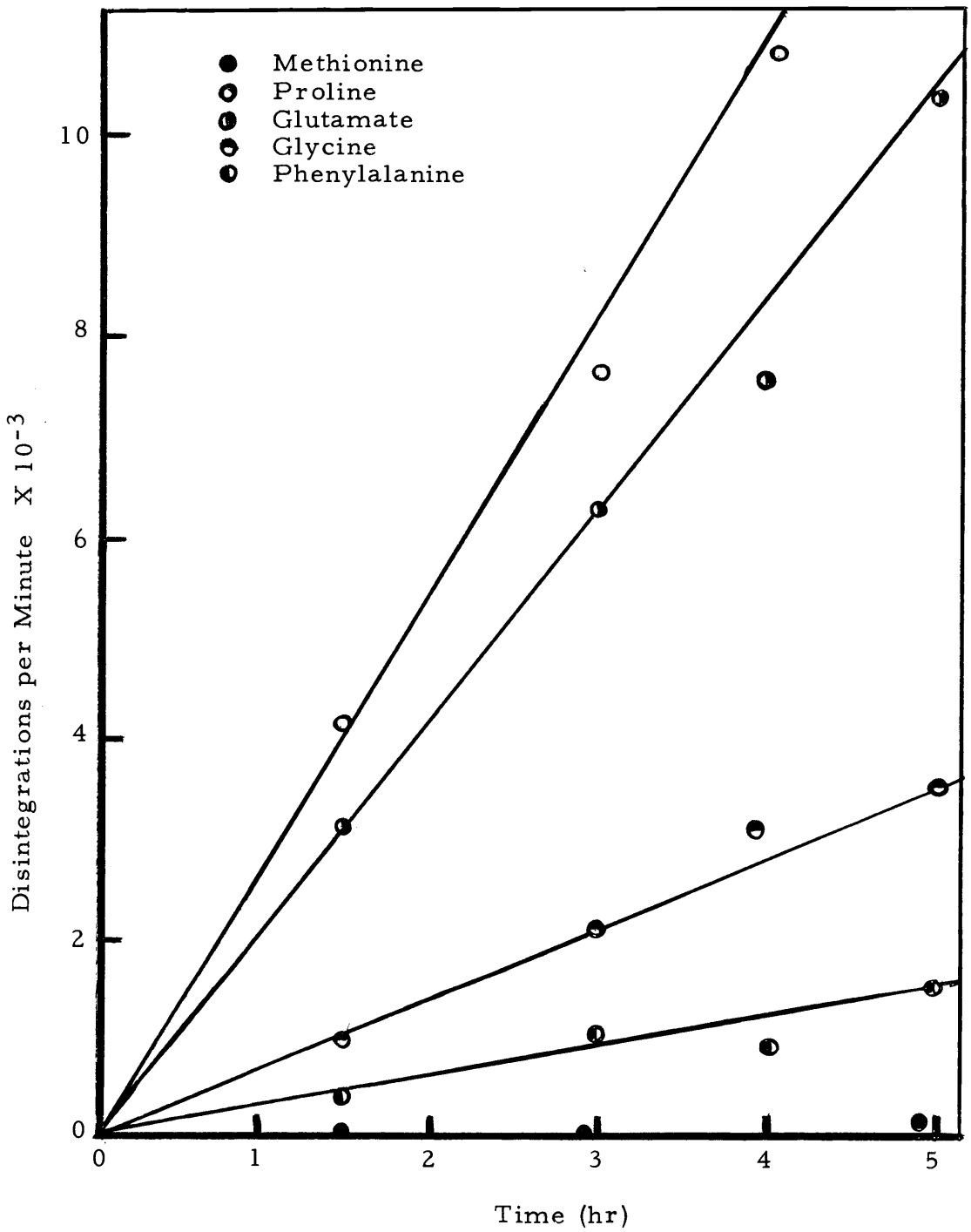

Figure 5. Time course of total uptake of ^{14}C-methionine, ^{14}C-phenylalanine, ^{14}C-glycine, ^{14}C-glutamate, and ^{14}C-proline by MP-38 at 5 C, 1 atm. All amino acid concentrations were 200 μg/L, with an activity of 0.02 μc/ml in 10 ml; the cell concentration was 6.0 X 10^5/ml.

organic substances presented.

Pressure studies were done with MP-38 using glutamic acid because of the key role this amino acid plays in cellular metabolism and because of the organism's pressure tolerance. Pressure-viability studies over the temperature range 5-25 C (Table 2) showed MP-38 remained viable for at least five hr at 5 C up to 400 atm. At 20 C, the organism was able to grow in three hr at pressures below 200 atm, but started to expire at 400 atm after two hr. At 25 C, MP-38 grew at atmospheric pressure, but died at 200 and 400 atm after three hr. The following experiments were run for a period of time at each temperature such that there was no change of cell numbers during the time of exposure. Table 2 also shows MP-38 to be more pressure resistant under the same conditions than MP-1, which started dying immediately above 100 atm.

Glutamate uptake by MP-38 was linear with concentration up to 200 μg/L, the highest concentration used (Figure 6). Uptake was also linear with time for the time interval and over the temperature and pressure range used in these experiments (Figures 7 and 8). This information, taken with the viability studies, indicates that there were no substantial numerical alterations in the bacterial population during the course of the experiment and that decreases in uptake were due to the effects of temperature and pressure. The linearity of uptake with time also indicates that the event or events which accounted for

Table 2. Survival of MP-38 and MP-1 under pressure.

Organism	MP-38			MP-38			MP-38			MP-1			
Temp. °C	5			20			25			5			
Pressure, ATM		200	500	1	200	500	1	200	500	1	100	300	500
Time, hr.													
0	463			71	55	60	237	434	372	205			215
	561			51	54	71	184	423	423	261			240
1	586	726		118	45	83	197	203	218				
		544		103	44	83	138	141	194				
2	780	302		259	70	43	184	237	219	181	294	101	58
		500		274	85	47	197	250	199	175	241	88	28
5	532	714	752	502	334	45	377	187	134	202	138	96	7
	512	650	481	537	356	45	331	150	164	212	160	95	70

Viability studies on MP-38 were done at 5, 20, and 25 C; viability for MP-1 was determined at 5 C. All plate counts are on dilutions of 10^{-3}. Cells were pressurized in syringes in ASW, 35 0/00.

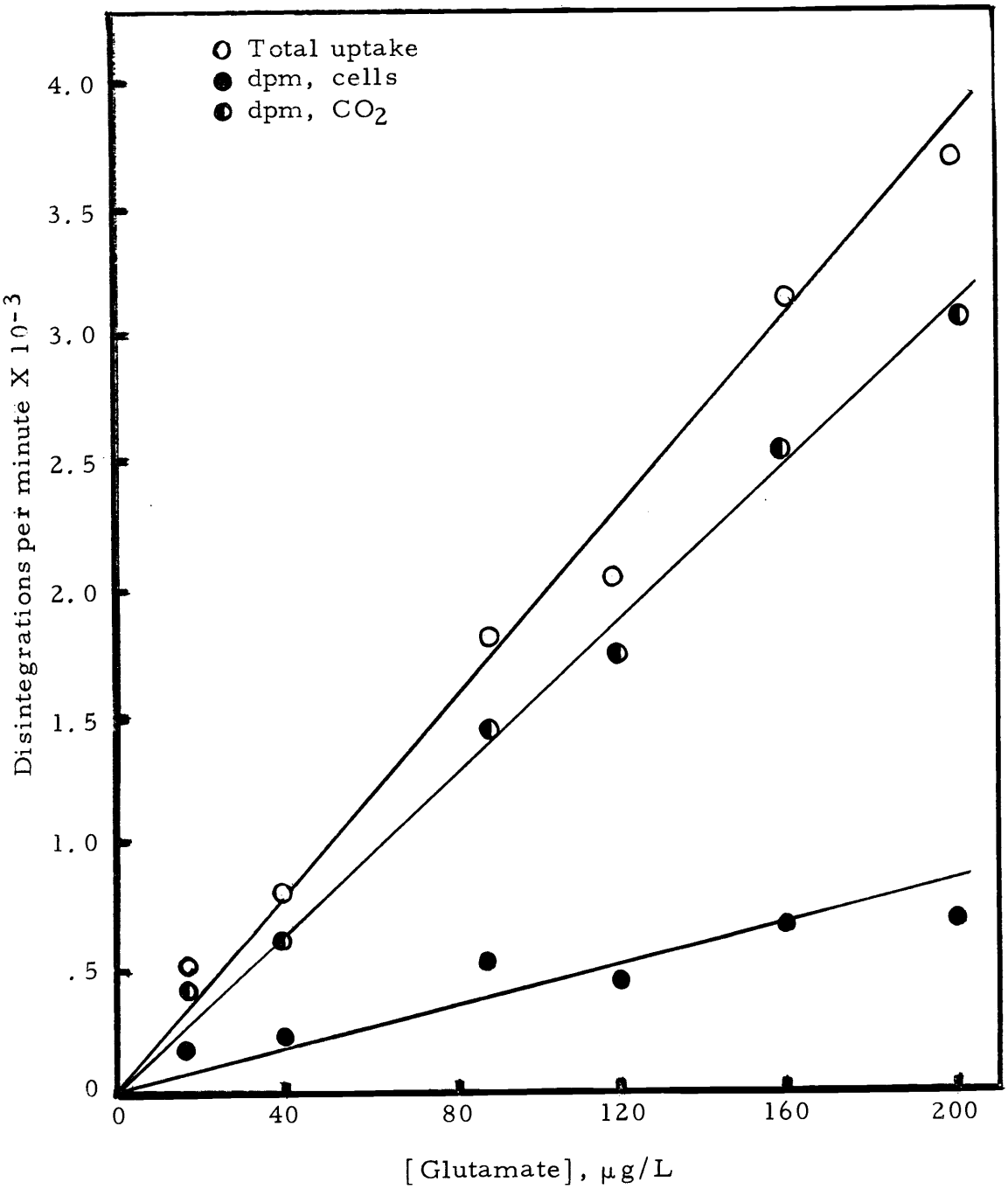

Figure 6. Uptake of ^{14}C-glutamic acid by MP-38 vs. concentration at 15 C, 1 atm. Cell concentration was approximately 6.0×10^5 cells/ml in 10 ml ASW.

