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Yi-shu Chiu

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RECOVERY OF HEAVY METALS BY MICROBES

by

Yi-shu Chiu

Faculty of Engineering Science

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
October 1972

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ABSTRACT

Microbes resistant to heavy metals (uranium, strontium, titanium, silver, and platinum) were isolated. The major isolates were filamentous fungi; two were used in the further studies. These were Penicillium C-1 and Penicillium C-2. The former culture was characterized by coarse pellet formation when grown on glucose medium, whereas the latter grew only in a dispersed or mycelial form. Both Penicillium cultures showed significant growth in basal medium or when grown on the same medium containing heavy metals, such as uranium and strontium, at concentrations of 100 ppm. These two cultures were able to take up 70% of the uranium when the uranium concentration in the medium was at 100 ppm. Of the two cultures, the most complete study was done with culture C-1 particularly as it relates to uranium. Uranium uptake by culture C-1 was affected by many factors such as pH, temperature, age of mycelia, external uranium concentration, etc. Among these factors, the relationship of temperature and age of mycelia to uranium uptake appeared to be most associated with physiological conditions of cells. Further studies indicated that the uranium uptake by culture C-1

involved adsorptive uptake as well as biologic involvement. Of the total uptake the rapid adsorptive uptake during the first 5 minutes accounted for about at least 50%. It may be concluded that uranium was also involved in certain biological function, and at least part of the uranium taken up formed some type of complex with the membrane structure, possibly in the form of uranium enzyme complex.

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CHAPTER 1
INTRODUCTION

For a long time mammalian cells have been known to concentrate ions and molecules, and many studies have been conducted upon this subject. Although microbes are also known to take up and concentrate ions, few studies have been made on this aspect. Some mechanisms for ion uptake are similar in animal and microbial cells, but there is one great difference. In most animal cells extracellular material, e.g. cell wall, is absent, whereas in microbes a rigid cell wall is usually present outside the cell membrane. The cell wall allows the maintenance of much higher concentration of salts within the cell than outside without osmotic rupture. For example, many marine algae are known to accumulate iodine until the internal concentration is more than 10^6 times that of the environment. If one defines the ratio of internal to external concentration as the concentration factor, higher concentration factors are found for both animal and microbial cells. In man for example, a higher concentration of potassium is always observed inside the red blood cell than in the blood plasma. In order to maintain a dynamic equilibrium,

the potassium ion has to be transported against a concentration gradient. This phenomenon of transporting ions or molecules against their concentration gradient has been termed active transport in contrast to passive diffusion or, simply, diffusion.

Active transport has also been used with reference to microbes such as marine algae which have high concentration factors for iodine. The iodine, however, may be adsorbed to the surface of the cells rather than osmotically free in the cytoplasm. In any event, there are many examples of microbes in the environment capable of taking up specific ions. If one exploits this characteristic in microbial process development, a new type of fermentation in which chemical elements are recovered by microbes, can probably be developed.

In addition to the exploration of space for new resources, the need to develop the resources of the oceans has been recognized. Oceans cover four-fifths of the surface of our globe, but our basic knowledge of its chemistry and biochemistry are quite limited. About 60 kinds of chemical elements are dissolved in ocean water, among which only a few elements such as sodium, magnesium, and iodine have been removed for use. The remaining elements, for instance uranium, manganese, etc., are rarely utilized, but offer potential for development. According to one estimation the amount of manganese in ocean is about

1.5×10^{12} tons. Ocean water also contains strontium, aluminum, nickel, cobalt, silver, gold, and radium. The recovery and conversion of these elements to utilizable forms by microbes is promising.

The possibility of applying microbes to recover metals is not limited to the oceans. Microbes can also be used to extract metals from mine water, and moreover, they may be used as agents to separate metals which are chemically too alike to be separated by present conventional chemical and physical methods. Ezekiel (Science News, Vol. 90, p. 135, 1966) reported zirconium and hafnium have been extracted from solution in amounts varying from zero to nearly 100%. The solution used contained the both metals. These metals have essentially identical chemical properties. Different microbes and specific conditions were required.

Another advantage in employing microbes is to make use of this potential for preserving our environment by taking up elements which are present in mining or industrial wastes. For example, in the light of universal concern, mercury appears to be one of the most important metal pollutants in the environment. However, recently there are reports concerning a mercury-resistant strain of Pseudomonas, which is capable of taking up mercurials and stimulating vaporization of mercury (Suzuki, et al., 1968; Tonomura et al., 1968). This organism removed mercurials from

industrial wastes (Suzuki et al., 1968).

Finally, the purpose of this study can be summarized as: (1) To seek microbes capable of taking up economically and/or industrially important metals; (2) To obtain information about the mechanism of uptake; (3) To develop a biological process for applying microbes to recover metals, and to develop information which might help to protect our environment from heavy metal contamination.

CHAPTER 2
HEAVY METALS UTILIZED

2.1 Uranium

Before the advent of atomic energy the use of uranium was limited to certain industries. Now it is an important metal to be used in atomic bombs and as a fuel in atomic energy generating plants. Although uranium mines are found in more and more places in the world, they may never be plentiful enough to supply the expanding atomic energy industry. The principal method for extraction of uranium from the ore concentrates is acid or alkali leaching. Further concentration of uranium from the leach solution is attained by either resin absorption or solvent extraction. At the end of the leaching step the dissolved uranium concentration in the leach solution is too low, e.g. below 2,500 ppm (Shankar et al., 1956). At such low concentration, the cost is high and the recovery is low when conventional processes are used. The use of bio-sorption method may find its greatest applicability when the uranium concentration is extremely low as seen in very lean leach solutions or waste mine water (at the level of 100-300 ppm or lower).

In the present studies emphasis is given to the bio-sorption method for uranium recovery from solutions of low uranium concentration.

Uranium is usually considered as a toxic element in most biological systems. However, there are few reports showing direct involvement of uranium in biological functions. Recently, Updegraff and Douros (1972) reported that small amounts (4-24 ppm) of uranyl compounds stimulate growth of certain bacteria.

In a study of the uptake of radioisotopes by microbes, it was shown that the alga Ochromonas sp. could concentrate U-238 (Morgan, 1961). The concentration factor was 330 at the end of a 2-day incubation period in a medium containing uranium.

2.2 Strontium

About 0.0002% of igneous rocks and about 0.02 to 0.03% of the earth's crust is strontium. In sea water strontium is relatively abundant (about 13 ppm) among the metals. There are miscellaneous uses of strontium in industry, however the industrial significance of the recovery of strontium is mainly based on its radioactivity. The development of a biological process would have a two-fold purpose: recovery of useful isotopes and removal of radioactive pollutants. To date the technology for

recovery of the isotopes from waste solution usually needs the application of classical methods such as precipitation, crystallization, extraction, or ion exchange. Most of these methods require many repeated steps and high chemical costs which make them unprofitable.

In the recovery of radioactive strontium from the environment many studies have been conducted by using bio-sorption methods, in which algae and higher organisms such as sea-weeds have been frequently used. Sr-90 is one of the important radioactive isotopes produced during fission. Uptake of Sr-90 was observed in Chlorella vulgaris (Avio and Rossi-Torelli, 1965), in Ulva rigida and other algae (Polikarpov, 1961). Sr-89 is also one of the fission products; it was taken up by several algae and marine bacteria (Morgan, 1961). The uptake of another isotope, Sr-85 was also studied (Williams, 1970). In Pithophora and Cladophora, living cells in medium with a low level (0.1 ppm) of calcium had a concentration factor for Sr-85 of more than 2,000, but the concentration factor decreased when the medium contained simultaneously high (30 ppm) calcium and low (0.1 ppm) potassium. When the medium contained high concentration (30 ppm) of both calcium and potassium, the concentration factor for Sr-85 increased to between 1,600 and 1,700 (Williams, 1970).

2.3 Titanium

Titanium is a corrosion-resistant, tough, and strong metal. It has a high melting point and relatively low density, falling between aluminum and iron.

Titanium is widely distributed in nature and ranks ninth in abundance of the elements making up the earth's crust. About 0.63% of the earth's crust and about 0.5-1.5% of the soil is titanium. Titanium is also found in minerals such as coals, in plants, in animals, and in human beings. The titanium content of plants and animals (both land and marine), and in fresh-water organisms varies from 0.1 to 100 ppm (Barksdal, 1966).

Though titanium is usually found in living organisms, its biological function is unknown. In bacteria utilizing aircraft fuels or hydrocarbons, titanium at 100 ppm was found to stimulate growth (Engel and Owen, 1969). At an initial concentration of 10 ppm, titanium was highly concentrated. The amount for various valences of titanium removed from the medium by the bacteria ranged from 30 to 80%.

2.4 Silver

Silver is a rather rare metal but has found places in every day life. Most of the uses are for the monetary purposes, silver plating, and photographic film.

Miscellaneous uses are found in chemical, mechanical, and electrical industries.

Most silver compounds are toxic. Salts of silver such as silver nitrate are very effective as an antiseptic to kill various kinds of microbes. In the sense of uptake of silver by microbes, our knowledge is limited.

2.5 Platinum

Platinum is one of the precious metals, and is more costly than gold. Platinum is about one hundred times as rare as gold; its existence in the earth's crust is estimated at about 0.01 g/ton. Needless to say, it is in great demand in jewelry manufacture. In industry there are many practical applications of platinum; the most important use is probably as a catalyst. There are no available data showing the interaction between platinum and microbes.

CHAPTER 3

ISOLATION OF MICROBES RESISTANT TO HEAVY METALS

3.1 Enrichment Culture

The microbes used in this study were isolated by an enrichment culture established by adding 1.5 liters sewage to 1.5 liters culture medium containing salts of the five heavy metals (Chapter 2). The medium components and metal salts are shown in Table 1. Methanol was the primary energy source in this medium. The culture vessel was aerated and fed continuously with basal medium plus metal salts except that PtCl_4 was added to the salt mixture only during the first week. The feeding rate was 0.5 liters/day, whereas the culture volume was maintained at 3 liters. The pH in the culture vessel was initially 5.0. After 6 days it increased gradually and at 12 days an almost constant pH of 7.0 ± 0.7 was observed. The continuous culture was maintained 80 days, and during this period a sample was collected from the outlet siphon and streaked every other day on agar plates containing basal medium. Microbes which grew were selected, restreaked and subcultured until pure cultures were obtained. Ten cultures were isolated by this procedure,

TABLE I. Composition of
Enrichment Culture Medium

Basal Medium		
<i>Methanol</i>	30	<i>g</i>
<i>Peptone</i>	1	<i>g</i>
$(\text{NH}_4)_2 \text{SO}_4$	1	<i>g</i>
K_2HPO_4	1	<i>g</i>
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.2	<i>g</i>
$\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$	0.2	<i>g</i>
Metal Salt		
$\text{SrCl}_2 \cdot 6 \text{H}_2\text{O}$	1	<i>g</i>
$\text{Ag}_2 \text{SO}_4$	0.5	<i>g</i>
$\text{UO}_2 \text{SO}_4 \cdot 3\text{H}_2\text{O}$	0.5	<i>g</i>
Ti Cl_4	0.5	<i>g</i>
Pt Cl_4	0.5	<i>g</i>
<i>Distilled water</i>	1000	<i>ml</i>
<i>pH = 5.0</i>		

of which most were filamentous fungi. These cultures were labelled as C-1, C-2, G-1, G-2, G-3, G-4, G-5, G-6, G-7, and Or-1. Except culture Or-1 which was orange in color in both agar and liquid culture, all the others were green molds.

3.2 Survey of Growth of the Isolates on Heavy Metals

Culture conditions: Growth was tested on individual heavy metals plus basal medium consisting of 0.1% peptone, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.1% K_2HPO_4 , 0.02% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.02% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 2.5% of either methanol or glucose. The pH of the basal medium was adjusted to 5.0 with hydrochloric acid before autoclaving. The medium was autoclaved at 115°C for 30 minutes. Methanol was sterilized by Seitz-filtration. The heavy metals were in the forms of $\text{UO}_2\text{SO}_4 \cdot 3\text{H}_2\text{O}$, $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, TiCl_4 , Ag_2SO_4 , and PtCl_4 . Solutions of these metal salts were autoclaved and added separately to the medium to give a final concentration of 100 ppm as individual metallic elements. The inocula were prepared by transfer of agar blocks containing spores and hyphal tips. The agar block measured about 0.5 cm square. The agar medium had the same composition as the glucose medium except that 2.5% (w/v) of Bacto agar was added. Culture flasks (500 ml), containing 100 ml of medium, were incubated at room temperature (25°C) on a rotary shaker (New Brunswick Scientific Co.) and shaken at 200 rpm. Cultures were incubated 5 days, and growth

was determined at the end of this incubation period.

Determination of dry weight: After incubation, mycelia were separated from the supernatant by filtration, washed with distilled water, and then dried to a constant weight at 100°C.

Results: Methanol was tested as a substrate for each culture (Table 2), and an additional ability of each culture to grow on uranium, strontium, titanium, platinum, and silver was measured. No heavy metals were added to the methanol medium used in Table 2. Growth is reported in dry weight (mg/100 ml) along with the final pH of the broth. Each metal was added separately to the methanol medium, and growth (Table 3) was evaluated by visual observation and compared with the control (Table 2). Growth was recorded as (a) no growth, (b) growth comparable to the control, and (c) growth slightly better than the control. With methanol as a substrate growth was sparse; the yield of dry weight in the control cultures ranged from 17 (culture C-1) to 60 mg/100 ml (culture Or-1). All the cultures showed an increase in pH during cell growth. At the end of a 5-day culture period, the final pH ranged between 5.2 (culture C-1) and 7.3 (culture Or-1) for the various cultures. The experiments were repeated and glucose was used as a substrate. In the glucose medium all the pH levels decreased. There

TABLE 2. COMPARISON OF GROWTH OF VARIOUS ISOLATES ON METHANOL MEDIUM IN SHAKE FLASK CULTURE. DRY WEIGHT AND pH WERE DETERMINED AT THE END OF A 5-DAY INCUBATION PERIOD (INITIAL pH= 5.0).

ISOLATE	FINAL pH	DRY WEIGHT (mg/100ml)
C-1	5.2	17±1*
C-2	6.5	46±8
G-1	6.2	43±5
G-2	6.4	35±9
G-3	6.1	18±7
G-4	5.7	19±6
G-5	5.7	43±7
G-6	5.9	33±2
G-7	6.1	34±1
Or-1	7.3	60±7

*STANDARD DEVIATION OF DUPLICATE VALUES.

Table 3

Comparison of growth of various isolates on methanol medium in the presence of 100 ppm uranium, strontium, titanium, platinum, or silver. Shake flask culture. The growth was observed at the end of 5-day incubation period.

METAL ISOLATE	U	Sr	Ag	Pt	Ti
C-1	++	+	+	+	+
C-2	+	++	++	+	+
G-1	+	+	+	+	+
G-2	++	+	+	+	+
G-3	+	+	+	-	+
G-4	+	+	+	-	+
G-5	-	+	+	-	-
G-6	++	+	+	+	+
G-7	+	+	+	+	+
Or-1	+	+	+	+	+

"-": No growth

"+": Growth comparable to the control.

"++": Growth slightly better than the control.

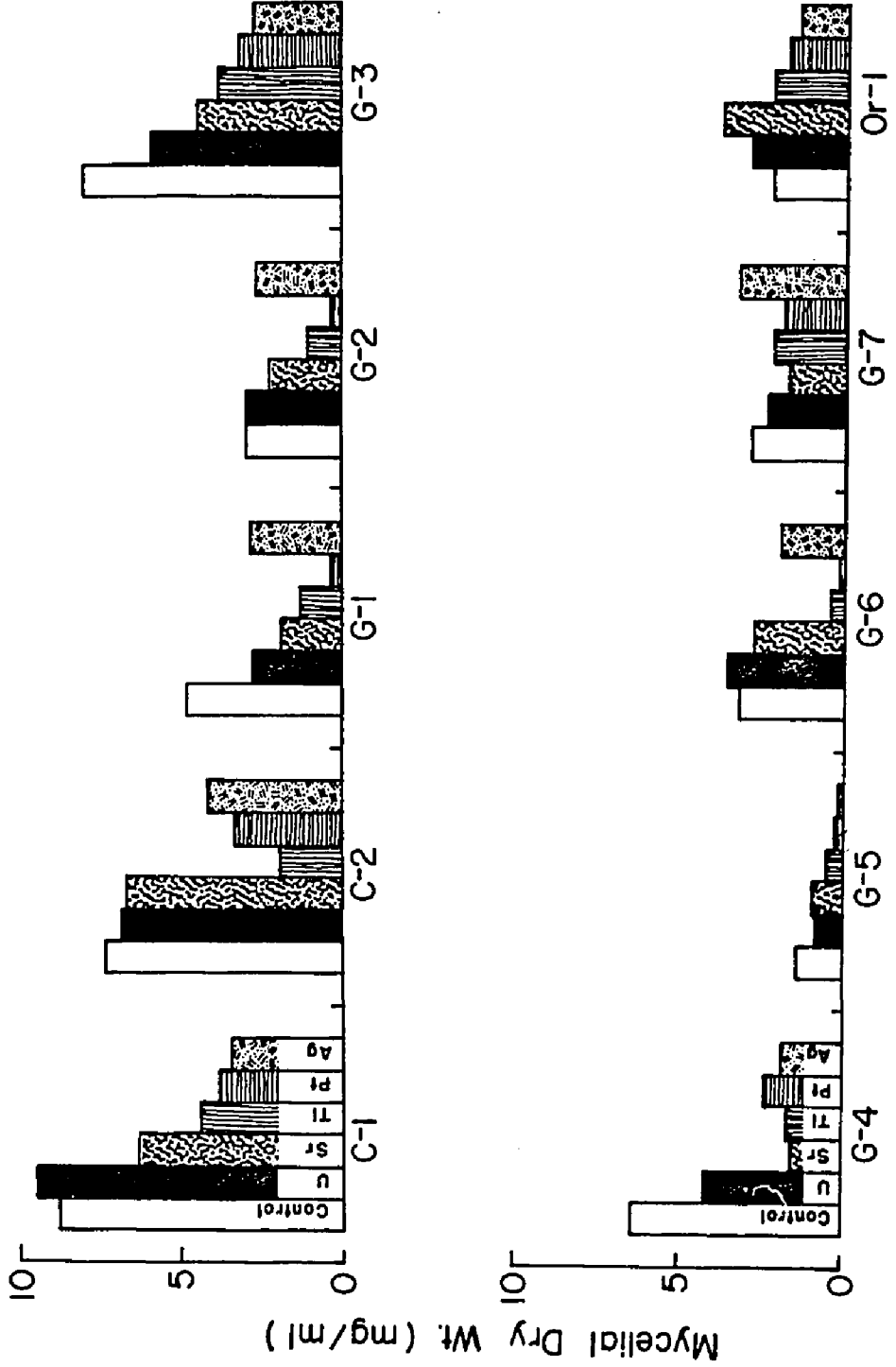
was a significant increase in yields of dry weight in most cultures grown upon glucose as compared with the methanol medium. The greatest increase in growth was shown by culture C-1, where the dry weight was 880 mg/100 ml in the control. Upon addition of the various heavy metals, the growth was more or less depressed in most cultures. In general, titanium, silver, and platinum depressed the growth more than uranium and strontium (Figure 1). Among the five metals, uranium appeared least toxic to most of the cultures. There were three instances (cultures C-1, G-6, or Or-1) where uranium gave better growth than a control in which no heavy metals had been added.

3.3 Culture Selection and Preliminary Test for Metal Uptake

Among the various isolates, cultures C-1 and C-2 showed significant growth on the basal glucose medium or when grown on the same medium containing heavy metals such as uranium and strontium at concentrations of 100 ppm (Figure 1). Cultures C-1 and C-2 were selected for preliminary testing for metal uptake. Morphological and culture characteristics are discussed in the next section. To test their potential for metal uptake, these cultures were grown separately on a glucose medium plus either uranium, strontium, titanium, silver, or platinum. Other culture conditions and the method of dry weight determination were the same as those stated in the preceding section (Section 3.2). For

Figure 1

Growth of various isolates in shake flask culture on glucose basal medium in the presence of 100 ppm of either uranium, strontium, titanium, platinum, or silver. Mycelial dry weight was measured at the end of 5-day incubation period. The graphs show the average of duplicate values.



analysis of metals, the cells were ashed at 700°C for 2 hours, and the ash residue was dissolved in either concentrated sulfuric acid (for uranium analysis), concentrated hydrochloric acid (strontium), concentrated hydrofluoric acid (titanium), concentrated nitric acid (silver), or aqua regia (platinum) according to the specific metal involved. Uranium was determined by a colorimetric method (Rodden, 1955), while the other metals, were determined by atomic absorption spectrophotometry (Beckman Model 979). The results are shown in Figures 2 and 3. From these figures it is seen that except for silver, of which only a very small fraction was recovered by the cells, more than 30% of all the other metals were recovered under optimum conditions. Of the five heavy metals, uranium was removed to the greatest extent. With culture C-1 about 50% of the added uranium was found in the mycelia at 3-days, however on continual incubation there was a slight release of uranium to the medium. With culture C-2 the initial uptake of uranium was low, but as cell growth continued the removal of uranium continued e.g., 20% uranium uptake was observed in 3-days and 90% in 9-days. Strontium showed a similar behavior to uranium during 9-day incubation (C-2). It is ranked next to uranium in metal uptake by both cultures C-1 or C-2. The third in importance was titanium which also was removed from the medium during growth.

Figure 2

Growth and metal uptake of culture C-1 in shake flask culture on glucose medium plus 100 ppm of either uranium, strontium, titanium, platinum, or silver. The precision of the analysis of uranium, strontium, titanium, platinum, and silver was respectively 0.1, 0.2, 1, 2, and 0.1 ppm.

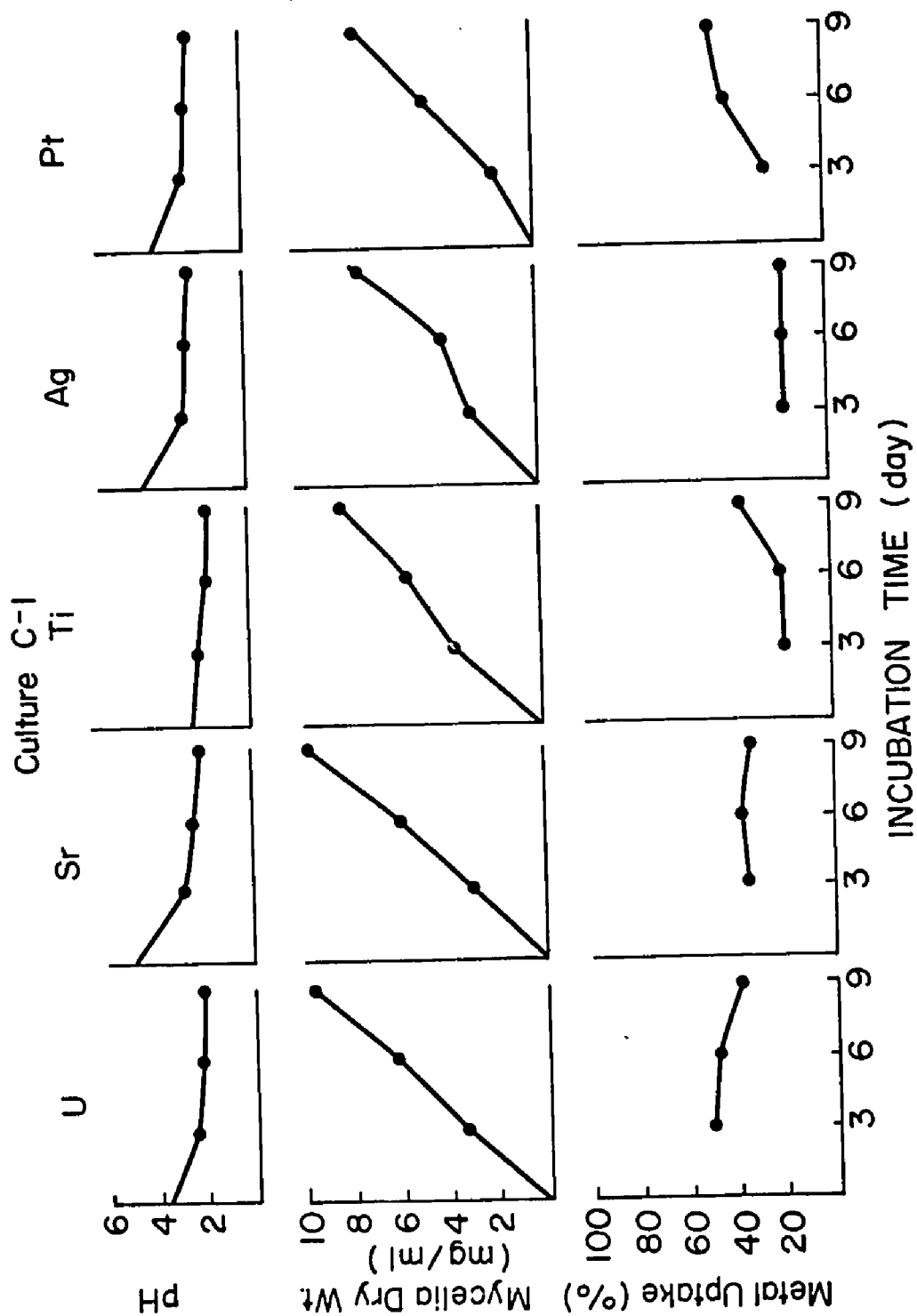
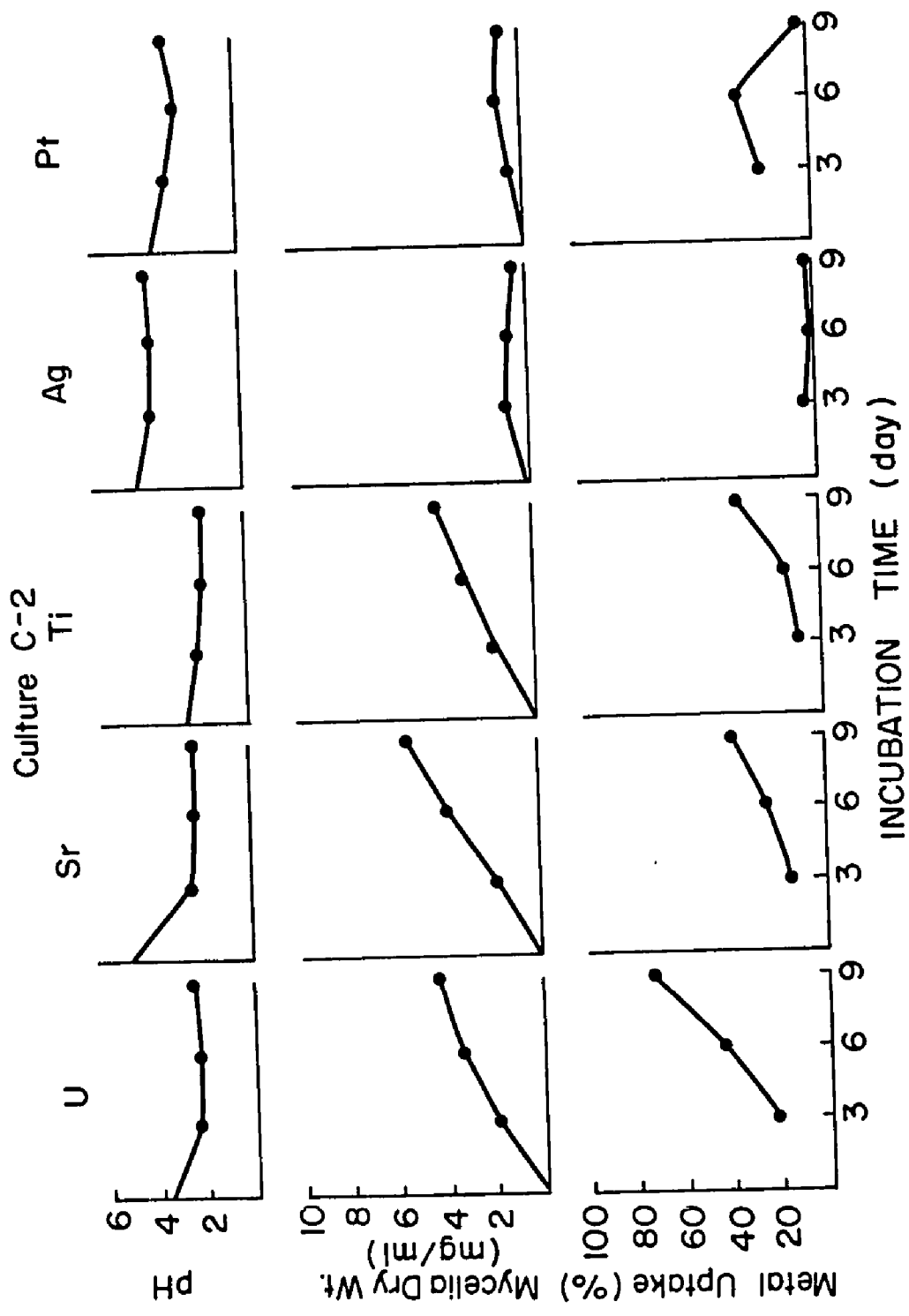


Figure 3

Growth and metal uptake of culture C-2 in shake flask culture on glucose medium plus 100 ppm of either uranium, strontium, titanium, platinum, or silver.



3.4 Characteristics of Cultures C-1 and C-2

Of the two cultures, more complete studies have been done with culture C-1 particularly as it relates to uranium uptake and metabolism. Both cultures C-1 and C-2 belong to the genus Penicillium. At the surface of methanol agar medium (methanol basal medium plus 2.5% Bacto agar), colonies were faintly grey and growth was sparse. Spore formation was inhibited. On glucose agar (glucose basal medium plus 2.5% Bacto agar), the colonies were white, turning faintly yellow with age. Growth was improved on glucose as compared with methanol. Culture C-1 was a hardier culture than C-2. In culture C-1 sporulation was strong, whereas in culture C-2 sporulation was relatively weak. The color of the spores in C-1 was deep green and yellowish green in C-2. Upon addition of uranium to the glucose agar medium it was found that in C-1 up to 500 ppm uranium did not inhibit the growth of colonies significantly, but at concentrations of 1,000 ppm there was about 30% inhibition in colony growth. At this concentration, the margin of the colonies remained white, and spore formation was inhibited. To test its tolerance to NaCl, culture C-1 was grown on glucose agar medium in which 10% NaCl was added. There was about 3 days' lag in the growth of colonies, but after this lag period colonies did develop. The size of colonies was relatively small and with less sporulation. Colony diameters after 10 days were

1 cm, whereas in the control a diameter of 7 cm was observed.