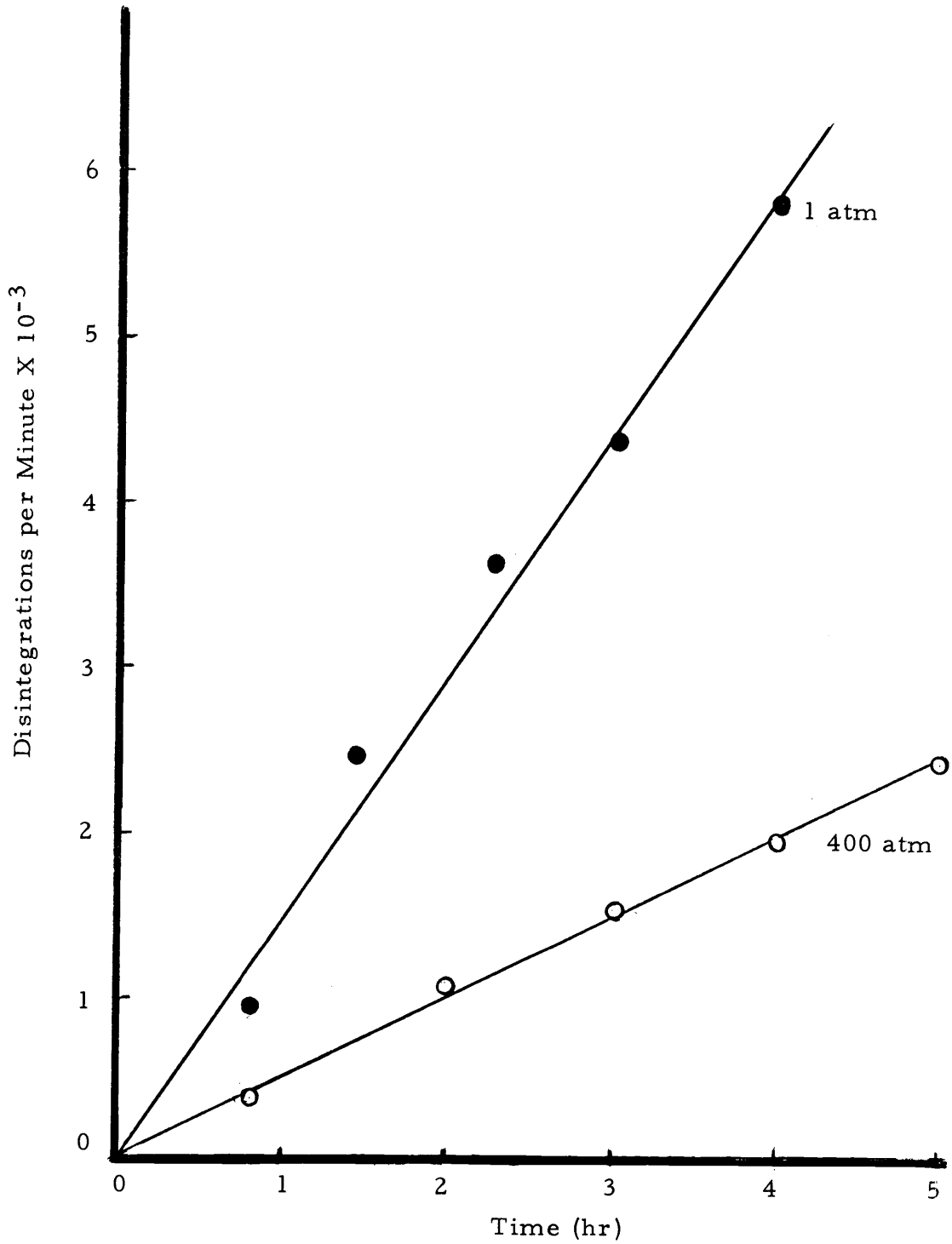

Figure 7. ^{14}C-glutamic acid uptake in artificial seawater by MP-38 at 5 C, 1 atm. Cell concentration was 6.0 X 10^5/ml, ^{14}C-glutamic acid concentration was 200 μg/L with an activity of 0.02 μc/ml in 10 ml.

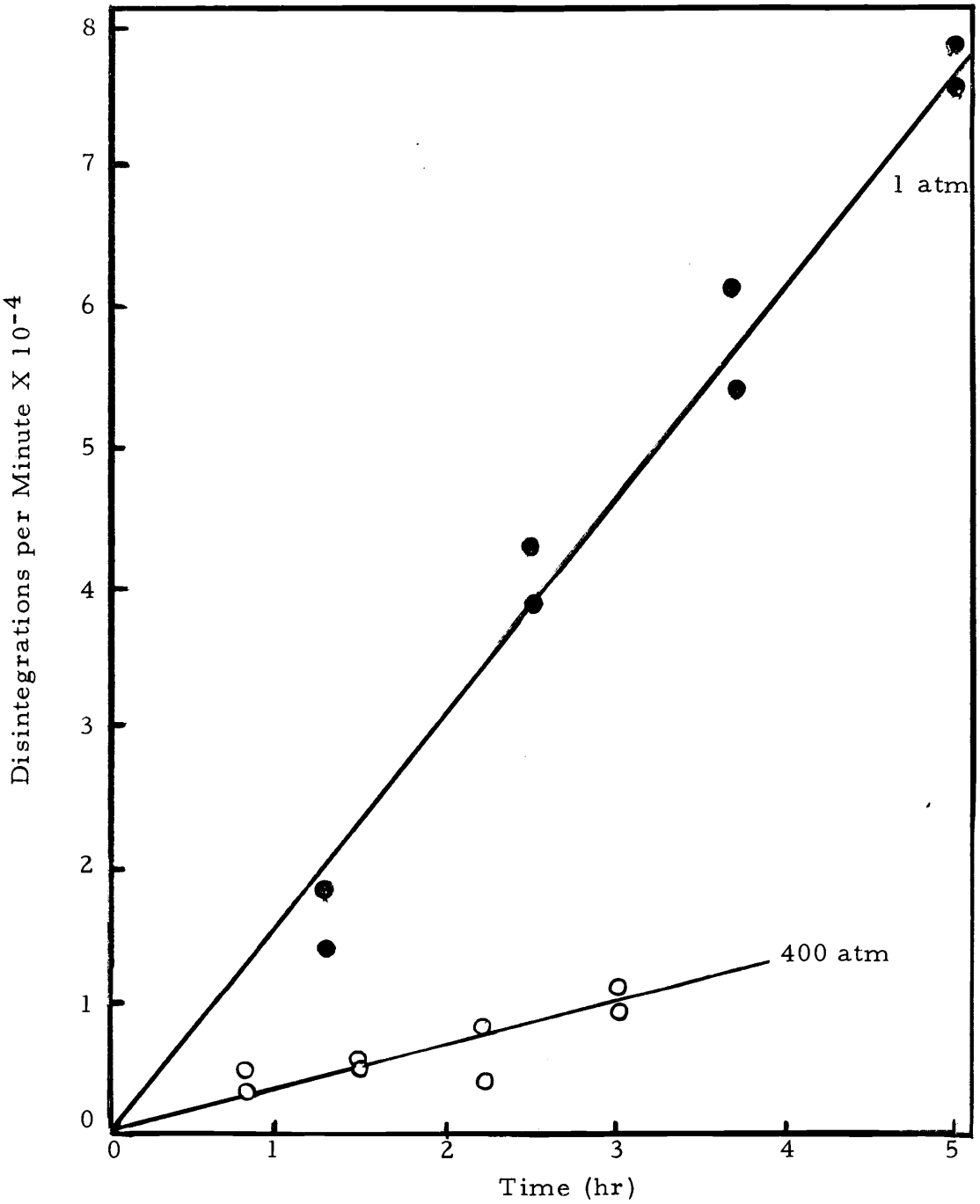

Figure 8. ^{14}C-glutamic acid uptake in artificial seawater by MP-38 at 20 C, 1 atm and 400 atm. Cell concentration was approximately 6.0 X 10^5/ml, ^{14}C-glutamic acid concentration was 200 μg/L, with an activity of 0.02 μc/ml in 10 ml.

decreased uptake with application of pressure happened at the outset of timed period. There was no progressive pressure effect indicated for the time period observed; the uptake rate is constant for each time interval.

Figures 9 through 13 present a comprehensive picture of glutamic acid transport over the temperature range 5 C to 25 C and the pressure range of 1 atm to 500 atm. It can readily be seen that increased pressure decreased the total amount of glutamate taken up by the cells in a given amount of time. The continued production of CO_2 at a constant or increasing proportion of the total, indicates that the cells respired under pressure. It also indicates that glutamate respiration and incorporation into cellular material are affected differently by pressure. The proportion of CO_2 increases as pressure increases at all temperatures up to 400 atm. At temperatures below 25 C, the percent CO_2 dropped or levelled off between 400 and 500 atm; but at 25 C, the proportion of CO_2 increased at 500 atm. The overall increase in CO_2 production may be due to the cell's failure to maintain a balance in the proportion of substrate which goes in to any of the metabolic or synthetic pathways. The factors which alter the distribution of radioactivity may reflect the different effects of temperature and pressure on the enzymes of those pathways. Any set of conditions which favor the activity of the enzymes of the respiratory pathway and/or inhibit those of the synthetic pathways would result in an

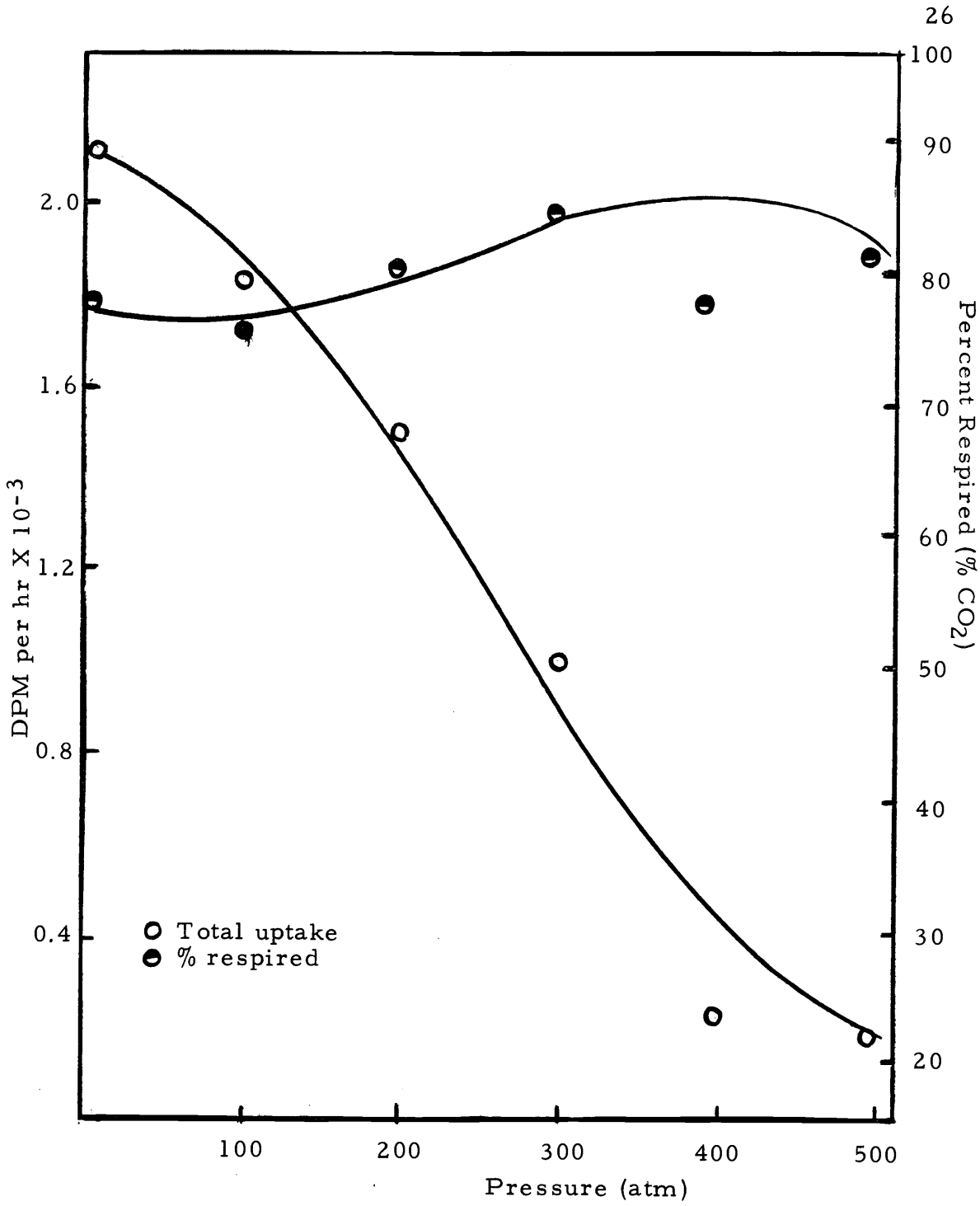

Figure 9. Uptake and respiration of ^{14}C-glutamic acid by MP-38 at 5 C. Cell concentration was approximately 6.0 X 10^5/ml in 10 ml ASW. Glutamate concentration was 200 µg/L with an activity of 0.02 µc/ml. Total exposure time was 5 hr.

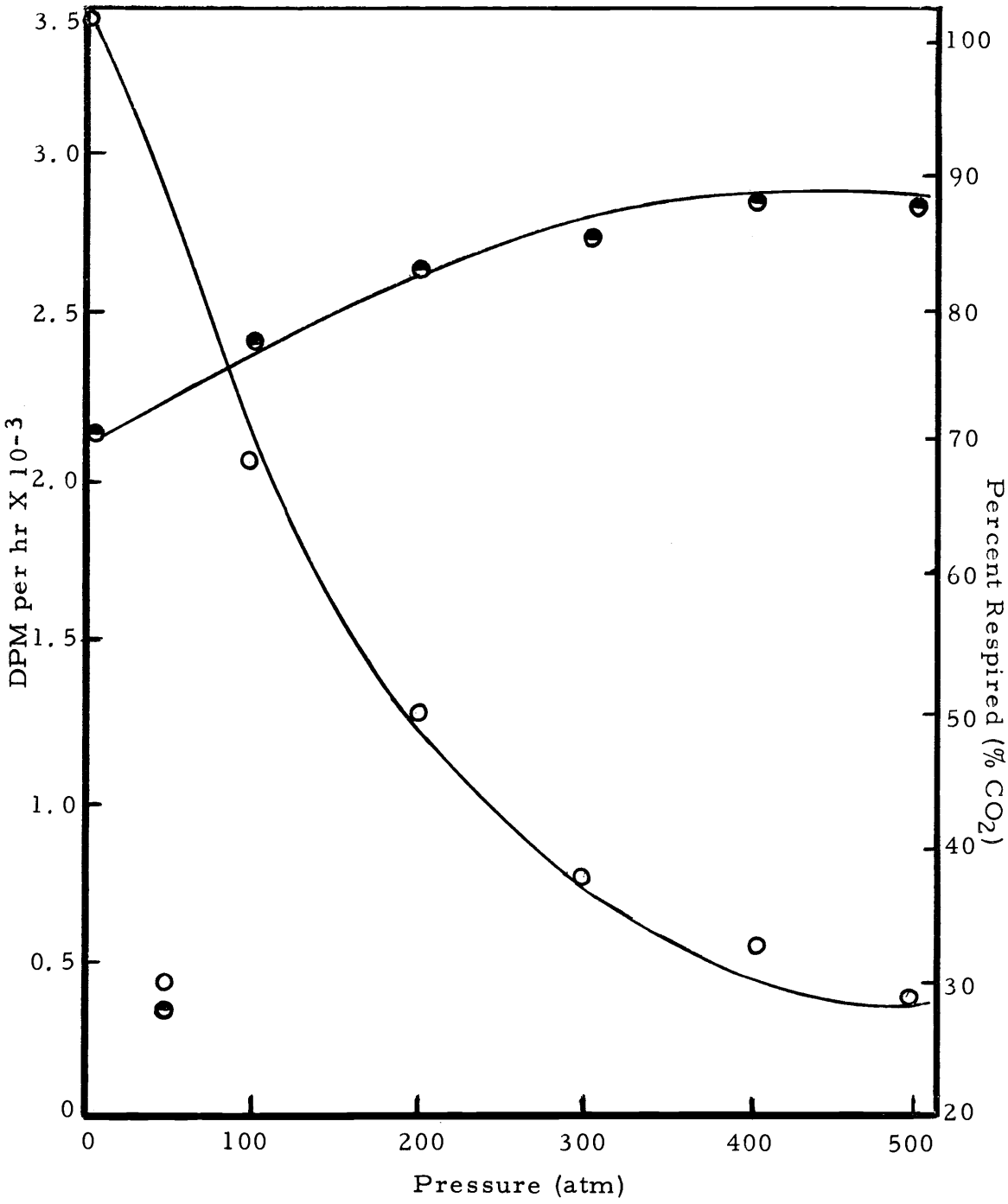

Figure 10. Uptake and respiration of ^{14}C-glutamate by MP-38 at 10 C. Cell concentration was approximately 6.0 X 10^5/ml. in 10 ml ASW; 200 μg/L glutamate with an activity of 0.02 μc/ml. Total exposure time was 3 hr.

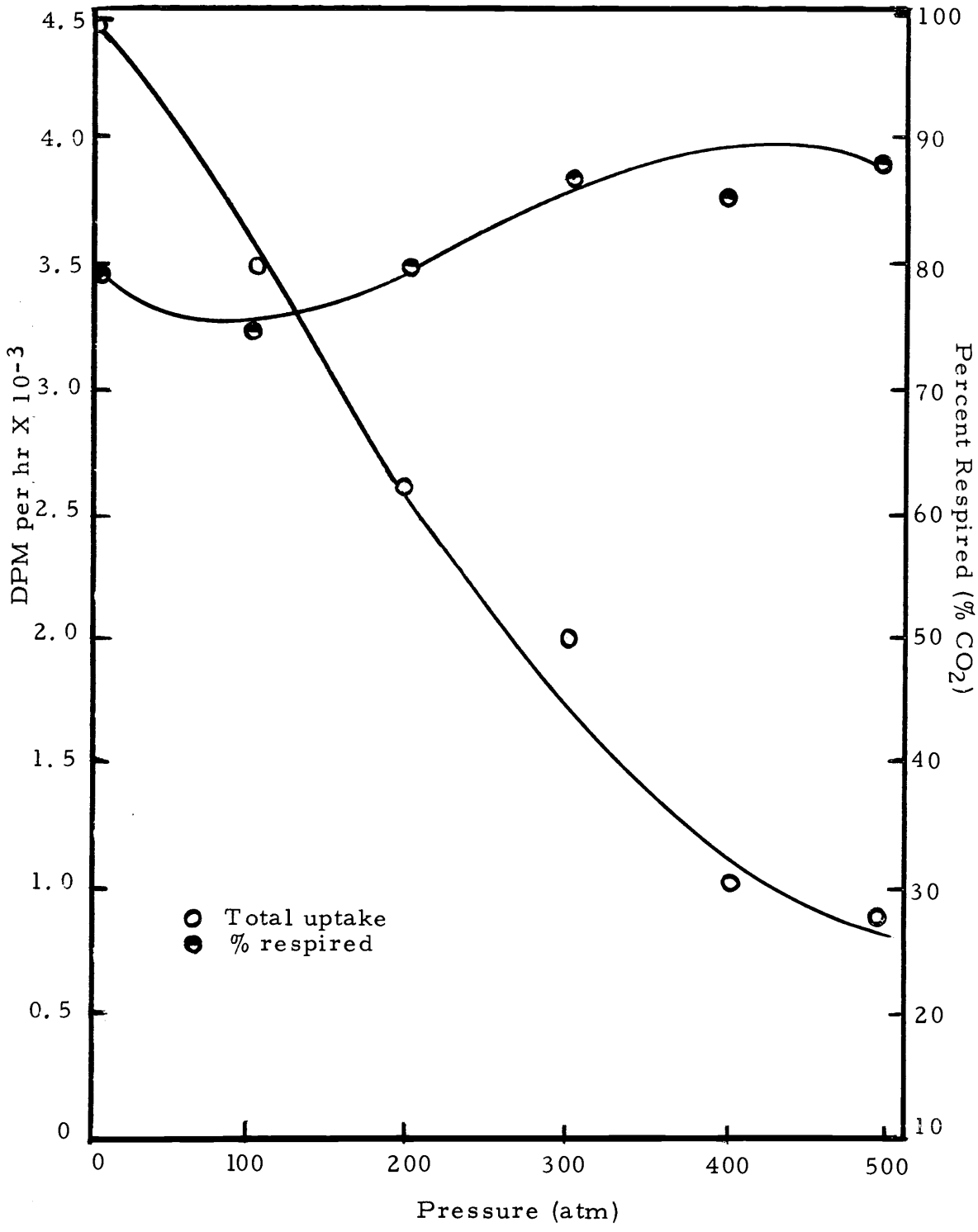

Figure 11. Uptake and respiration of ^{14}C-glutamate by MP-38 at 15 C. Cell concentration was approximately 6.0 X 10^5 cells/ml in 10 ml ASW; 200 μg/L glutamate, with an activity of 0.02 μc/ml. Total exposure time was 2 hr.

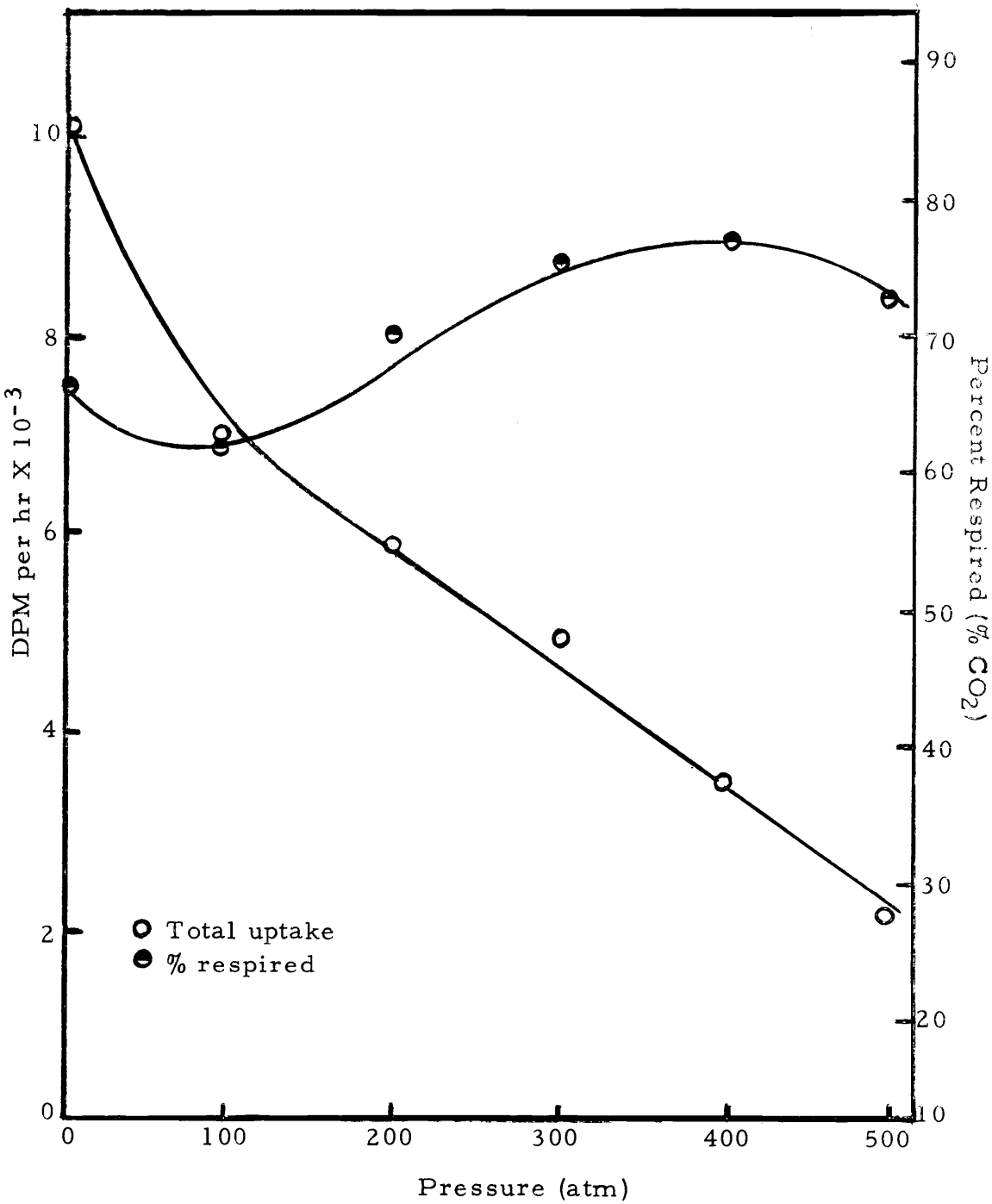

Figure 12. Uptake and respiration of ^{14}C-glutamate by MP-38 at 20 C. Cell concentration was approximately 6.0 X 10^5 cells/ml in 10 ml ASW; 200 μg/L glutamate with an activity of 0.02 μc/ml. Total exposure time was 2 hr.