In submerged culture (shake flask) using common monosaccharides as substrates the hyphae were white, turning slight yellow with age in both cultures C-1 and C-2. In C-1 the mycelia clumped together to form discrete pellets, whereas C-2 grew in dispersed form. In further growth tests with C-1, hexoses such as fructose, galactose, and mannose supported good growth. Ethanol gave a moderate growth. Growth on lactose was poor. When uranium was added to the liquid medium at a concentration of 100 ppm, there were two observed effects of uranium on the growth of C-1 in terms of mycelial dry weight: (a) there was neither significant stimulation nor inhibition when grown on the above mentioned carbohydrates; (b) uranium stimulated growth with ethanol, i.e. there was about 30% increase in the growth of mycelial dry weight. In the test of its resistance to the other common toxic heavy metals, C-1 was found to be able to grow on glucose medium plus either cadmium, chromium, or copper in concentrations up to several hundreds ppm. Mercury at concentration of 10 ppm also permitted growth.

Slide cultures were made on a glucose medium and the photomicrographs of cultures C-1 and C-2 taken (respectively Figures 4 and 5). Conidiophores in the micrographs show penicillate branching. Figure 4a shows a fairly young

conidiophore in which one can see clusters of phialides bearing chains of conidia. Figure 4b shows a simple unbranched conidiophore while Figure 4c shows a greater complexity of branching. Conidiophores and vegetative hyphae structure are shown in Figure 4d. Hyphae are about 2μ thick, branched and septate.

Conidiophores normally show one or two degrees of branching and are not strongly divaricate. Phialides are typically bottle shaped, bearing chains of ellipsoid, unicellular conidia from their narrow apical tips (sterigmata).

Figures 5a and 5b show conidiophores of culture C-2 which are very similar to C-1. It differs in having shorter conidiophores and relatively short stout phialides.

Figure 4

Photo-micrographs of slide culture C-1. Figures 4a, 4b, and 4c, respectively, show the young stages of sterigmata formation and conidial development (a), a cluster of sterigmata with conidial chains produced by an unbranched conidiophores (b), and branched conidiophores (c). All are 2,500x magnification. Figure 4d is the same slide with a lower magnification (1,000x).



a



b



c



d

Figure 5

Photo-micrographs of slide culture C-2. Branched conidiophores with cluster of sterigmata and conidial chains. 3,600x magnification.



b



a

CHAPTER 4
FACTORS AFFECTING GROWTH OF
Penicillium CULTURES C-1 AND C-2

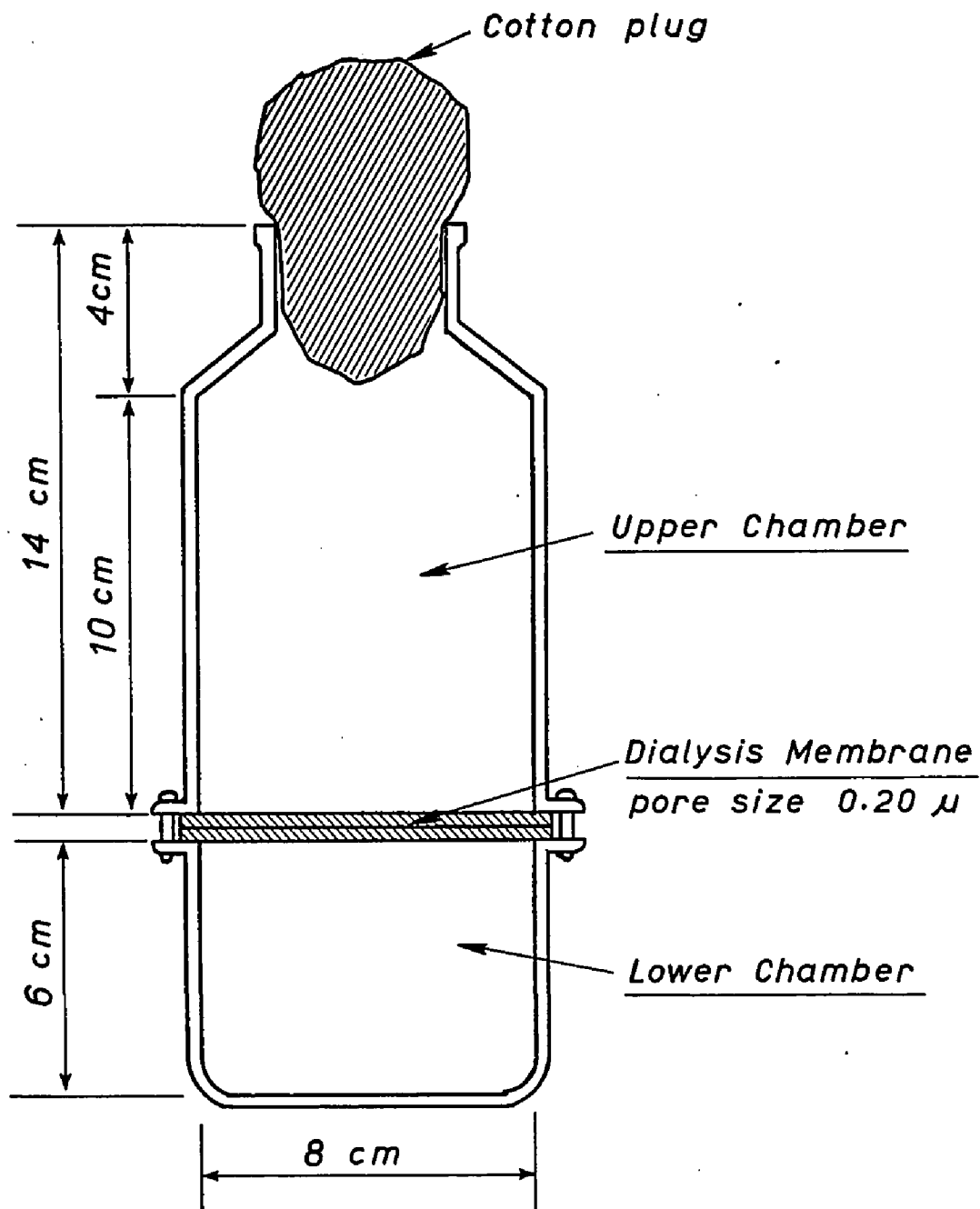
4.1 Materials and Methods

The effects of pH, temperature, medium, cultivation methods, the heavy metals such as uranium and strontium, and other metals on growth were investigated. Except as otherwise shown the experiments were completed with glucose medium (pH 5.0) over a period of 6 days. The cultures were grown in shake flasks (500 ml size with a working volume of 100 ml), and incubated at room temperature (25°C) on rotary shaker (New Brunswick Scientific Co.) at 200 rpm. For fermentor culture, a magnetic bottom-driven fermentor (Virtis Co.) of 12-liter capacity was used. This unit was operated at a working volume of 6 liters. Two six-blade, paddle-type impellers fixed on the central shaft of the jar were rotated at 300 rpm. Air flow was introduced at the rate of 2 liters/minute and dispersed into the liquid through the sparger located near the bottom. The temperature was controlled at 25°C. For dialysis culture, dialysis flasks as shown in Figure 6 (modified from Gerhardt and Gallup, 1963) were used. The flask consisted of two

Figure 6

Dialysis flask (modified from Gerhardt and Gallup, 1963).

The flask consisted of two chambers separated by a dialysis membrane.



chambers separated by a dialysis membrane (pore size 0.2μ). The lower chamber (280 ml) was filled with basal medium. The upper chamber contained 100 ml of basal medium with inoculum. The dialysis flasks were incubated and handled in a manner similar to shake flask culture. When temperature control was required the flasks were incubated in a controlled environmental incubator shaker (New Brunswick Scientific Co.) and shaken at 200 rpm. The inoculum size was 5% (v/v), and was prepared from mycelia of 5-day-old cultures. For culture C-1 the mycelia were first homogenized in a Waring blender for 30 seconds prior to inoculation. The seed culture for both C-1 and C-2 was prepared by transfer of spores and hyphal tips. The inocula had dry weights of 8-9 mg/ml and 7-8 mg/ml, respectively, for culture C-1 and C-2. Growth in terms of mycelial dry weight represents the average of two separate determinations. In each determination, the dry weight of inoculum (assumed 8.5 and 7.5 mg/ml for C-1 and C-2, respectively) has been subtracted. For additional materials and methods see Section 3.2 (Chapter 3).

4.2 Results

pH: To test the effect of pH on growth, the initial pH of the medium was adjusted to 1.0, 2.0, 3.0, ..., and 9.0 with either hydrochloric acid or sodium hydroxide. The

results (6 days' growth) are shown in Figure 7. For culture C-1 maximum growth (10 mg/ml) was observed at pH 3.0, whereas for C-2 there were two maxima at pH 3.0 (dry weight 7 mg/ml) and pH 5.0 (9 mg/ml).

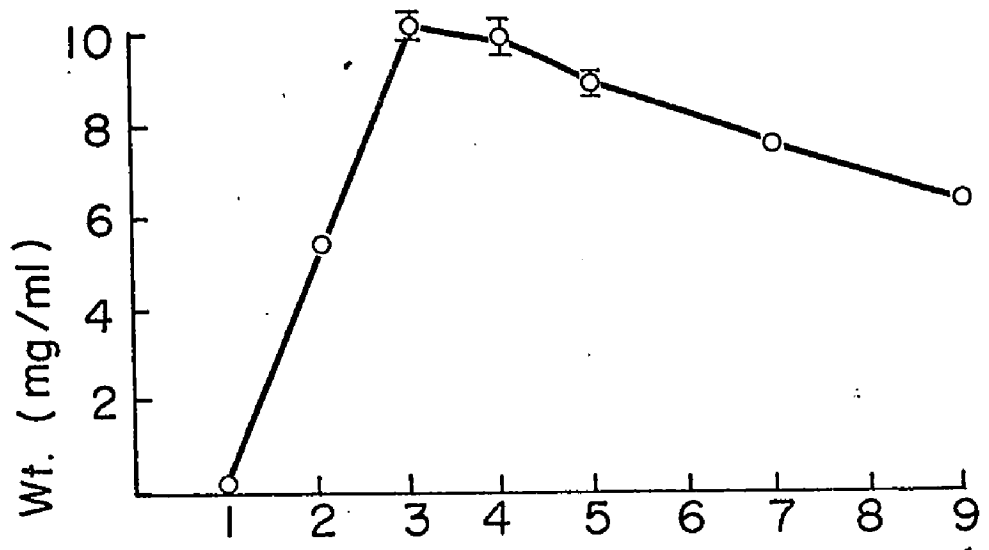
Temperature: The incubation temperature was controlled at either 10, 20, 25, 30, or 40°C. The temperature effect on growth (6 days) is shown in Figure 8. The optimum temperature for culture C-1 was 30°C, and for culture C-2, 25°C. Mycelial dry weights obtained at these temperatures were 12 and 9 mg/ml respectively.

Medium: The basal medium with different carbon sources at concentration of 2.5% (w/v) was tested for growth. The carbon sources included various monosaccharides, dextran, and alcohols. The pH of the basal medium was adjusted to 5.0, and then the test substrate was added. One commercial beer (10x dilution, pH 4.4) and several commercial nutrient broths (Bacto) such as sabouraud dextrose broth (pH 5.7), wort broth (pH 4.8), and malt extract broth (pH 4.7) were also tested as growth substrates. The results (6 days' growth) are shown in Figure 9. The pattern of utilization of these substrates was similar for the two cultures. Hexoses were good carbon sources. Ethanol was only moderately utilized. Of the carbohydrates tested, lactose gave the poorest growth. The best growth

Figure 7

Effect of initial pH on growth (6 days) of Penicillium
cultures C-1 and C-2 on glucose medium in shake flasks.

Culture
(a) C-1



Culture
(b) C-2

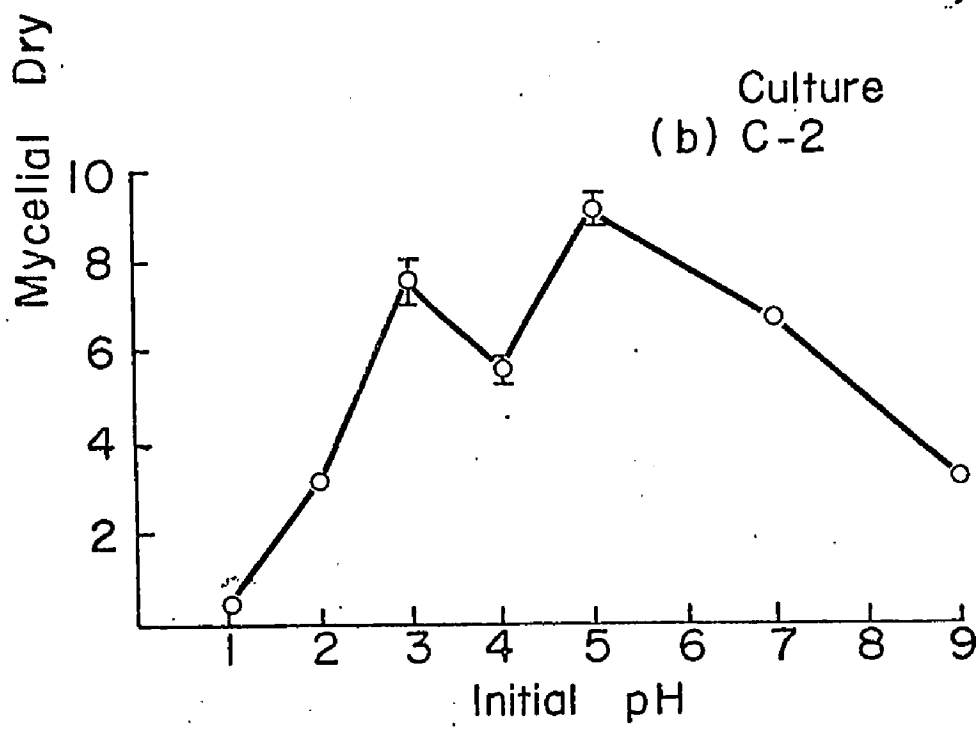


Figure 8

Effect of temperature on growth (6 days) of Penicillium cultures C-1 and C-2 on glucose medium in shake flasks.