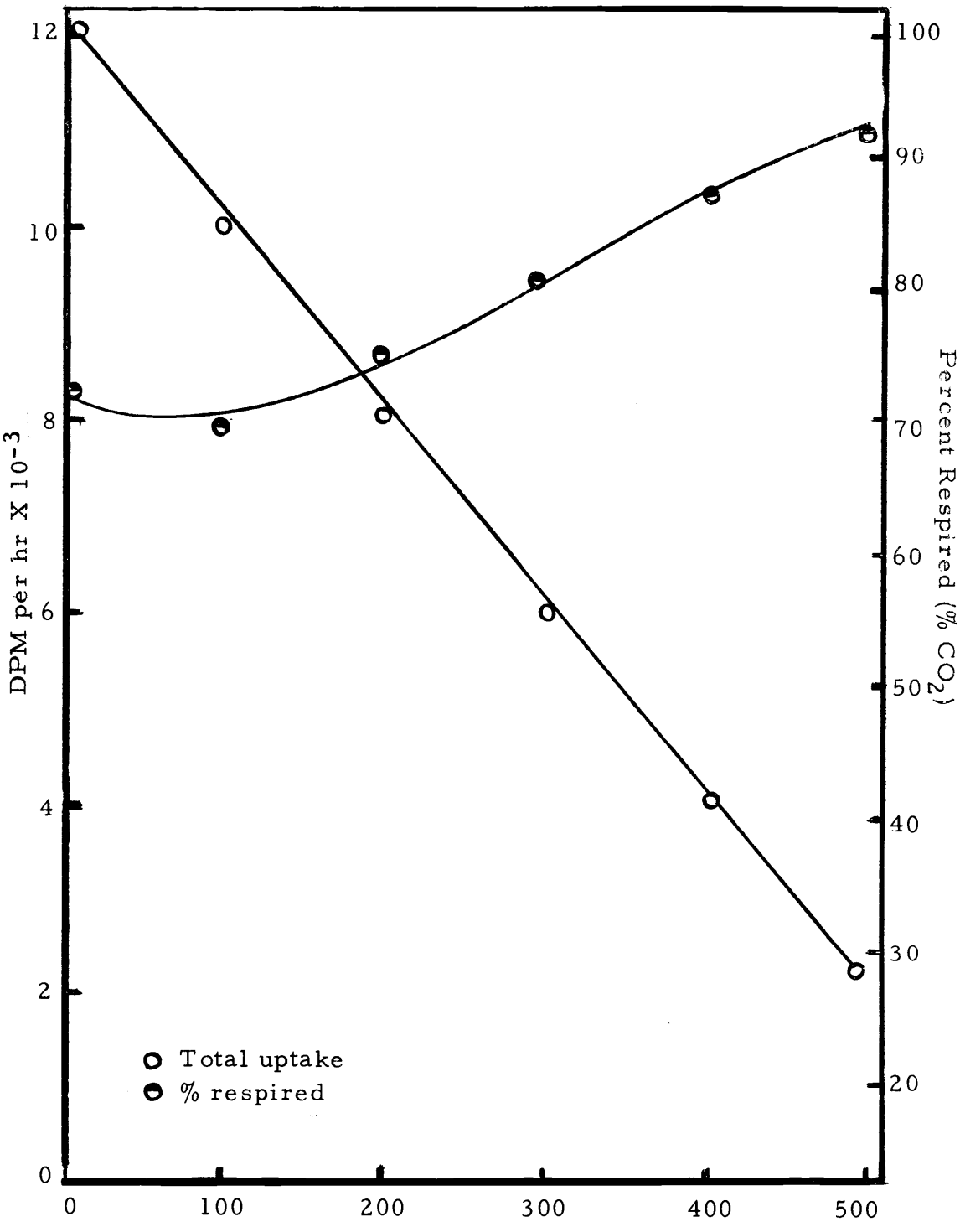

Figure 13. Uptake and utilization of ^{14}C-glutamic acid by MP-38 at 25 C. Cell concentration was approximately 6.0 X 10^5 cells/ml in 10 ml ASW; 200 μg/L glutamate, with an activity of 0.02 μc/ml. Total exposure time was 3 hr.

increased proportion of CO_2. If pressure depressed incorporation more than respiration, the availability of substrate for the respiratory enzymes would be effectively increased; that is, the competition for glutamate is decreased. It is possible that the effects of pressure on enzyme structure, solvent structure, substrate ionization, etc., might result in increased enzyme-substrate affinity which would permit more effective competition for substrate on the part of the respiratory enzymes.

Carbon dioxide production may not be as affected by pressure as incorporation due to a possible intrinsic ability of respirational enzymes to resist the molecular volume decrease affected by high pressure. If these enzymes, or a rate-limiting enzyme, undergo only a small molecular volume increase on forming the E-S complex, the effects of pressure may be minimized. As the temperature is increased, the effect of pressure should be further minimized and finally offset. This may have been the case for glutamate uptake between 400 and 500 atm. At the lower temperatures, 500 atm may have been sufficient to inhibit respiration by preventing molecular volume increase. At 25 C, the effects of heat may have compensated for the effects of pressure, permitting continued or increased activity of respiration enzymes.

Although the proportion of CO_2 increases, the actual CO_2 production decreases, as does the actual amount of glutamate incorporated.

The actual amount of glutamate respired or incorporated decreases with pressure because the total amount of glutamate taken up decreases with increasing pressure.

It is unlikely that glutamate uptake was influenced by amino acid pool levels. The high proportion of respired substrate indicates that the glutamate was rapidly utilized and spent little time in the pool. Using similar amino acid concentrations, Kay and Gronlund (1969a) found that pool levels did not influence amino acid uptake for Pseudomonas aeruginosa also because of rapid utilization.

Figure 14 shows the uptake of glutamate as a function of temperature at 1 atm and 400 atm pressure. A comparison of the temperature-growth and temperature-uptake curves at atmospheric shows that the optimum temperature of growth is lower than the optimum and maximum of the transport system which have not been reached at 25 C. The temperature-pressure optimum effect discussed by Johnson (1970) cannot be completely demonstrated with this data since the optimum temperature at atmospheric pressure was not attained. However, it can be seen that, as the optimum temperature is approached from a lower temperature by raising the incubation temperature, the effect of pressure is decreased. The values for uptake at low temperatures are more depressed as compared with atmospheric values than are those at temperatures closer to the optimum for the glutamate transport mechanism. The inhibiting effect of pressure should be

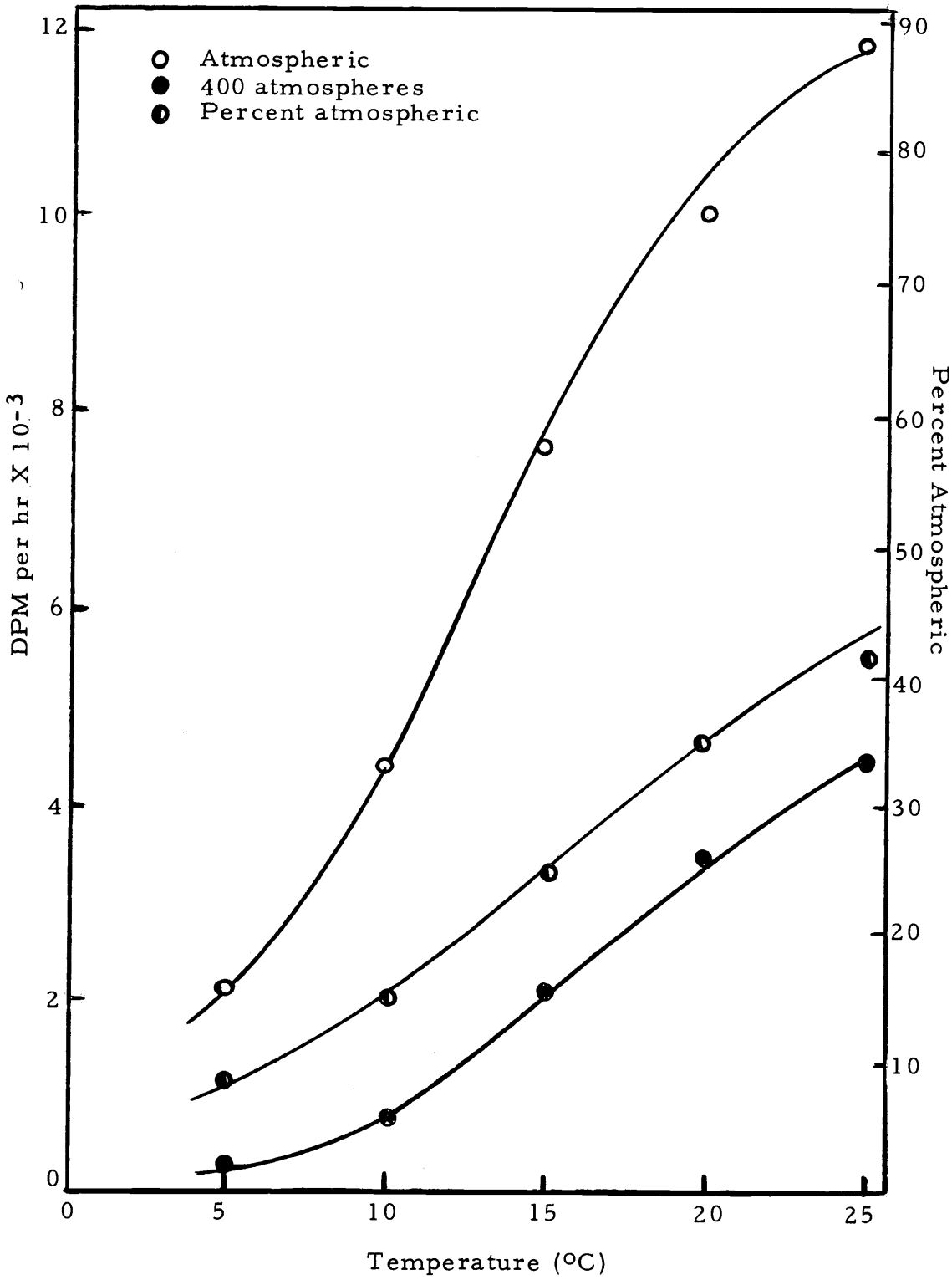

Figure 14. Total uptake of ^{14}C-glutamate by MP-38 at atmospheric and 400 atm pressure, over the temperature range 5 C to 25 C. Uptake of glutamate at 400 atm is plotted as percentage of the uptake at 1 atm at the same temperature.

minimized at the optimum temperature; and at higher temperatures, pressure should accelerate transport and protect transport proteins from heat damage (Johnson, 1970; Morita and Haight, 1962). Studies on the transport of glycine, phenylalanine, and proline were also done at 5 C (Figures 15 through 17). As with glutamate, increased pressure reduced the total amount of amino acid taken up by the organism. However, the respiration patterns at 5 C are different for all four amino acids. Where glutamate respiration increased up to 400 atm and then decreased slightly, glycine respiration was depressed strongly at higher pressure, proline respiration increased, and phenylalanine CO_2 production was relatively unaffected. Since pressure affected amino acid incorporation into protein the same amount for each amino acid, the differences in respiration patterns are probably due to intrinsic differences in the respiration enzymes for each amino acid.

Proportional glycine respiration showed little increase with increasing pressure and was depressed above 400 atm. The relative amounts for incorporation and respiration may not be altered because both systems are equally pressure resistant up to 400 atm. Another possible explanation is that, regardless of the response of the incorporation system, the respiration enzymes are limited by pressure, so that effective substrate levels have no influence. Above 400 atm these enzymes are overcome by pressure effect and strongly inhibited.

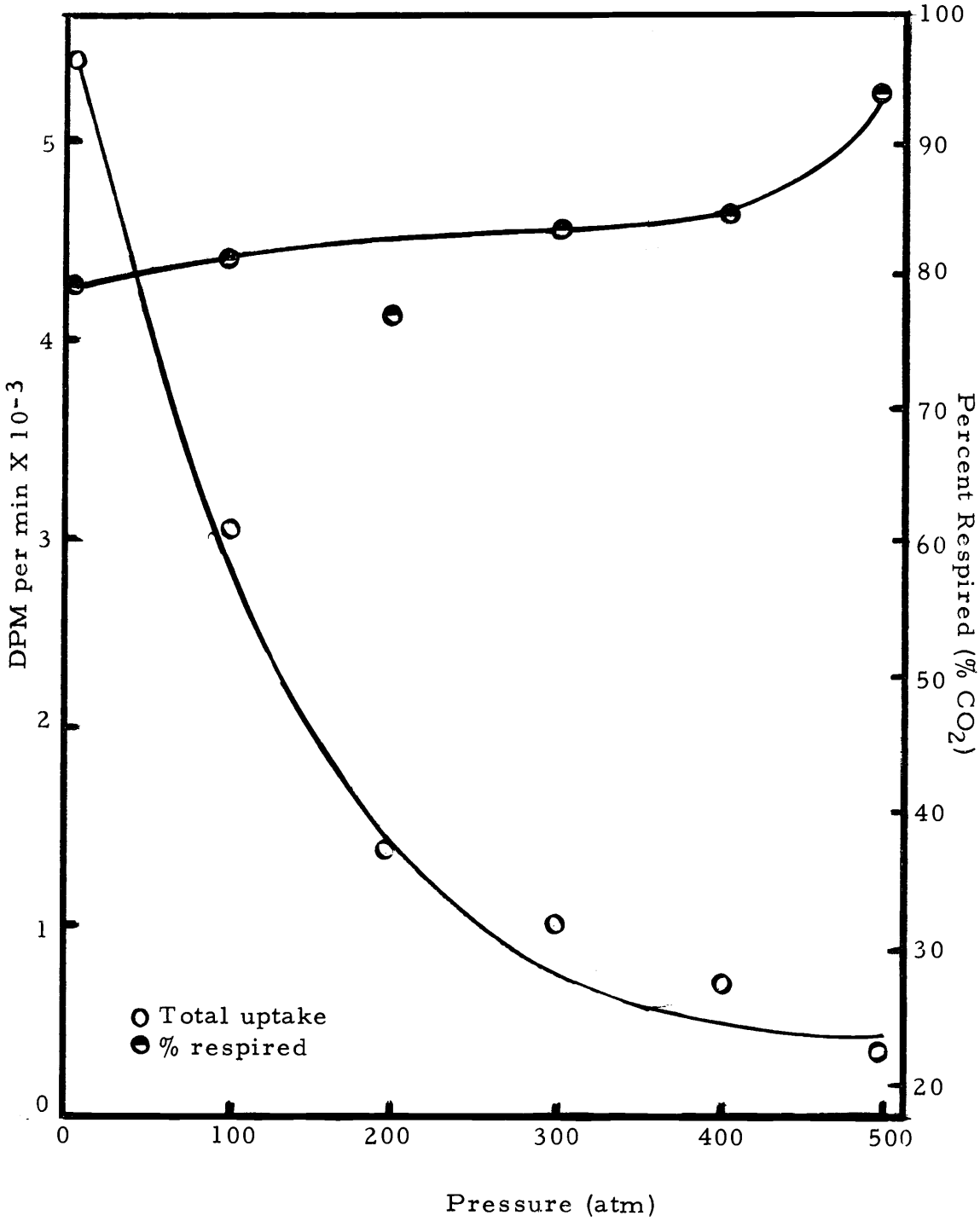

Figure 15. Uptake and respiration of ^{14}C-proline by MP-38, 5 C, five hr exposure. Cell number was approximately 6.0 X 10^5ml in 10 ml ASW; proline concentration was 200 μg/ml, with an activity of 0.02 μc/ml.

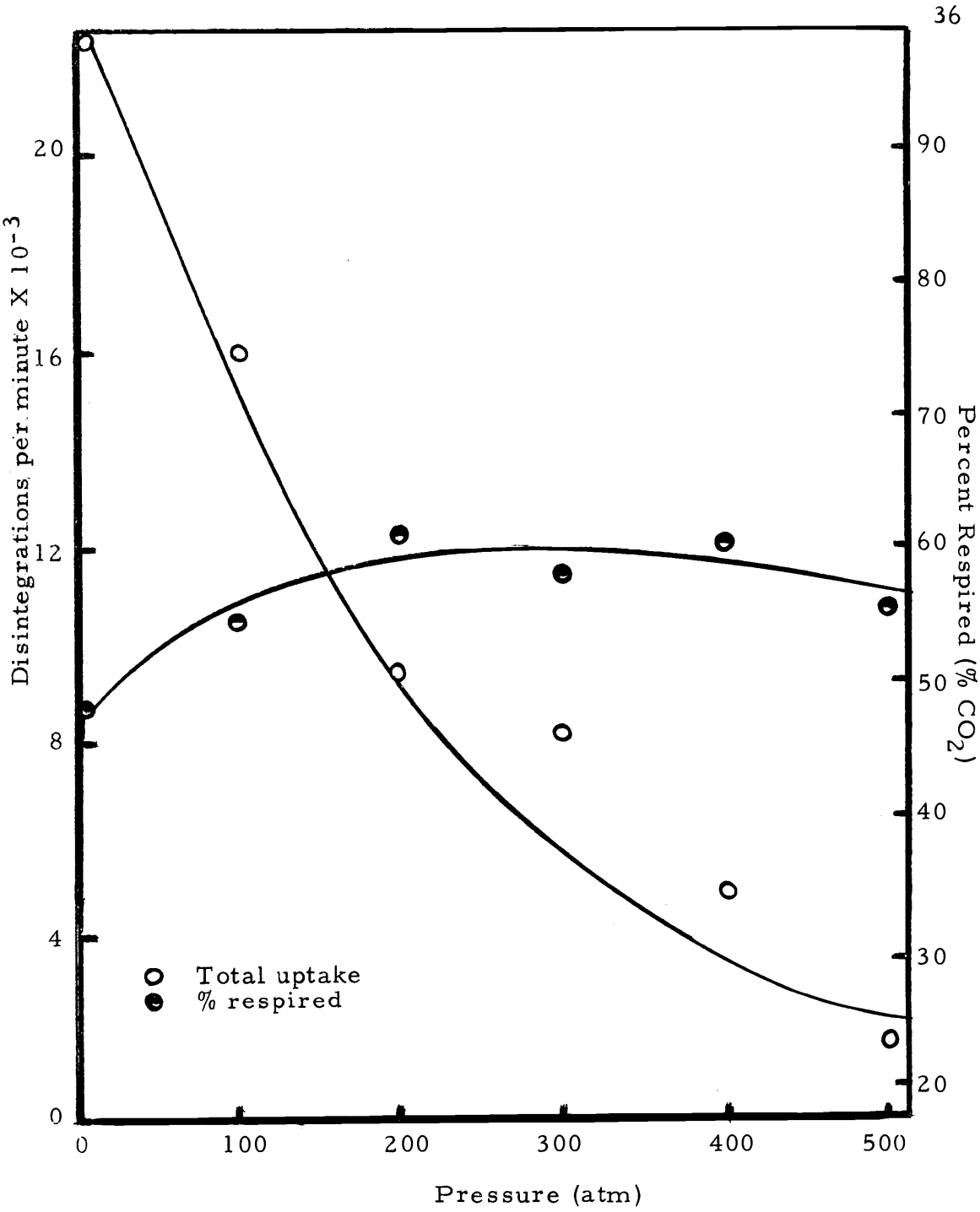

Figure 16. Uptake and respiration of ^{14}C-phenylalanine by MP-38, 5 C, five hr exposure. Cell number was approximately 6.0 X 10^5/ml in 10 ml ASW; 200 µg/L phenylalanine, with an activity of 0.02 µc/ml.

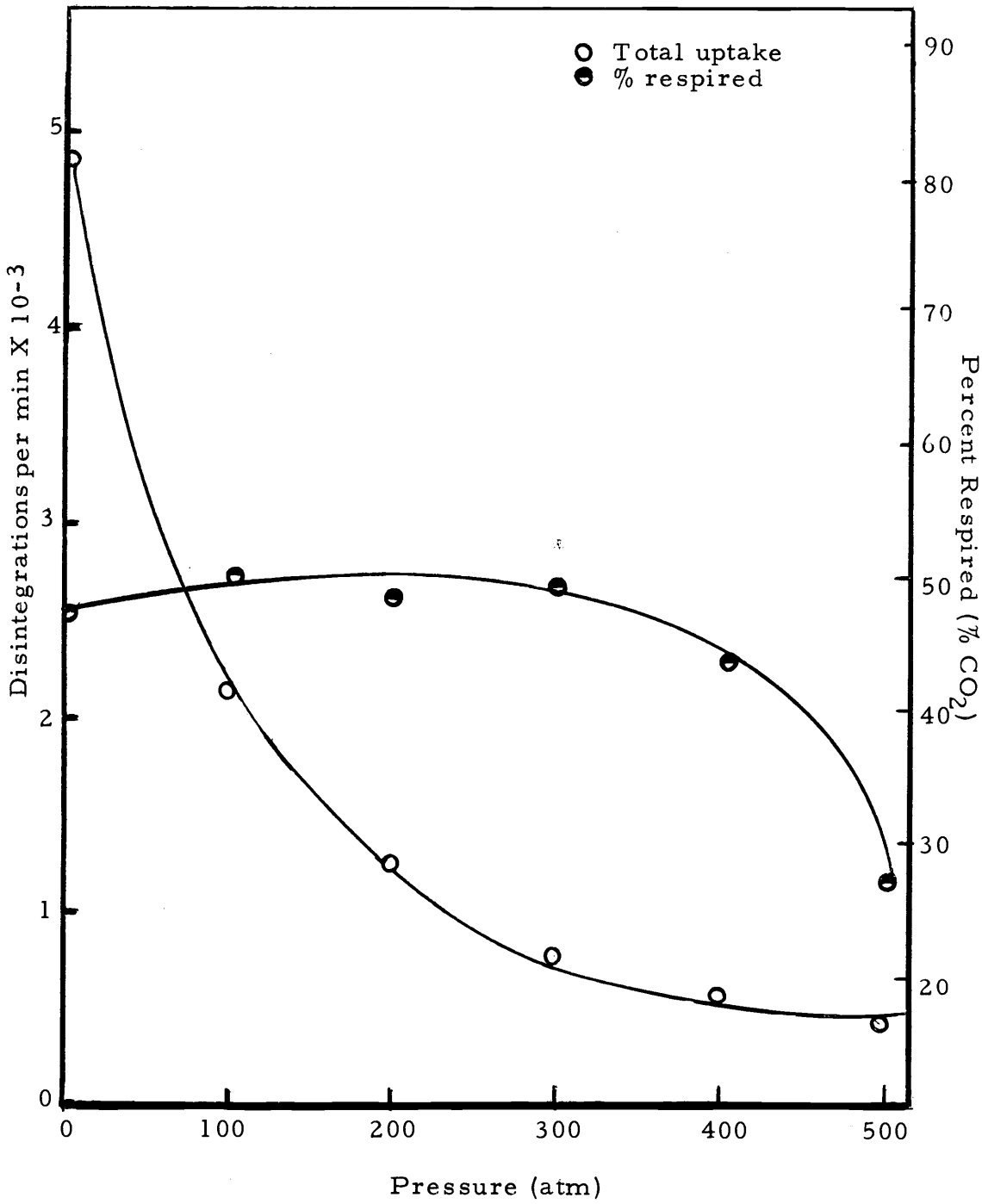

Figure 17. Uptake and utilization of ^{14}C-glycine by MP-38, 5 C, five hr exposure. Cell number was approximately 6.0 X 10^5/ml in 10 ml ASW; 200 μg/L glycine, with an activity of 0.02 μc/ml.