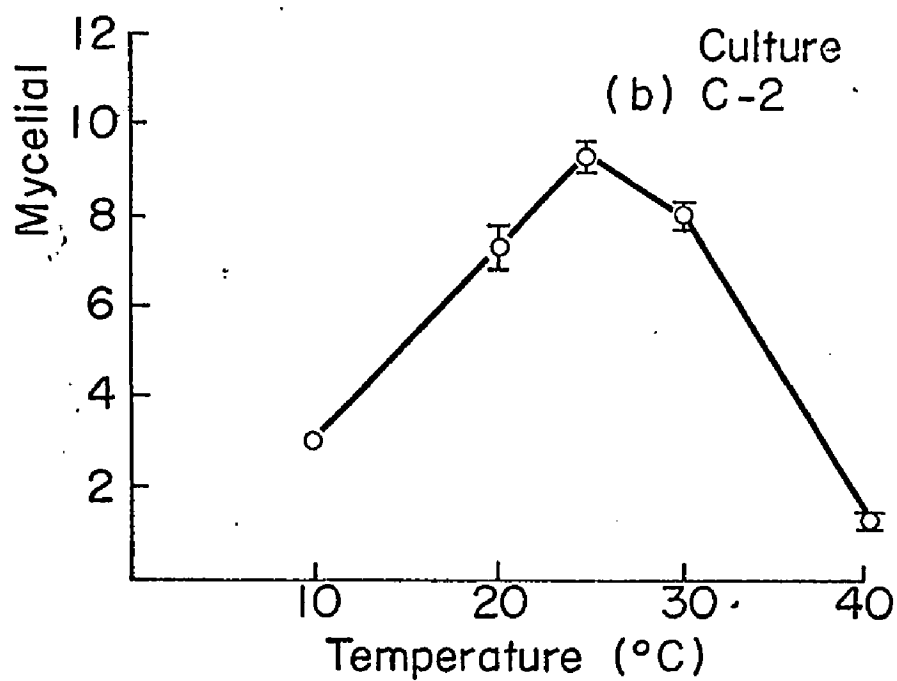
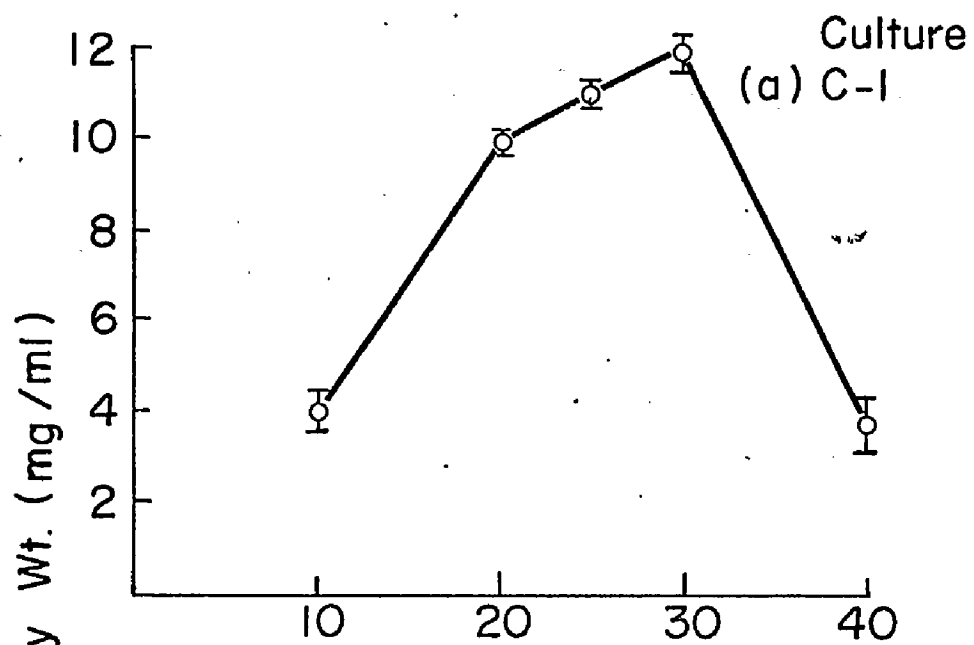
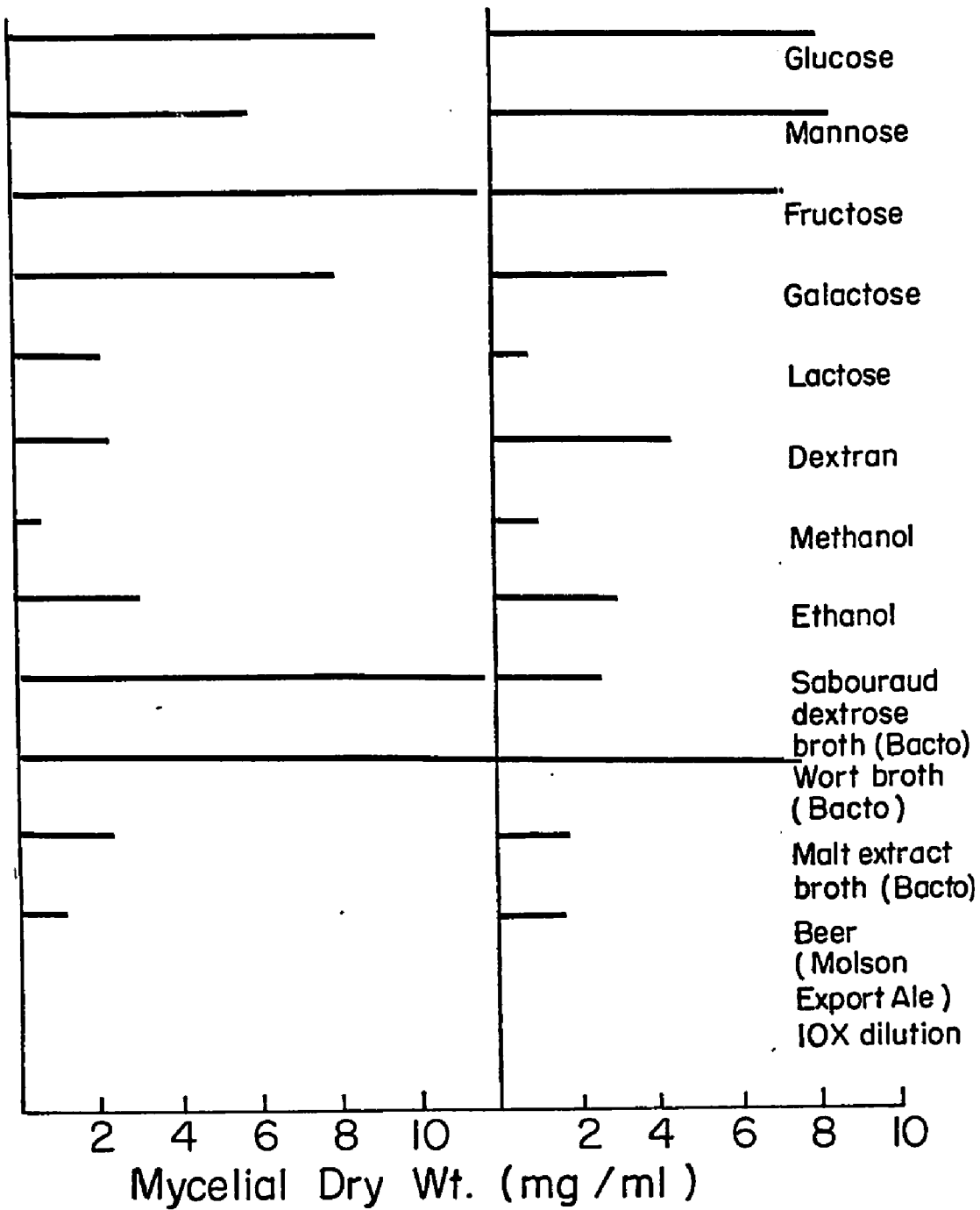


Figure 9

Growth (6 days) of Penicillium cultures C-1 and C-2
on various sources of carbon.

Culture
(a) C-1

Culture
(b) C-2



for culture C-1 was obtained with fructose (12 mg dry weight/ml), whereas for C-2 both glucose and mannose gave comparable growth (about 8 mg/ml).

Types of Cultivation: A comparison of growth under shake flask, fermentor, and dialysis cultivation was made with cultures C-1 and C-2. The results are shown in Figure 10. Of these cultivation methods, dialysis gave the best growth. For instance in culture C-1, the dry weight at the end of a 6-day incubation in dialysis culture was as high as 25 mg/ml, whereas in shake flask it was about 10 mg/ml. In fermentor culture the dry weight decreased to 8 mg/ml. In culture C-2, the growth decreased in the same order as seen in culture C-1, but the dry weight obtained in each cultivation method was lower than the corresponding values in culture C-1.

Uranium and strontium: Experiments were conducted in which specific levels of either uranium or strontium were added to the basal medium, and the effect on cultures C-1 and C-2 was determined. The results are shown in Figures 11 and 12. For culture C-1 over the range of 100 to 1,000 ppm, uranium gave no significant inhibition of growth during the 6-day period of incubation, but with strontium at 1,000 ppm significant growth retardation was seen after 4 days. Retardation of growth became more significant at longer

Figure 10

Growth of Penicillium cultures C-1 and C-2 under shake flask, fermentor, and dialysis cultivation on glucose medium.

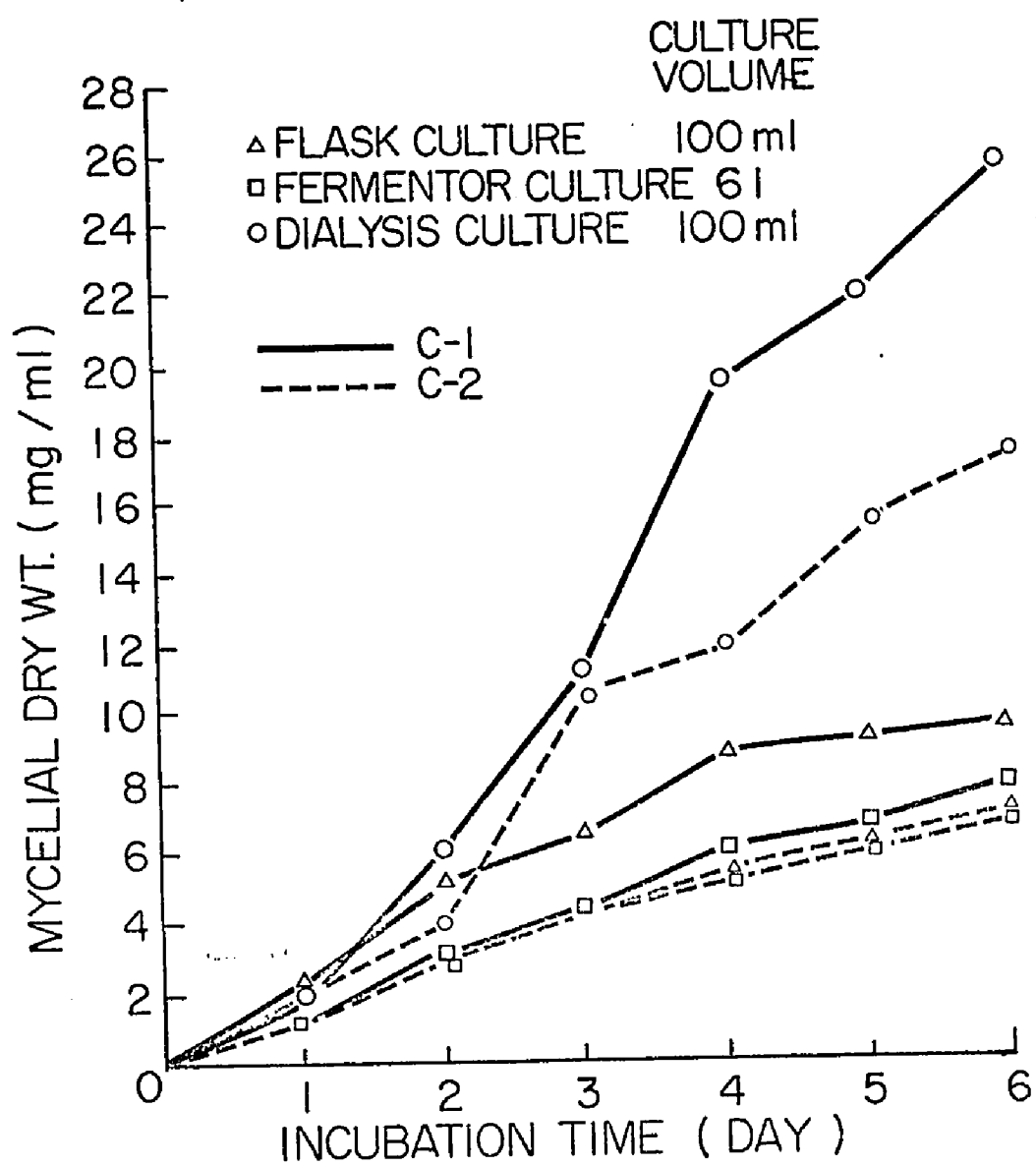
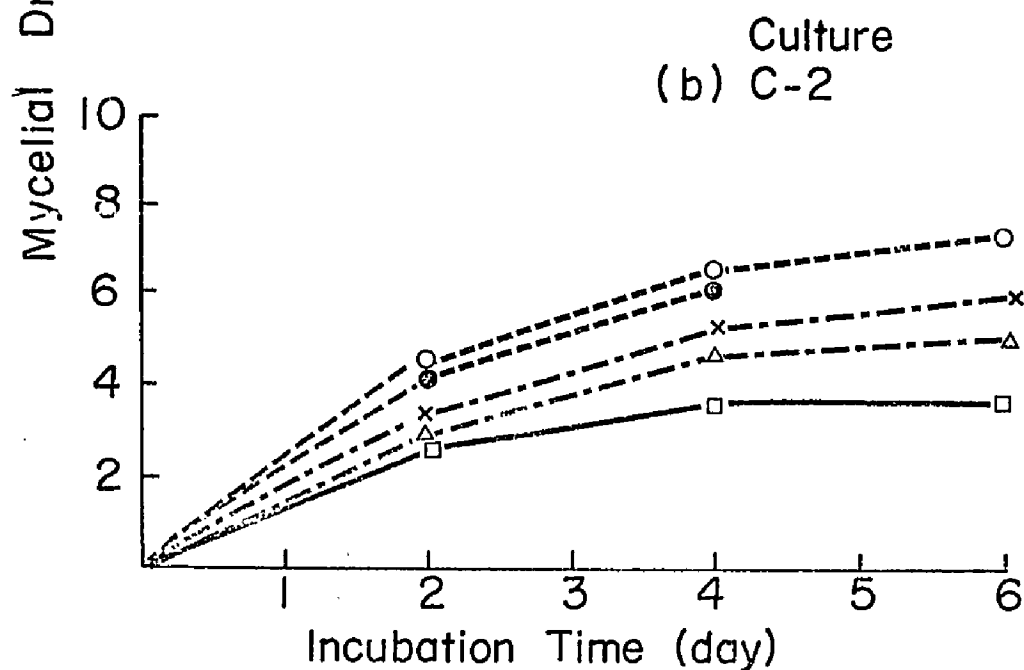
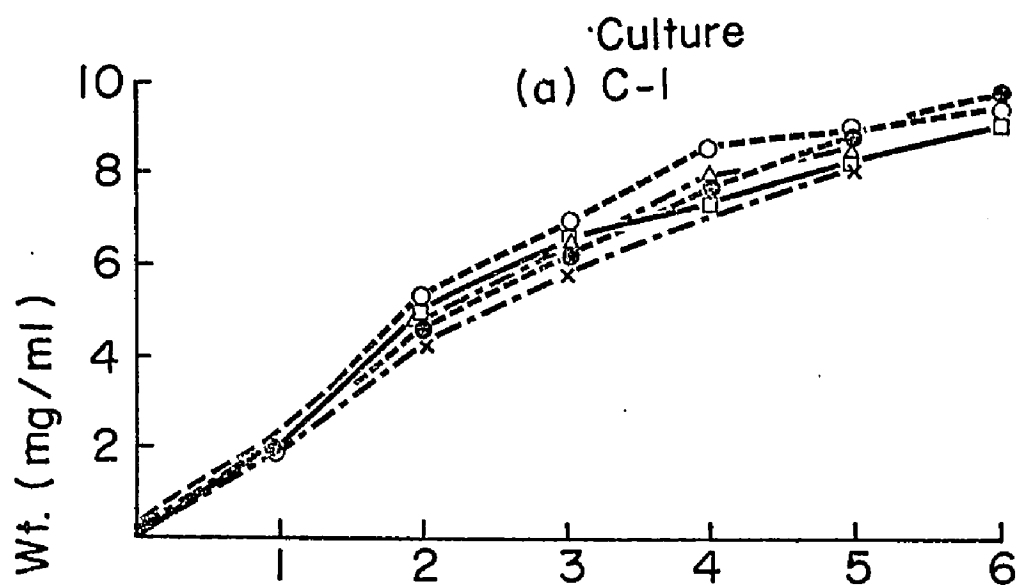