Phenylalanine respiration was also at a fairly constant proportion of the total taken up. This may indicate that the relative abilities of the metabolic pathways using phenylalanine to compete for substrate were maintained under pressure. It is possible that the respiration enzymes were functioning at their maximum capacity at each pressure level with respect to the total substrate concentration, a pseudo-saturation condition.

Explanations for the increase in proline respiration are the same as those for glutamate, with some exceptions. If pressure inhibited the enzymes of proline respiration less than those of other pathways, available proline may have been metabolized at all pressures, indicating a higher degree of pressure resistance at this temperature than the glutamate enzymes. A similar curve would be expected if pressure stimulated proline respiration, either by increasing enzyme-substrate affinity or enzyme activity. If an enzyme underwent a molecular volume decrease at atmospheric, the effect of pressure would be to enhance enzyme activity, increasing CO_2 production. Enzyme activity could also be stimulated by formation of an active protein aggregate (quaternary structure) or assumption of a more active configuration. The pH changes and ionization increase of the solvent may promote such variations.

However, it must again be noted that the increase in CO_2 production is only a relative increase. The actual amount of amino acid

metabolized or incorporated in all cases is decreased with pressure. Figure 18 indicates the differences in MP-38's response to these four substrates. It is possible that the four amino acid transport and metabolic systems have different temperature, pressure, and concentration optima, varying pool levels, and that any given amino acid may have more than one transport mechanism.

These figures, taken together, indicate that the essential effect of high pressure is to reduce the organism's supply of nutrients at low temperature by inhibiting its transport system. Such an effect would have tremendous ecological ramifications for nutrient material in the deep sea where the combined effects of cold and pressure may essentially prevent bacterial activity.

Figures 19 through 22 give the reversibility patterns for each amino acid; Table 3 presents the rate of uptake at atmospheric pressure with and without previous pressurization. All four transport systems exhibit reversible pressure inhibition with differing degrees and kinds of pressure damage. The proline transport system appears to be completely reversible in that the post-pressurization uptake rate is approximately the same as that at 1 atm without pressure. However, there is a substantial decrease in the proportion of CO_2 respired compared to atmospheric conditions. The percent respired is also lower at 1 atm than at 500, pointing up the relative increase in CO_2 production with pressure for proline. The decrease of CO_2 production

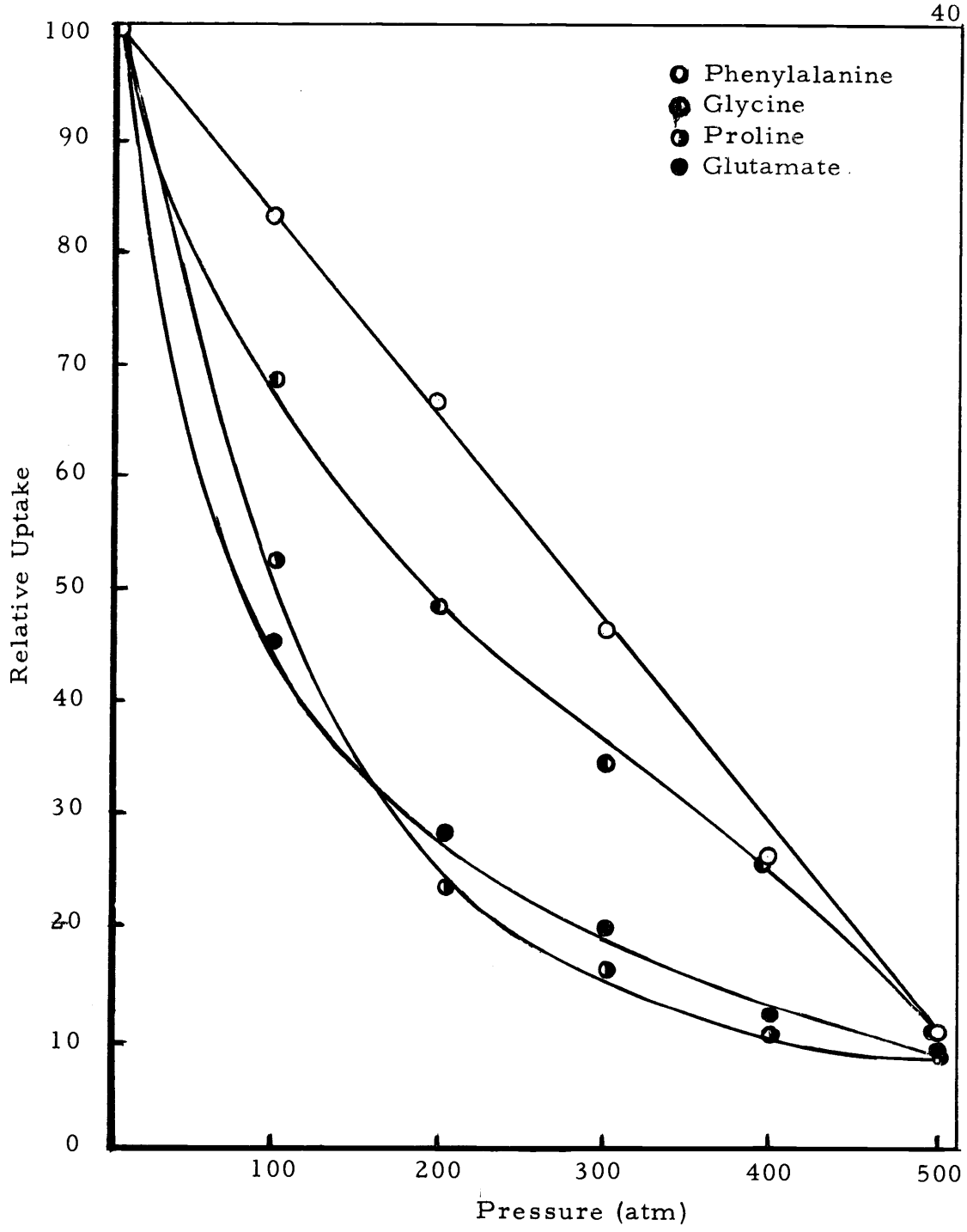

Figure 18. Relative uptake of four amino acids by MP-38, expressed as percent atmospheric, at 5 C, five hr exposure. Cell concentration was approximately 6.0 X 10^5/ml in 10 ml ASW. Amino acids were 200 µg/l with an activity of 0.02 µc/ml.

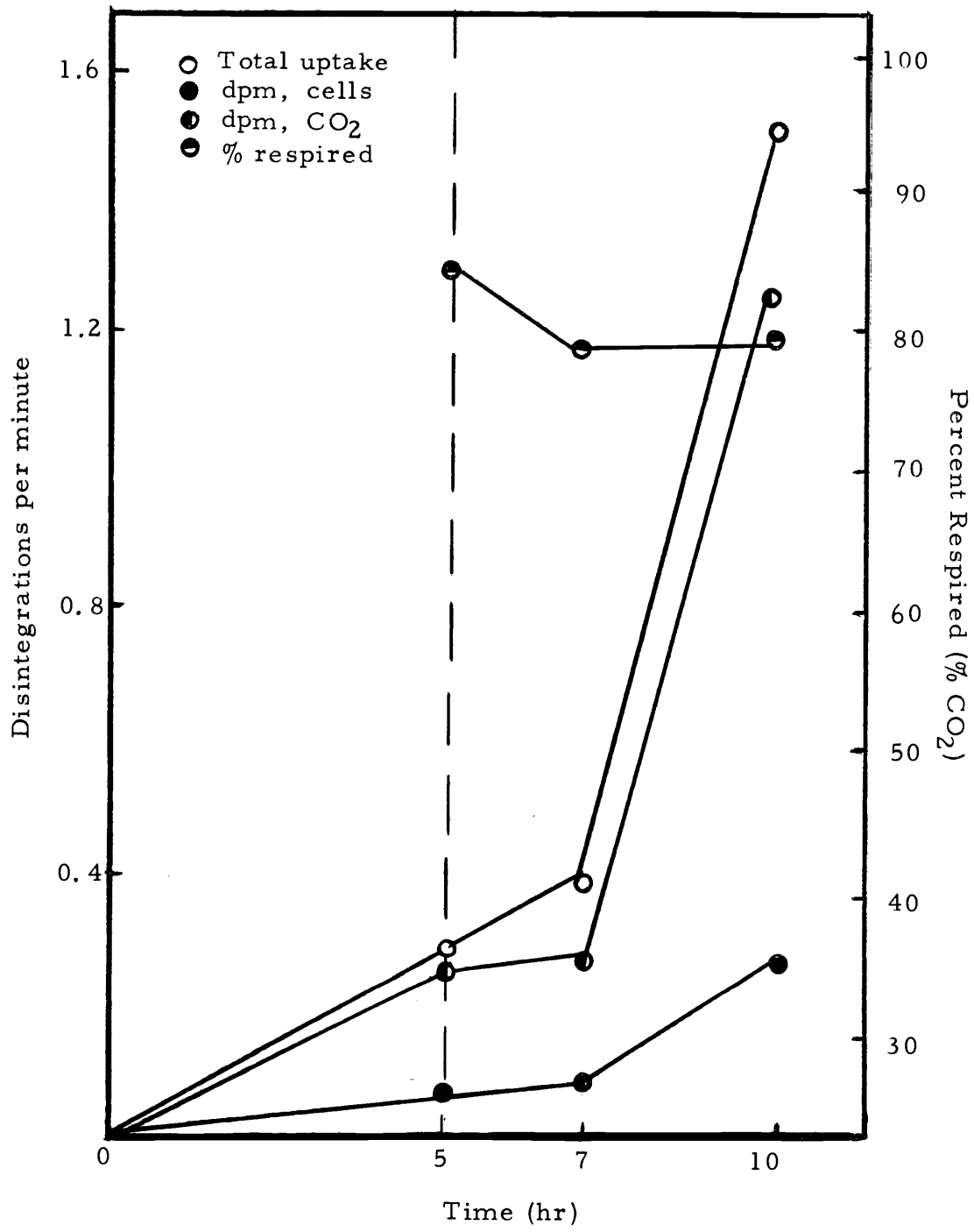

Figure 19. Reversibility of pressure inhibition of ^{14}C-glutamate uptake after 5 hr exposure to 500 atm pressure, 5 C. Cells were incubated at 1 atm at 5 C for 6 hr after release of pressure. Cell count was 6.0 X 10^5 cells/ml in 10 ml ASW; 200 µg/L glutamate with an activity of 0.02 µc/ml.

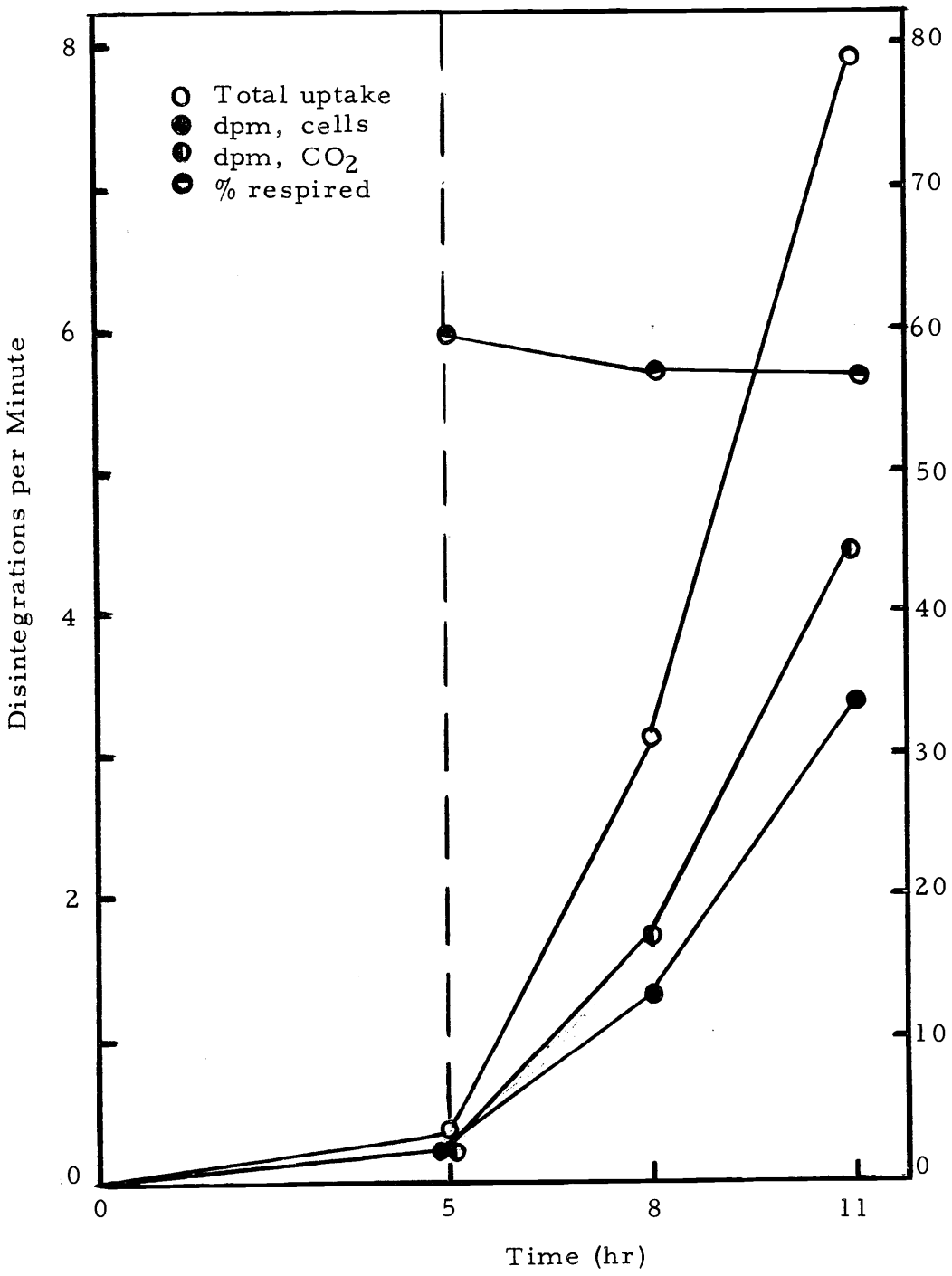

Figure 20. Reversibility of pressure inhibition of ^{14}C-proline uptake after five hr exposure to 500 atm, 5 C. Cells were incubated at 1 atm, 5 C for six hr after pressure release. Cell count was 6.0 X 10^5 cells/ml in 10 ml ASW, 200 µg/L proline with an activity of 0.02 µc/ml.

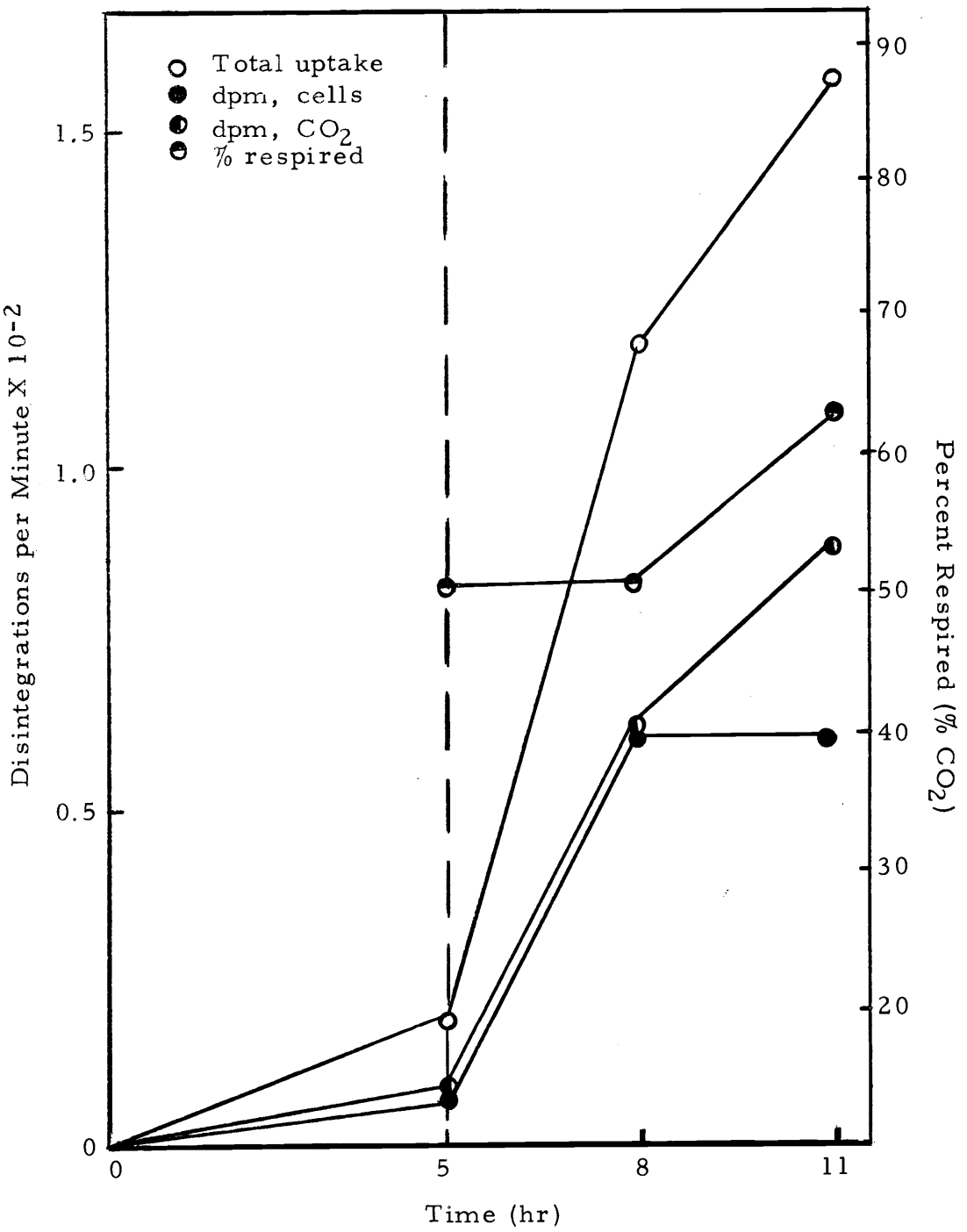

Figure 21. Reversibility of pressure inhibition of ^{14}C-phenylalanine uptake after five hr exposure to 500 atm pressure, 5 C. Cells were incubated at 1 atm, 5 C, for six hr after pressure release. Cell count was 6.0 X 10^5 cells/ml in 10 ml ASW, 200 µg/ml phenylalanine with an activity of 0.02 µc/ml.

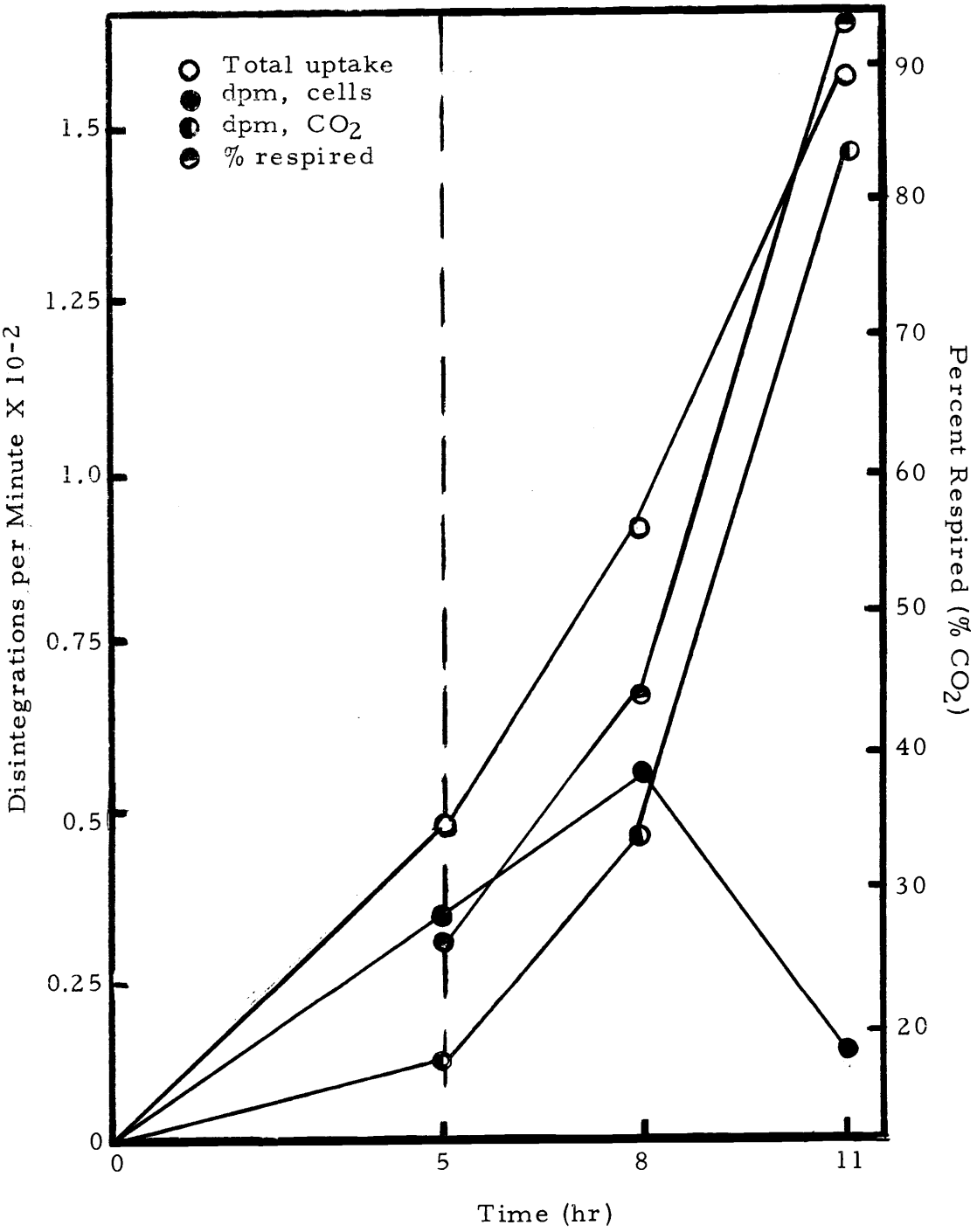

Figure 22. Reversibility of pressure inhibition of ^{14}C-glycine uptake after five hr exposure to 500 atm pressure, 5 C. Cells were incubated at 1 atm, 5 C, for six hr after pressure release. Cell count was 6.0 X 10^5/ml in 10 ml ASW, 200 µg/L glycine with an activity of 0.02 µc/ml.

Table 3. Reversibility of pressure inhibition on uptake and respiration of four amino acids by MP-38.

Amino acid	Uptake rate atm. dpm/hr	Uptake rate atm after pressure release dpm/hr	% CO_2 atm.	% CO_2 atm. after pressure release
glutamic	2500	400	76	80
glycine	1000	280	48	82
proline	1100	1200	80	54
Phenylalanine	580	280	48	64

Cells were exposed to substrate at 500 atm for 5 hr at 5 C. After pressure release, cells were incubated at atmospheric for 6 hr. Cell concentration was 6.0×10^5 cells/ml in 10 ml, amino acid concentration was 200 µg/L with an activity g 0.02 µc/ml.

relative to a non-pressurized sample may indicate that some non-reversible damage was done to the respiration pathway. It may be that under pressure hydrogen bonds responsible for tertiary and/or quaternary protein structure were disrupted. On release of pressure, the molecule may undergo a molecular volume increase, and lose its specificity or activity due to configurational changes. Solvent ionization and increased solubility of inorganic cations and anions (Morita, 1967) may provide a means of altering tertiary and quaternary protein structure under pressure through intramolecular bonding and intermolecular interaction. It is possible to postulate a situation in which a molecular volume decrease due to pressure, accompanied by increased dissociation of a protein's carboxyl groups, allows charged groups to get close enough to one another to permit a salt bridge to form using available divalent cations. On pressure release, the salt bridge may remain intact, inactivating the transport protein, or other enzymes.