Figure 11

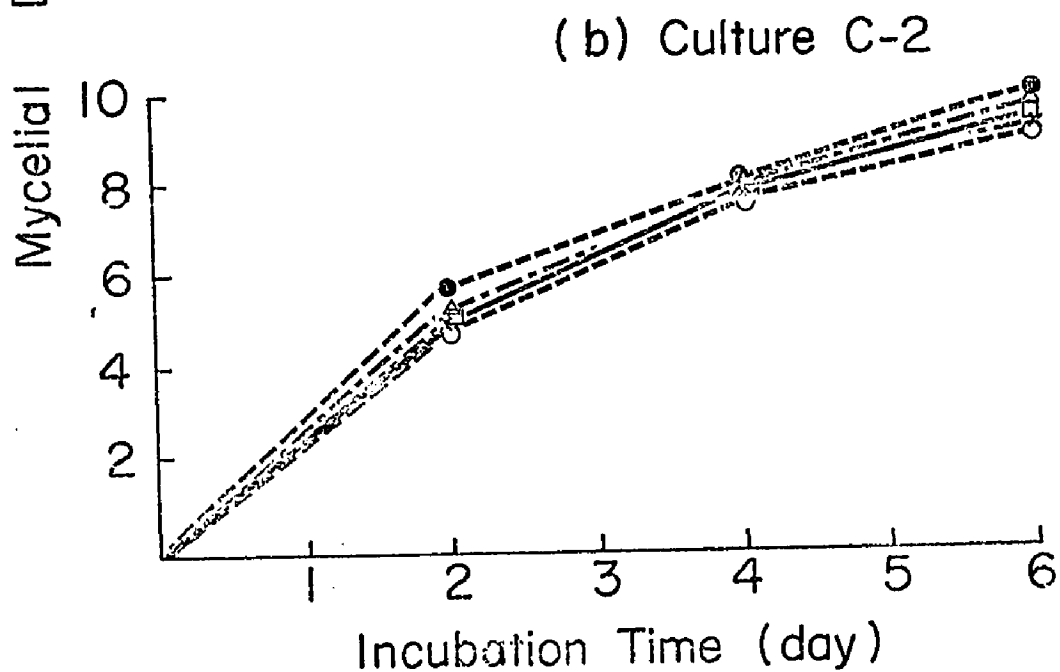
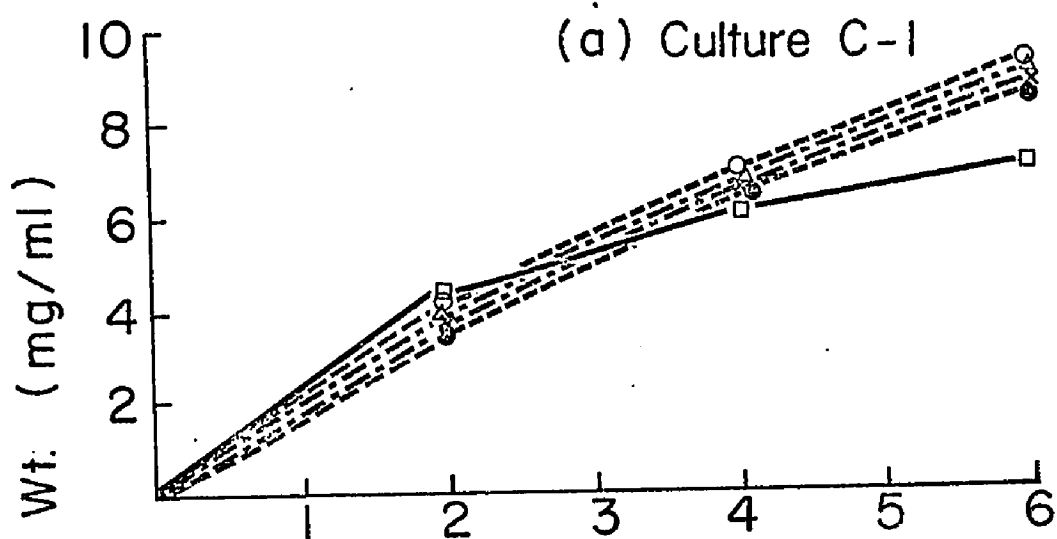
Effect of concentration of uranium on growth of Penicillium cultures C-1 and C-2. Shake flask culture on glucose medium plus uranium at various concentrations (100, 200, 600, and 1,000 ppm).



- | | |
|------------|--------------------------------|
| ○- - - - ○ | Basal glucose medium (control) |
| ●- - - - ● | " plus 100 ppm Uranium |
| x- - - - x | " plus 200 ppm Uranium |
| △- - - - △ | " plus 600 ppm Uranium |
| □- - - - □ | " plus 1,000 ppm Uranium |

Figure 12

Effect of concentration of strontium on growth of Penicillium cultures C-1 and C-2. Shake flask culture on glucose medium plus strontium at various concentrations(100, 200, 600, and 1,000 ppm).



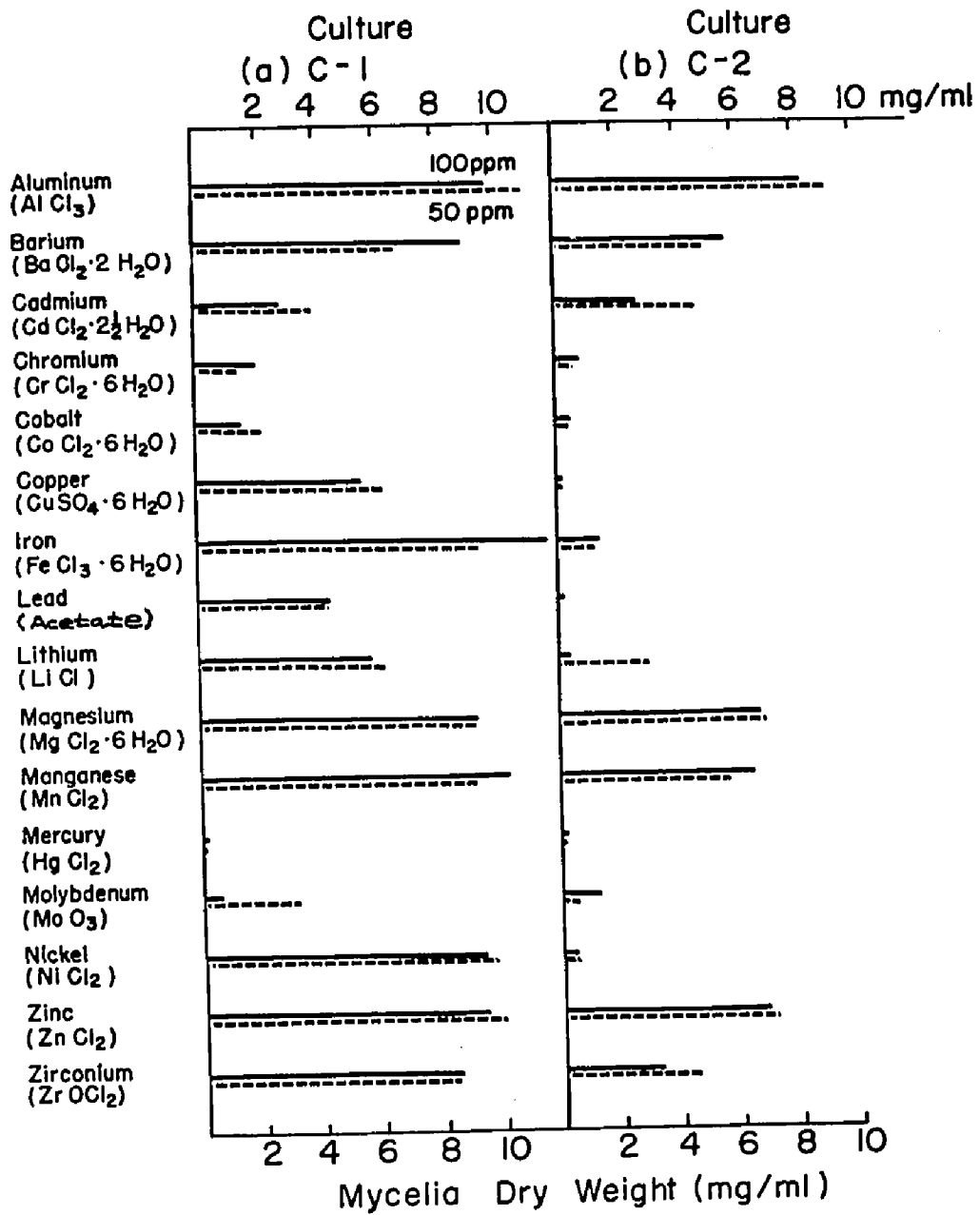
- Incubation Time (day)
- Basal glucose medium (control)
 - " plus 100 ppm Strontium
 - ×-----× " plus 200 ppm Strontium
 - △-----△ " plus 600 ppm Strontium
 - " plus 1,000 ppm Strontium

incubation periods, e.g., at the end of a 6-day period of incubation the dry weight was 7 mg/ml, whereas in the control or where less than 600 ppm strontium was added the dry weight was 8.5 - 9 mg/ml. An identical experiment was conducted with culture C-2 with opposite results. With uranium the dry weight of culture C-2 decreased steadily as the concentration increased. For instance at the end of 6-day incubation period, the dry weight decreased from 7 to 4 mg/ml as the uranium concentration increased from zero to 1,000 ppm (Figure 11b). With strontium, there were no significant differences among the dry weight obtained with various concentrations ranging from zero to 1,000 ppm (Figure 12b).

Other metals: In addition to the five heavy metals mentioned earlier, various other metals were also used to test the growth of cultures C-1 and C-2. The metal salts were autoclaved separately and added to the glucose basal medium. Each metal was tested at 50 and 100 ppm, the results (6 days' growth) are shown in Figure 13. Aluminum, barium, and zinc were less toxic to both cultures. In contrast, cadmium, cobalt, chromium (Cr^{3+}), mercury, and molybdenum were more toxic. The most toxic metal was mercury. The individual culture showed markedly different behavior to several of the metals. For instance copper and nickel, which inhibited the growth of C-2, had little effect

Figure 13

Effect of various metals on growth (6 days) of Penicillium cultures C-1 and C-2. Shake flask culture with glucose medium plus specific metal. Each metal was added at concentrations of 50 and 100 ppm.



on the growth of C-1. Also iron (Fe^{3+}) inhibited growth of C-2, but stimulated growth of C-1. To compare the toxicity of these metals with the five heavy metals, an evaluation was made on fungistatic concentration of the metals. By analogy with the definition of pH, the metal concentrations were converted to "pM" values as shown in Figures 14 (culture C-1) and 15 (culture C-2). The smaller pM, the less toxicity.

4.3 Discussion

Both Penicillium cultures were acid tolerant and mesophiles. In culture C-1 it seems unusual that it had two maxima in the growth-pH curve, but this type of curve has been found in several other fungi (Webb, 1919; Webb, 1921; Hopkins, 1922; Lindfors, 1924; Tilford, 1936; Brock, 1951). Both cultures C-1 and C-2 grew readily on hexoses but poorly on lactose. The growth on ethanol was moderate, and sparse on methanol.

Of the metals tested at 100 ppm, uranium, strontium, aluminum, barium, and zinc did not affect the growth significantly; but growth was retarded by cadmium, chromium, cobalt, mercury, and molybdenum. There were several significant differences in growth characteristics between cultures C-1 and C-2 as affected by metals: (a) At a concentration range up to 1,000 ppm, C-1 was more resistant

Figure 14

Fungistatic concentration of various metals (in the forms as shown in Figures 1 and 13) on Penicillium C-1. The metal concentrations are expressed in "pM" values and plotted according to the chemical group. "pM" is defined as negative of the log of the metal concentration.

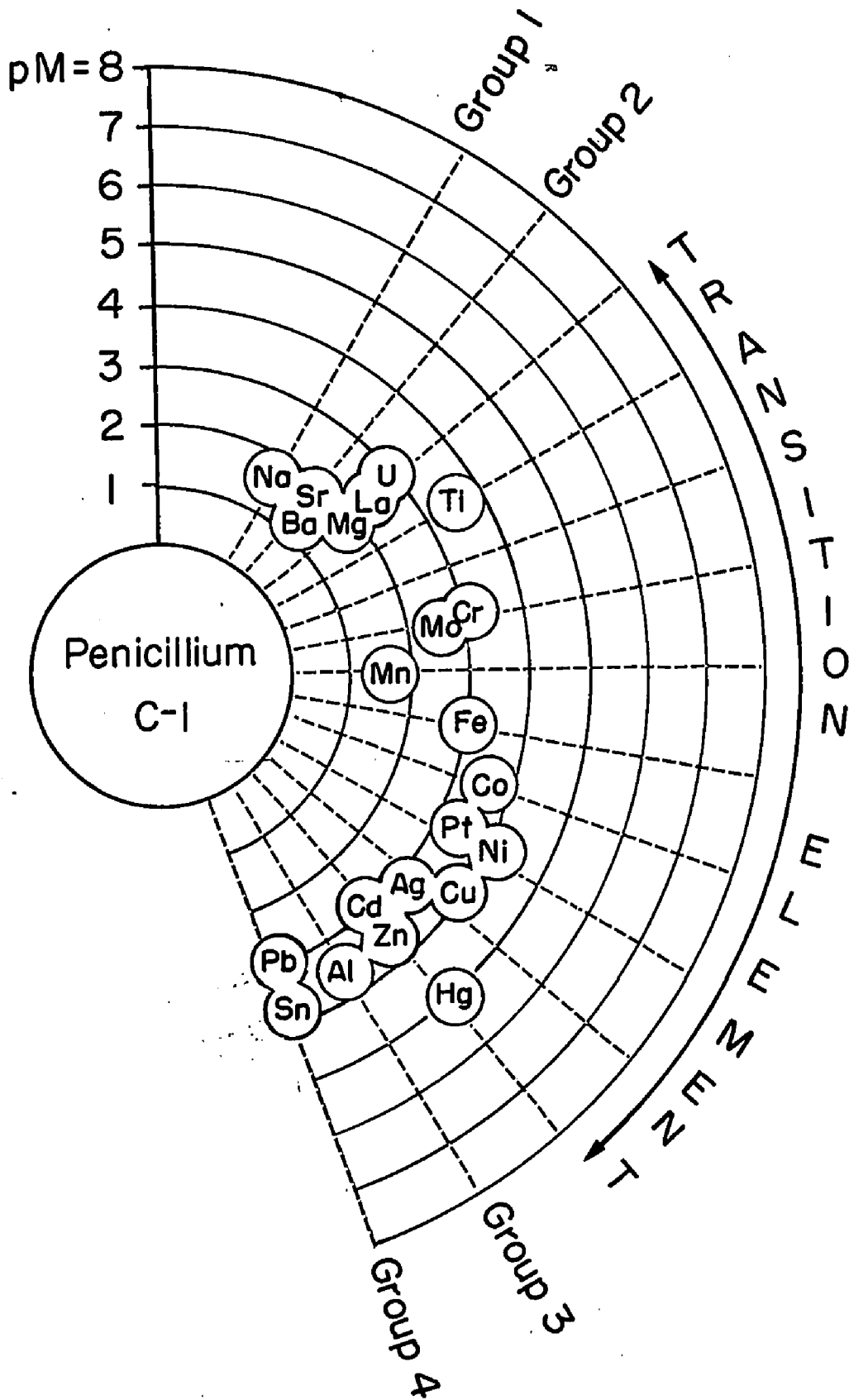
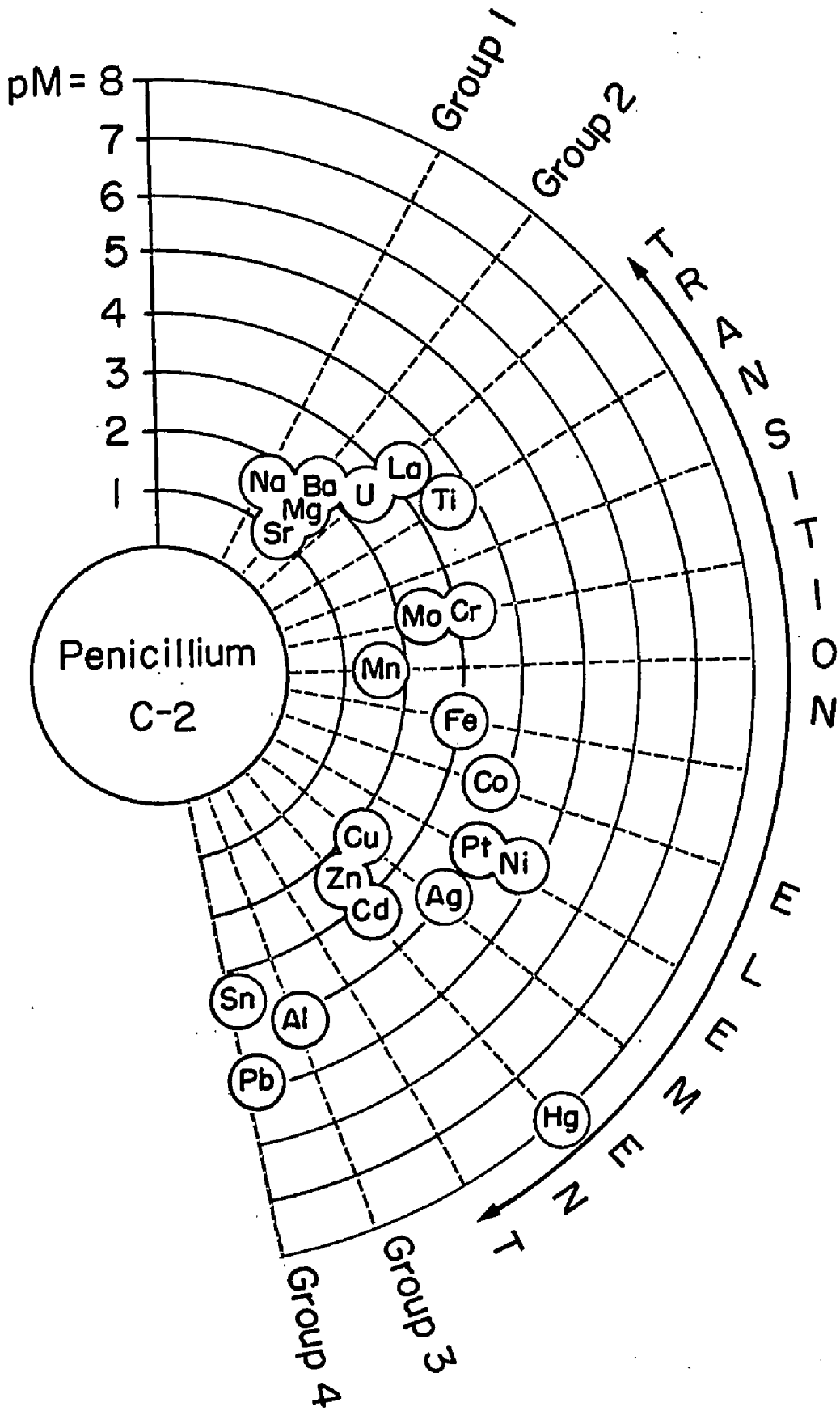