Glutamate, glycine, and phenylalanine transport systems all show impairment to the uptake mechanism, as evidenced by lowered uptake rates. The increased (percentage respired) CO_2, relative to non-pressurized samples, indicates that the respiratory mechanisms are still functional and relatively undamaged after pressure release. Only glutamate transport exhibited a lag or recovery time after release of pressure (depressurization). The impairment of uptake or

transport proteins may be due to alteration of the tertiary structure of the transport protein.

Figures 23 and 24 show the result of exposing cells to substrate at 1 atm for three hr before pressurization. Counts from samples taken after exposure to 500 atm for given amounts of time indicate that both proline and glutamate were able to protect their transport proteins from pressure inhibition. The uptake rate at 500 atm without pre-incubation was 200 dpm per hr for glutamate and 85 dpm per hr for proline. With pre-incubation the rates at 500 atm were 980 dpm per hr for glutamate and 280 dpm per hr for proline. Such substrate protection may be due to the formation of a stable enzyme configuration in the enzyme-substrate complex. With a large amount of available glutamate or proline, the active site of the transport protein may remain charged with substrate and protected from pressure inhibition for a period of time. The presence of co-factors combined with the protein in its active configuration may also help protect the transport mechanism (Morita, 1970).

The ability of substrates to protect their transport proteins from pressure damage and the altered uptake pattern this provides raises some questions about data on intracellular processes under pressure. The pressure profiles of the amino acids studies indicated that limited uptake was responsible for the decrease in absolute amount of CO_2 produced or amino acid incorporated with increasing

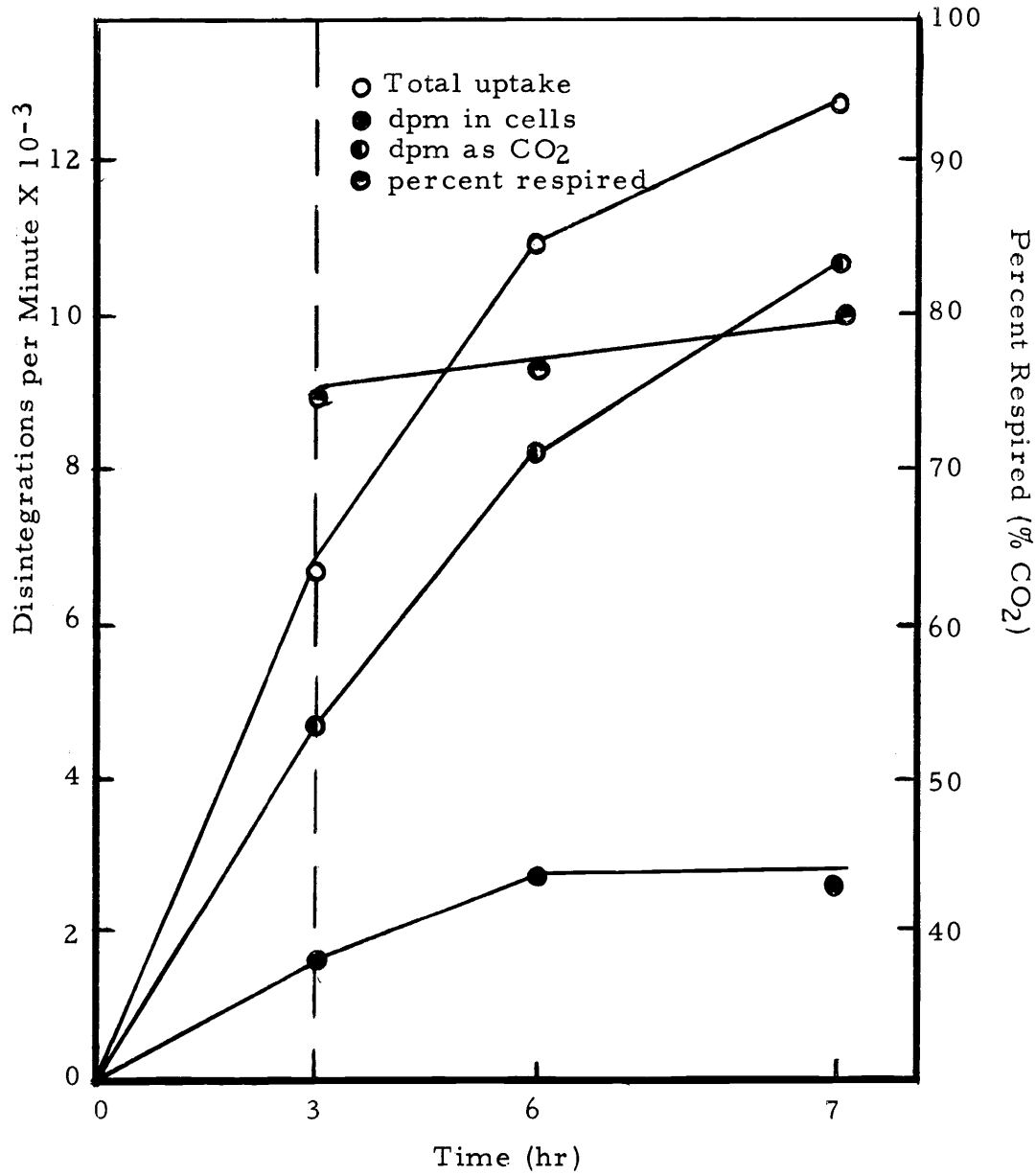

Figure 23. Uptake and utilization of ^{14}C-glutamic acid under pressure by MP-38 at 5 C. Cells were pressurized to 500 atm after exposure to substrate for three hr at 1 atm. Cell concentration was 6.0 X 10^5 cells/ml in 10 ml ASW; 200 µg/L glutamic acid, with an activity of 0.02 µc/ml.

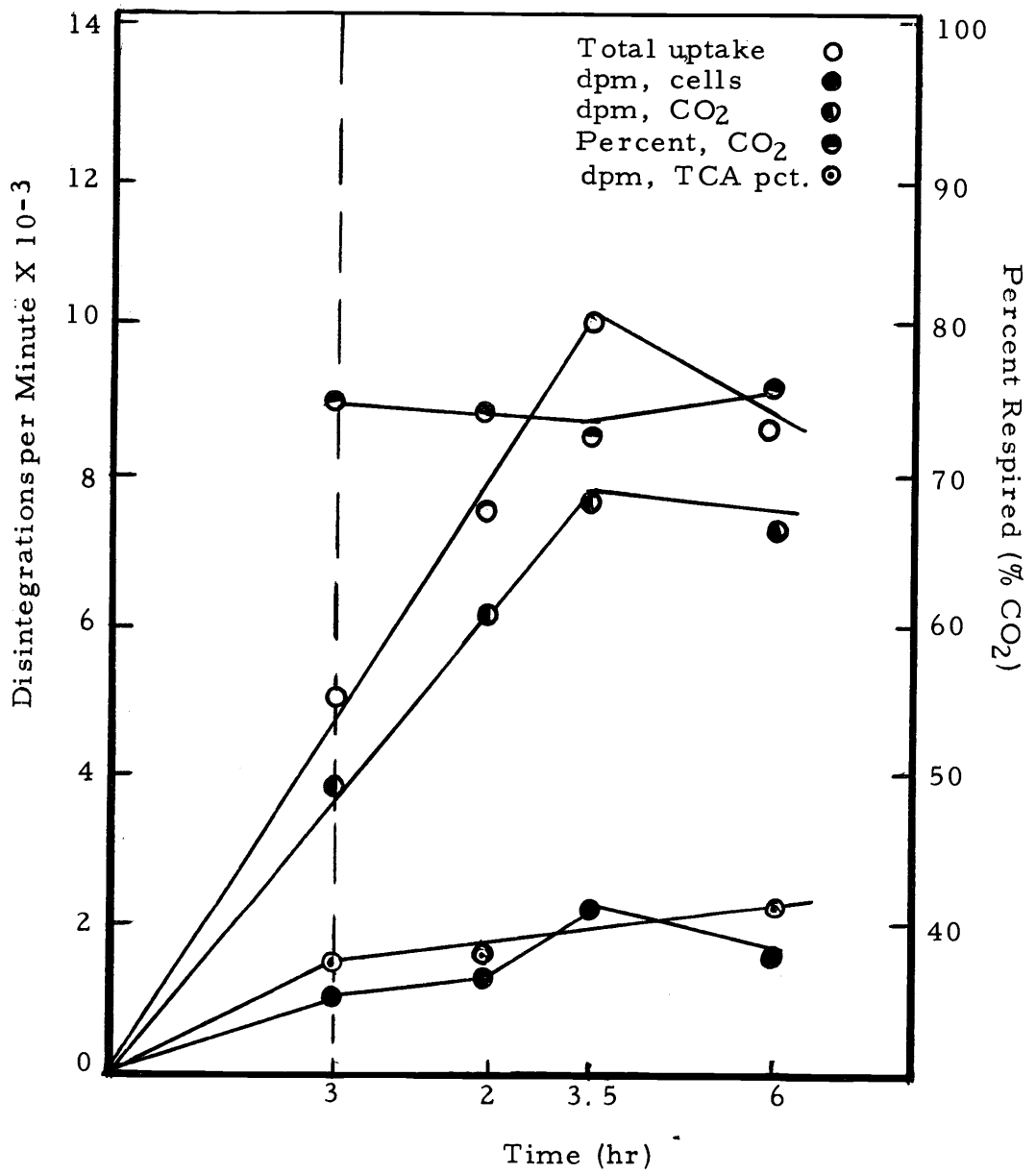

Figure 24. Uptake and utilization of ^{14}C-proline under pressure by MP-38 at 5 C. Cells were pressurized to 500 atm after three hr exposure to substrate at 1 atm. Cell concentration was 6.0 X 10^5 cells/ml in 10 ml ASW; 200 μg/L proline, with an activity of 0.02 μc/ml.

pressure. Those experiments on macromolecular synthesis previously discussed may have reflected the actual pressure response of the biosynthetic pathways studied. It is possible, however, that those experiments demonstrated the order of pressure sensitivity of the various transport systems involved rather than the synthetic systems. Comparison of proline uptake and incorporation for MP-38 at 5 C, 500 atm with and without pre-incubation indicate the effect the transport system can have on data from incorporation studies. At atmospheric pressure at 5 C, MP-38 took up and incorporated into cellular material approximately 2×10^{-13} mg proline/cell/hr. At 500 atm, this figure was reduced to 2×10^{-15} mg/cell/hr. If cells were pre-incubated with proline, the incorporation at 500 atm, as a TCA precipitate, was approximately 1.6×10^{-13} mg/cell/hr, equivalent to the amount found in whole cells (Figure 24). The protection of the transport system by pre-incubation substantially changed the picture of incorporation under the same conditions. One would have to allow substrate to enter the cell before pressurization, to see what effect pressure has on its use, not its uptake. Temperature variation, which also affected amino acid transport (Figure 14) would be reflected in incorporation data. Cellular uptake of glutamate was approximately 2×10^{-12} mg/cell/hr at 20 C, 1 atm. At 5 C, the value was only 3×10^{-13} mg/cell/hr. Comparison of data for whole cells and that from TCA precipitates might help indicate if transport or incorporation

were the more sensitive process.

Studies of pressure effects on cellular systems using whole cells must take into consideration the effect of pressure on the transport system of compounds used for measuring incorporation and intracellular enzyme activity. Protective effects of substrates and cofactors should also be taken into account in intra-cellular and cell-free enzyme studies.

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