Figure 15

Fungistatic concentration of various metals (in the form as shown in Figures 1 and 13) on Penicillium C-2. The metal concentrations are expressed in "pM" values and plotted according to the chemical group. "pM" is defined as the negative of the \lg of the metal concentration.



to increased uranium concentrations, but less resistant to strontium as compared with C-2 (Figures 11 and 12). (b) At concentrations of 100 ppm, either copper, lead, or nickel inhibited the growth of C-2 almost completely, but allowed relatively good growth of C-1. Also iron inhibited growth of C-2, but stimulated growth of C-1 (Figure 13).

(c) Mercury at 10 ppm allowed the growth of C-1, but inhibited the growth of C-2 completely. With regard to developing criteria for metal toxicity, "pM" defined as negative of the log of maximum metal concentration allowable to growth of particular organism was used. With most of the metals, culture C-1 gave lower "pM" values than C-2 (Figures 14-15).

CHAPTER 5

FACTORS AFFECTING PELLET GROWTH OF Penicillium C-1

Since it was noted in earlier experiments that uranium affected the pellet size of Penicillium C-1, experiments were made to compare the sizes of pellets in Penicillium C-1 as affected by such factors as pH, inoculum size, surfactants, and metallic ions. Particularly, interest was given to the type of pellet growth as affected by the five heavy metals including uranium, strontium, titanium, silver, and platinum.

5.1 Materials and Methods

Experiments were done in shake flasks (500 ml size, 100 ml working volume) incubated at room temperature (25°C) on a rotary shaker (New Brunswick Scientific Co.) at 200 rpm. Except as otherwise stated, a 5% (v/v) inoculum of culture C-1 (homogenized in Waring blender) was added to the glucose medium (pH 5.0) and incubated 5 days (see Chapter 3).

Pellets were separated from the liquid by filtration. The size of the pellets retained on the filter was determined by using a calibrated 10x microscope. Pellets were selected at random and measured in one direction, and the

average diameter of 50 pellets was calculated. The coefficient of variation was 10-20%. Average pellet volume was calculated from the average diameter by assuming a spherical shape of pellet.

In order to develop information about the mechanism of hollow core formation, subcellular studies on the pellet were conducted with the electron microscope. The pellet was fixed overnight in 0.2 M cacodylate-buffered formaldehyde-glutaraldehyde mixture at pH 7.2, washed in 0.1 M cacodylate over 2 hours, and postfixed in 1% OsO_4 for 6 hours (Karnovsky, 1965). The material was stained overnight in 0.25% uranyl acetate, and dehydrated by a graded series of solvents: ethanol and acetone ratio 2 to 1, 1 to 1, and absolute acetone each for half hour. Pellets were then embedded in araldite and sectioned.

5.2 Results

pH: To test the effect of pH on pellet growth, initial pH of the medium was adjusted to 2.0, 3.0, 4.0, ..., and 9.0 with either hydrochloric acid or sodium hydroxide. The results are shown in Figure 16. As seen in the figure the largest pellets were obtained at pH 3.0. The pellet volume at this pH was 4.2 mm^3 , as the pH shifted to higher or lower values the pellet volume decreased. To investigate the relationship between pellet volume and growth in mycelial dry weight, the dry weight was plotted in the lower

part of the figure (Figure 16). Of note is that the maximum dry weight was also obtained at pH 3.0 which gave the largest pellets. Above and below pH 3.0, pellet size decreased; likewise growth decreased.

Inoculum size: 1, 8, and 15% (v/v) of inoculum were added to flasks containing basal medium. Pellet size was determined. Results are shown in Figure 17. As the inoculum size increased from 1, to 8, to 15%, i.e. the number of units of growth per flask was increased, the pellet volume decreased respectively from a maximum of 3.9 mm³, to 1.8 mm³, to 1.3 mm³.

Surfactant: The commercial antifoams either HODAG F-28 (Hodag Chemical Co.) or Nalco 27 (Alchem Ltd.) were added to the basal medium at concentrations of 0.5, 1, and 5% (v/v) prior to inoculation. The results showed that F-28 at 5% gave the best growth and also caused dispersed growth of mycelium.

Uranium, strontium, titanium, silver, and platinum: An inoculum was added to a series of 6 flasks. Each series contained a control with only basal medium. To each of the remaining flasks containing basal medium, either uranium, strontium, titanium, silver, or platinum was added to a

Figure 16

Effect of initial pH on pellet size of Penicillium C-1.
Shake flask culture on glucose medium. Pellet size and
dry weight were determined at the end of a 5-day incubation
period.

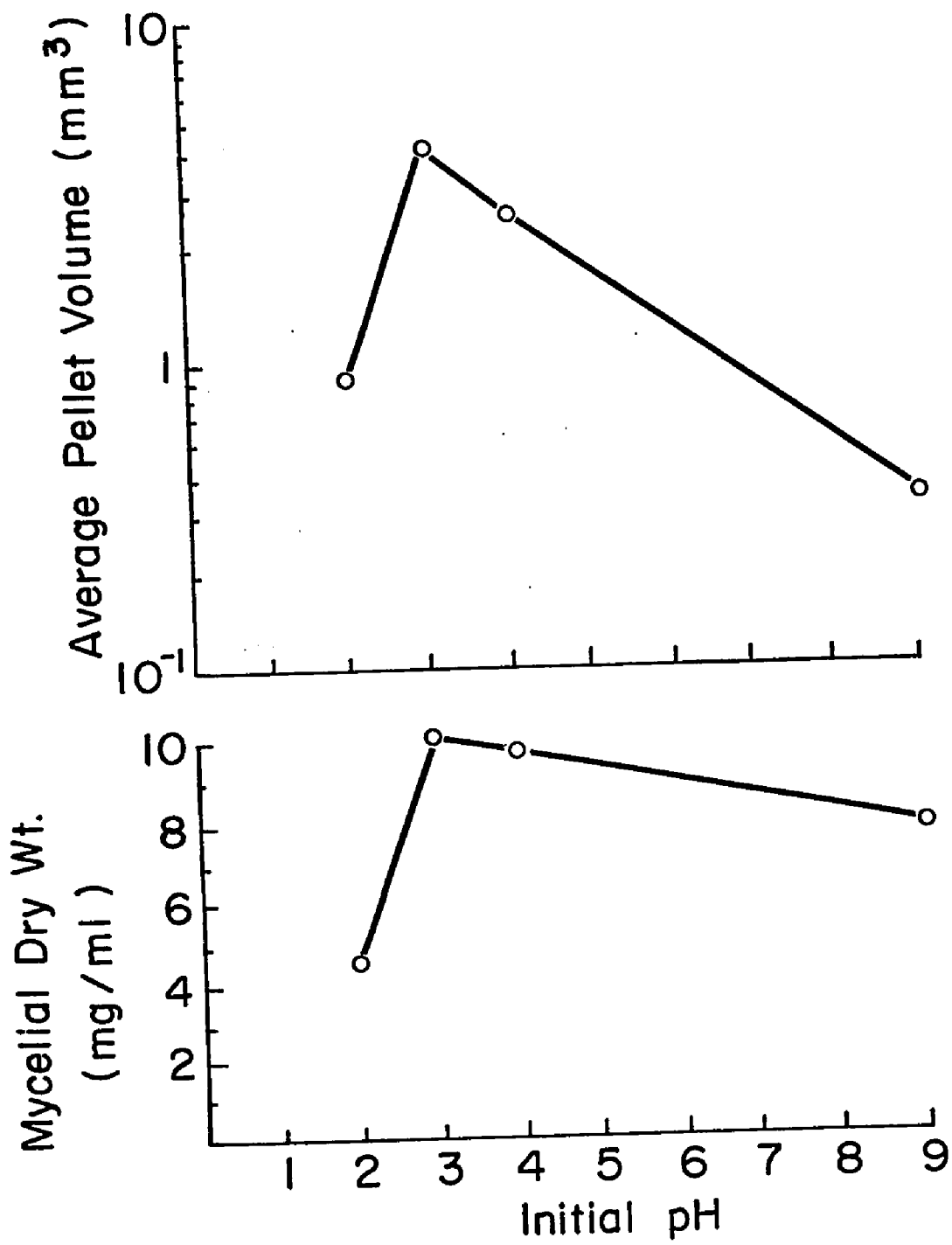
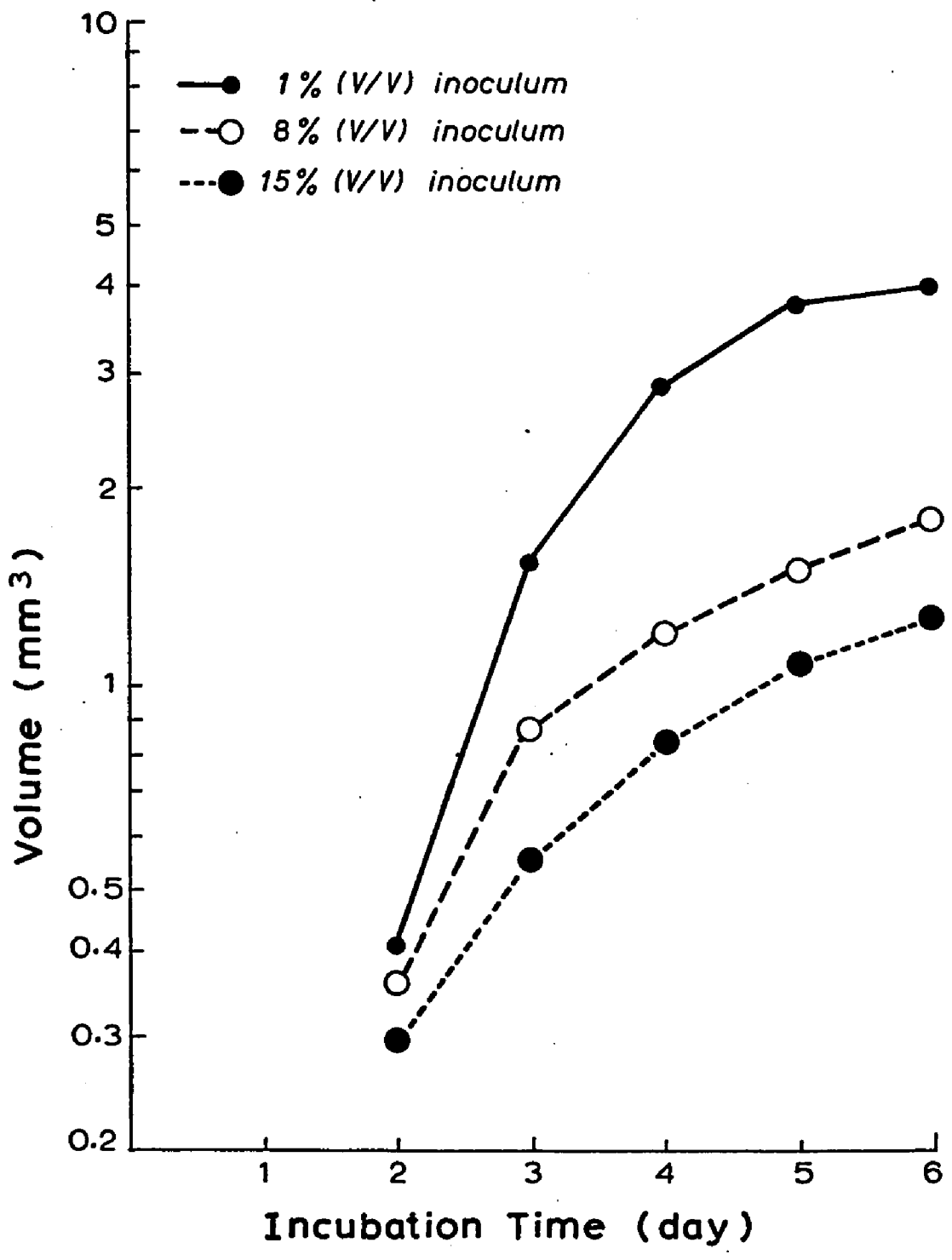


Figure 17

Changes in pellet size of Penicillium C-1 as affected by inoculum size. Shake flask culture on glucose medium.



final concentration of 100 ppm. After a specific time of incubation, pellets were separated for analysis. The change of pellet size during growth upon addition of each metal is shown in Figure 18. Changes in dry weight of mycelia with time are indicated in the lower part of this figure. Of the five metals tested, uranium gave pellets (average volume 5.1 mm^3) which were larger than the control culture (average volume 2.5 mm^3) which received no special supplement of metals. A comparison of the effect of strontium addition with the control shows no significant difference. In comparing the individual metals, the average size of pellets decreased in the order of uranium, strontium, titanium, platinum, and silver.

Other metals: In addition to the preceding five metals, the effect of various other heavy metals on pellet formation was evaluated. Metal salts were autoclaved separately and added at various concentrations to the basal medium. The average pellet volume was plotted against metal concentration using a log-log scale as shown in Figure 19. Generally, in the range (0.1 - 1,000 ppm) of concentration tested, pellet size decreased in volume as the metal increased in concentration. For individual metals, the rate of decrease became greater as the concentration increased. When a comparison was made among the metals, it

Figure 18

Growth of pellets of Penicillium C-1 on glucose medium
in the presence of 100 ppm uranium, strontium, titanium,
platinum, or silver. Shake flask culture.

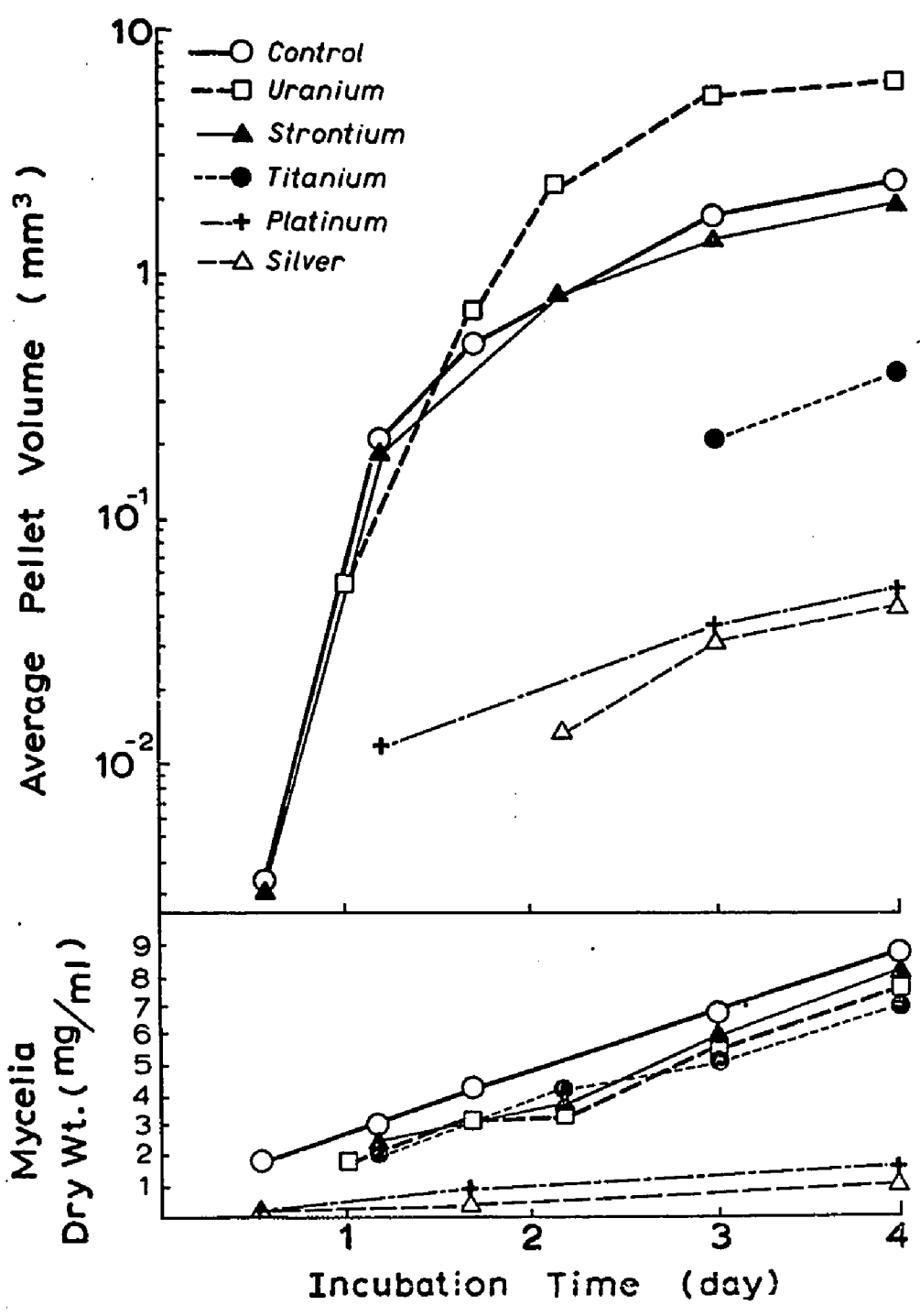
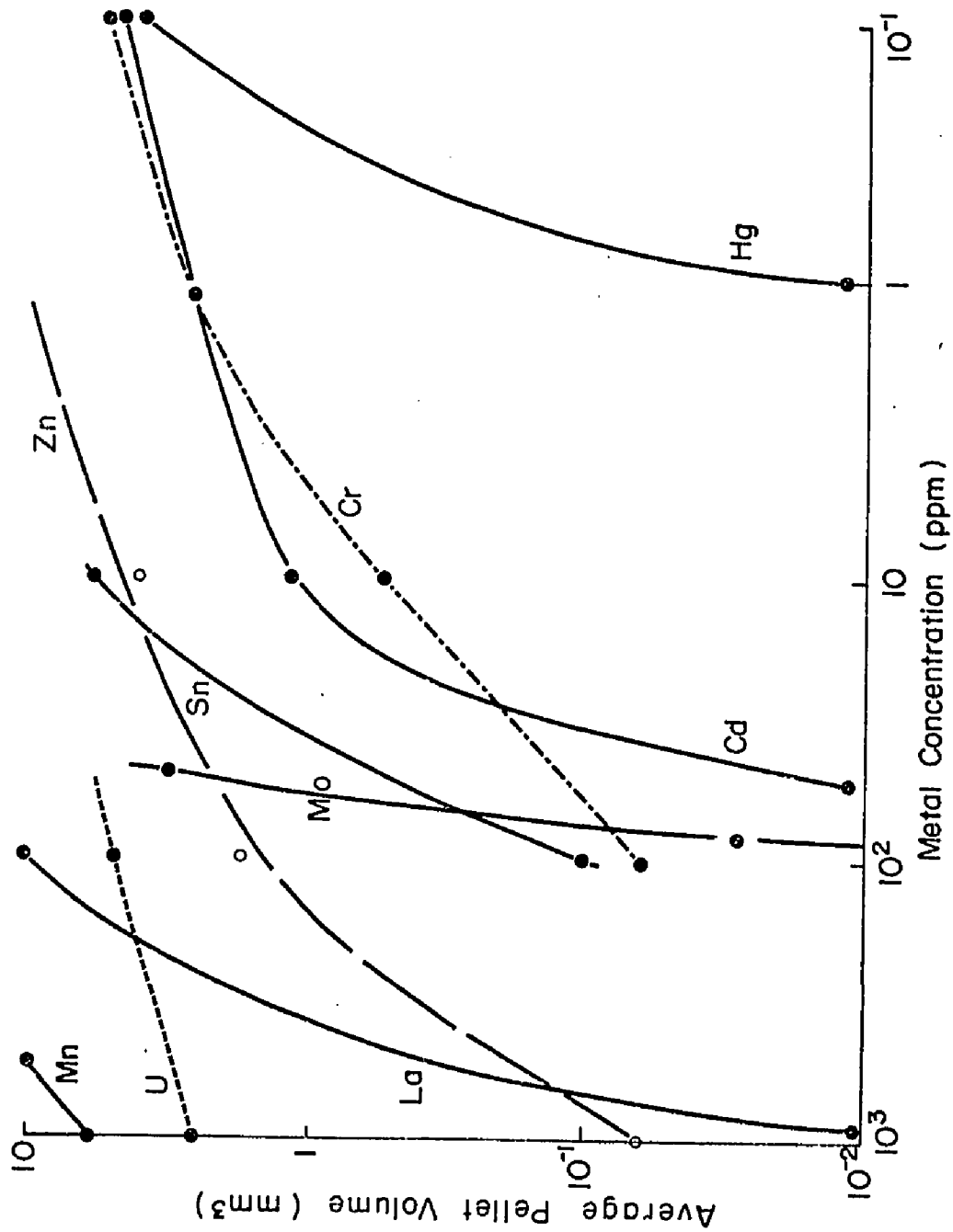


Figure 19

Effect of concentration of various heavy metals (in the forms as shown in Figures 1 and 13) on pellet size of Penicillium C-1. Shake flask culture on glucose medium. Pellet size was determined at the end of a 5-day incubation period.



was more difficult to obtain a discrete pellet with the more toxic metals. For instance when comparing the three most toxic metals, mercury, cadmium, and chromium, at concentration 0.1 ppm each metal gave pellets similar in size. The average pellet volume varied between 4.6 to 5.2 mm³. However when the concentration was increased from 0.1 to 10 ppm of mercury and to 100 ppm of cadmium, there was no pellet formation. To compare uranium with other heavy metals, an identical experiment was conducted with uranium at 100 and 1,000 ppm. The average pellet volumes obtained at these concentrations were respectively 4.8 and 2.5 mm³ which were much larger compared to those obtained with other metals at the same concentration (Figure 19). The corresponding mycelial dry weight obtained with these metals is not shown in the figure, but repetition of these experiments showed that when certain growth characteristics are weakened by metal addition, a smaller size of pellet is obtained.

5.3 Discussion

There are many factors, e.g. pH, inoculum size, heavy metals, affecting pellet growth of Penicillium C-1. Of particular interest is the effect of heavy metals on pellet formation. Of five heavy metals (uranium, strontium, titanium, silver, and platinum) tested, uranium gave the

largest size of pellet which was even larger than a control receiving no heavy metal treatment.

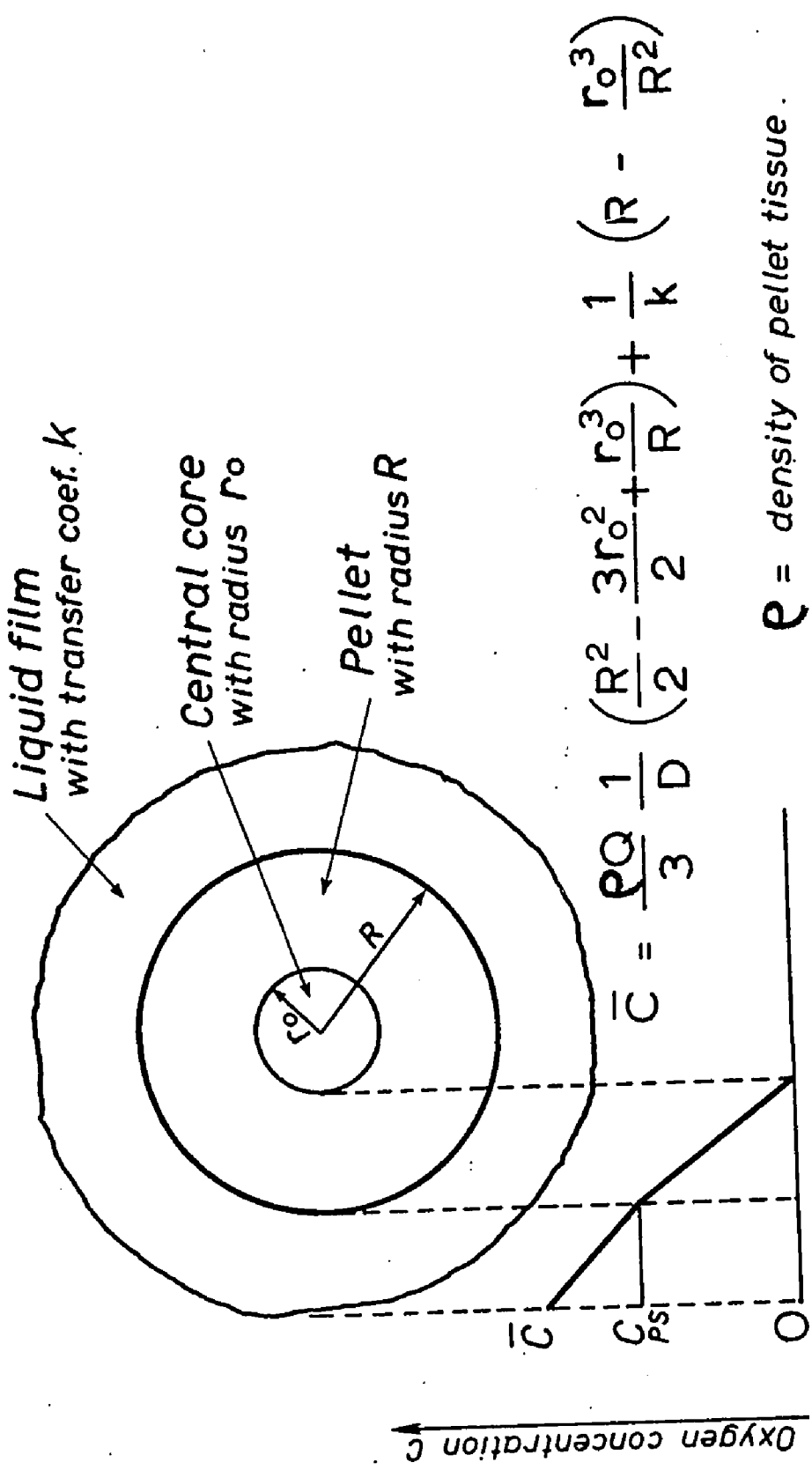
The size attainable in pellet growth can be explained by the diffusion-limitation theory. A schematic diagram of the pellet of fungus and an overall equation based on diffusion phenomena are shown in Figure 20. Important factors supporting the use of diffusion-limitation theory are: (a) One can demonstrate that a central hollow core forms in the pellet when it attains a certain size (Figure 21). Furthermore, the electron micrographs showed that the intracellular structure adjacent to the hollow core appears "loose" and more granular with part of the cellular material lysed away (Figure 22), whereas the cell from the peripheral zone of the pellet shows a compact and continuous intracellular structure (Figure 23). (b) A larger size of inoculum gives smaller pellets (Figure 17). (c) When growth as determined by mycelial dry weight was weakened by certain factors such as pH or heavy metals, pellet growth was inhibited (Figure 16). The theory of diffusion-limitation as related to pellet growth has also been suggested by a number of authors (e.g. Yano et al., 1961; Phillips, 1966; Pirt, 1966; Zajic and Chiu, 1972b).

Based on the diffusion-limitation theory it is suggested that uranium, which caused pellets larger than the control without uranium, facilitated the diffusion of the

growth-limiting substances into the cells of the pellet of Penicillium C-1. It is presumed that at least part of the uranium was transported to the cell membrane to form some type of complex with the membrane structure.

Figure 20

A schematic diagram of pellet of fungus and the overall equation based on diffusion phenomena (for derivation of the equation, see Appendix 1).



ρ = density of pellet tissue.

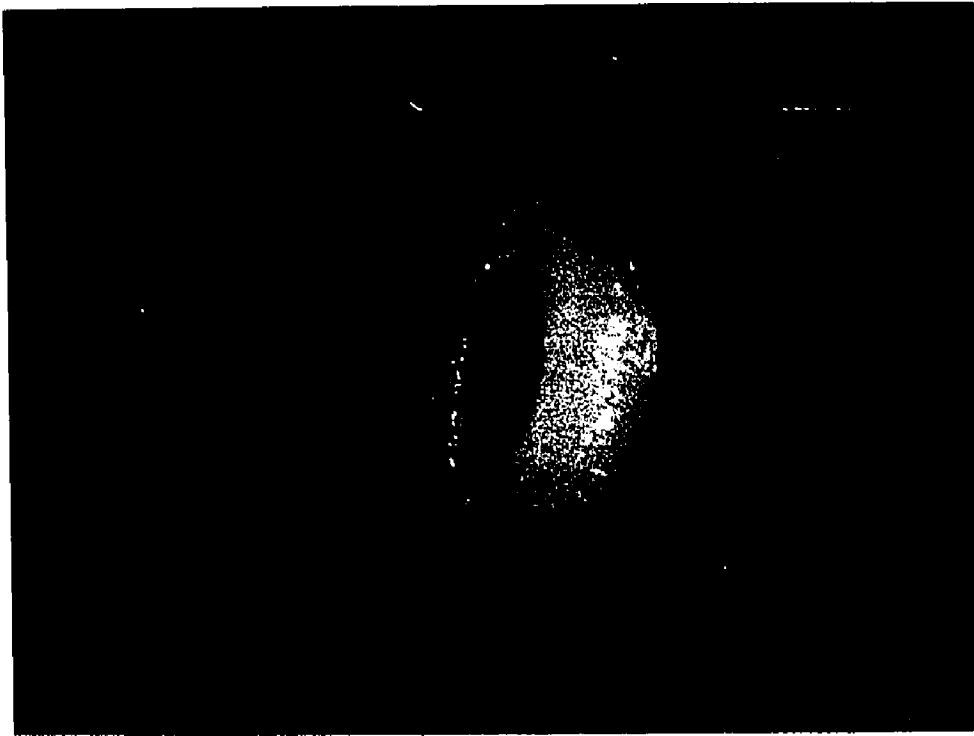
Q = metabolic demand rate per unit weight of pellet tissue.

D = diffusion coefficient

path of oxygen diffusion

Figure 21

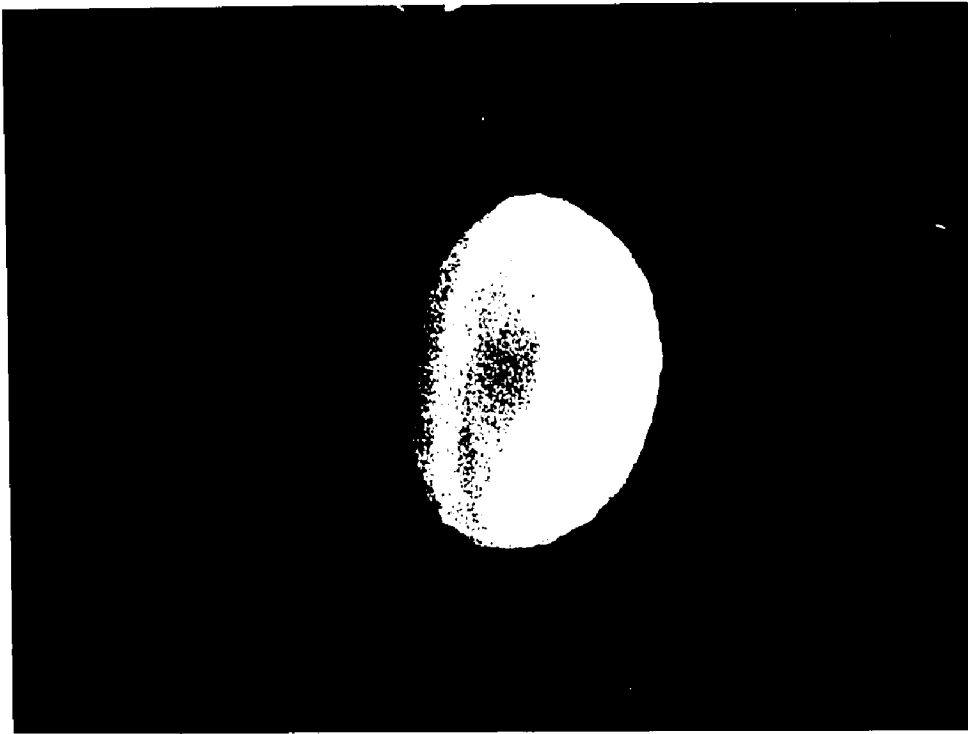
Photo-micrographs demonstrating the hollow central core
of pellet in Penicillium C-1.



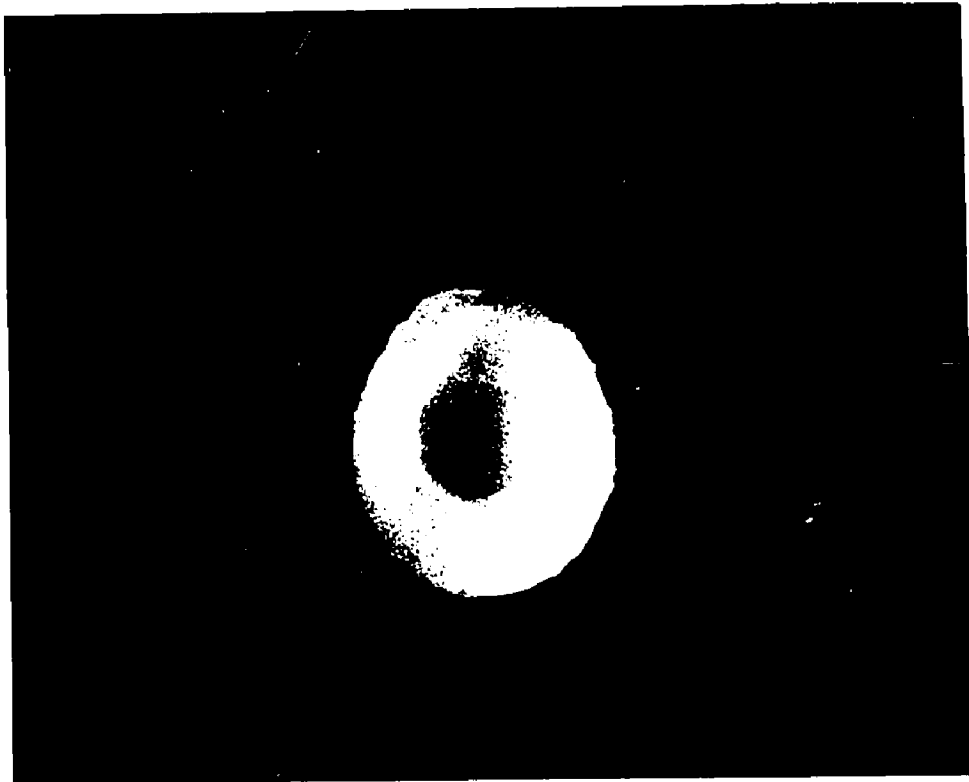
b



a



b



a

Figure 22

Electron micrograph of hyphae adjacent to the central
hollow core of the pellet of Penicillium C-1. Shake flask
culture on glucose medium for 5 days.









Figure 23

Electron micrograph of hyphae at the periphery of the pellet of Penicillium C-1. Shake flask culture on glucose medium for 5 days.

CHAPTER 6

KINETICS OF GROWTH OF Penicillium C-1

6.1 General Growth Kinetics

In single cell organisms such as bacteria, growth may be represented by either cell number or total cell mass which in turn can be expressed as dry weight. Under ideal conditions these two expressions are identical. Therefore during exponential growth, the specific growth rate μ can be calculated from either

$$n = n_0 e^{\mu t} \quad (6.1)$$

where n = cell number at time t

n_0 = initial cell number

or

$$M = M_0 e^{\mu t} \quad (6.2)$$

where M = total cell mass at time t

M_0 = total initial cell mass

However, in filamentous organisms such as fungi grown under submerged culture conditions, mycelial dry weight is the best method of expressing growth. In studying the growth kinetics of fungi in submerged culture, some workers (Zalokar, 1959; Pirt and Callow, 1960; Borrow et al., 1964; Yu, 1972) showed that the exponential equation (Equation 6.2) could be used to describe the growth curve within a limited range; while others (Emerson, 1950; Machlis, 1957; Marshall and Alexander, 1960) found that the growth could be described by the cube root equation:

$$M^{1/3} = k't + M_0^{1/3} \quad (6.3)$$

where k' = constant

In view of these two types of growth, Pirt (1966) suggested that exponential growth may be associated with the dispersed mycelial form and the cube root growth with pellet form. The cube root equation which was previously empirically based, was derived from the diffusion-limitation theory for the growth of fungi in pellet form under certain conditions (Pirt, 1966). The theory took into account that the thickness of the active growing peripheral layer is small compared with the radius of pellet. Therefore use of Equation 6.3 can not be made until the pellet acquires a

certain theoretical size. In the batch growth of Penicillium C-1 in shake flask or fermentor, as seen in Figure 10 neither an exponential nor a cubic relation was followed. Instead, a simple linear relation exists in the growth approximately up to 4-days. Beyond this period, the growth was retarded. During the active growth of culture C-1 on glucose medium the doubling time was about one day, and the growth rates were 2.1 and 1.5 mg dry wt./ml/day respectively for both shake flask and fermentor culture systems.

6.2 Kinetics of Growth in Dialysis Culture

It is generally agreed that growth of microbes in batch culture is limited by either exhaustion of nutrients or accumulation of toxic products. Thus if the toxic products are removed from the broth of culture, a higher rate of growth and a higher concentration of cells can be obtained. Regarding these aspects, the recently developed dialysis culture method (Gerhardt and Gallup, 1963), was used to remove toxic products (Gerhardt and Gallup, 1963; Aida and Yamagucki, 1969). This in turn gives higher rates of growth and a higher concentration of cells. It was demonstrated that the capability of dialysis culture to affect cell concentration is by extending the active growth period. Culture C-1 was grown in dialysis flasks

(Figure 6, Chapter 4) on a glucose medium. The data obtained (Figure 10, Chapter 4) confirmed that the growth rate was higher as compared with the usual batch culture. The growth curve during the active growing phase was described by a square root equation:

$$M^{1/2} = M_0^{1/2} + k''t \quad (6.4)$$

The constant obtained with the growth curve of dialysis culture (Figure 10) is $k'' = 1.2$ (mg dry wt./ml)^{1/2}/day. The growth equations of dialysis, shake flask, and fermentor culture of Penicillium C-1 were compared and are shown in Figure 24.

6.3 Kinetics of Growth in Pellet Size

In the growth of a single pellet, at the initial stage it is believed that the supply of oxygen and nutrients can reach all regions in the pellet, therefore a simple exponential growth relation is able to describe the growth. The equations are:

$$dm/dt = \alpha'm$$

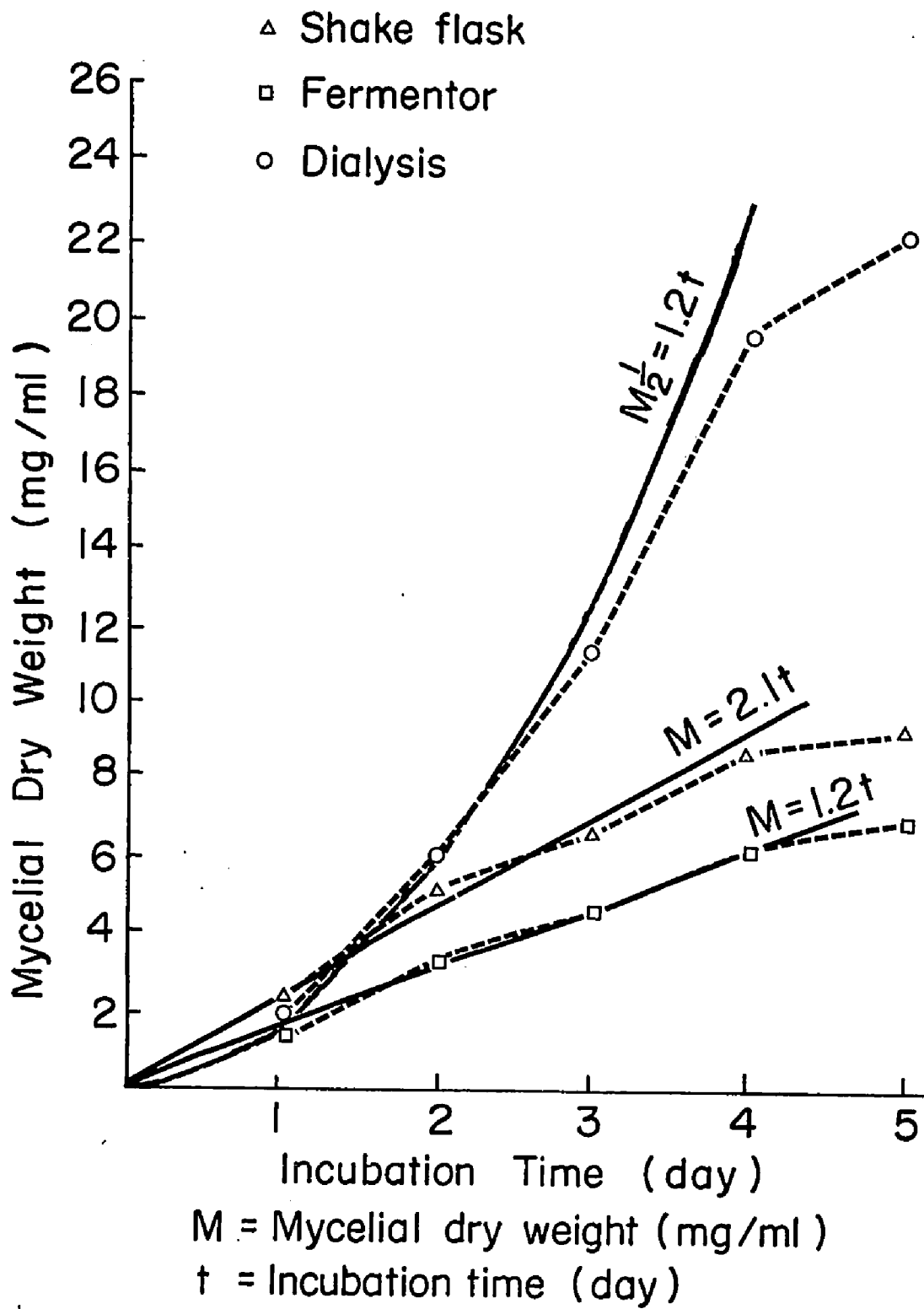
$$m = m_0 e^{\alpha't}$$

or

$$V = V_0 e^{\alpha't} \quad (6.5)$$

Figure 24

Growth of Penicillium C-1 under dialysis, shake flask, and fermentor culture on glucose medium were compared and expressed by the equations.



where m = mass of a pellet at time t

m_0 = initial mass of the pellet

V = volume of the pellet at time t

V_0 = initial volume of the pellet

α' = constant

However when the pellet exceeds a certain size, it is presumed that the growth is limited to a peripheral layer of constant thickness. As the pellet growth continues, the proportion of the peripheral layer to the whole pellet becomes smaller. Under these conditions there is a retardation in exponential growth. In view of this, it was suggested that the fundamental law governing the growth of most organisms as described by the Gompertzian function might describe the growth of a single pellet (Zajic and Chiu, 1972b).

The Gompertzian function is given in the following:

$$V = V_0 \exp A/\alpha [1 - \exp (-\alpha t)] \quad (6.6)$$

where V = volume at time t

V_0 = initial volume

A, α = constants

Differentiation of Equation 6.6 with respect to time gives:

$$dV/dt = A \exp (-\alpha t) V \quad (6.7)$$

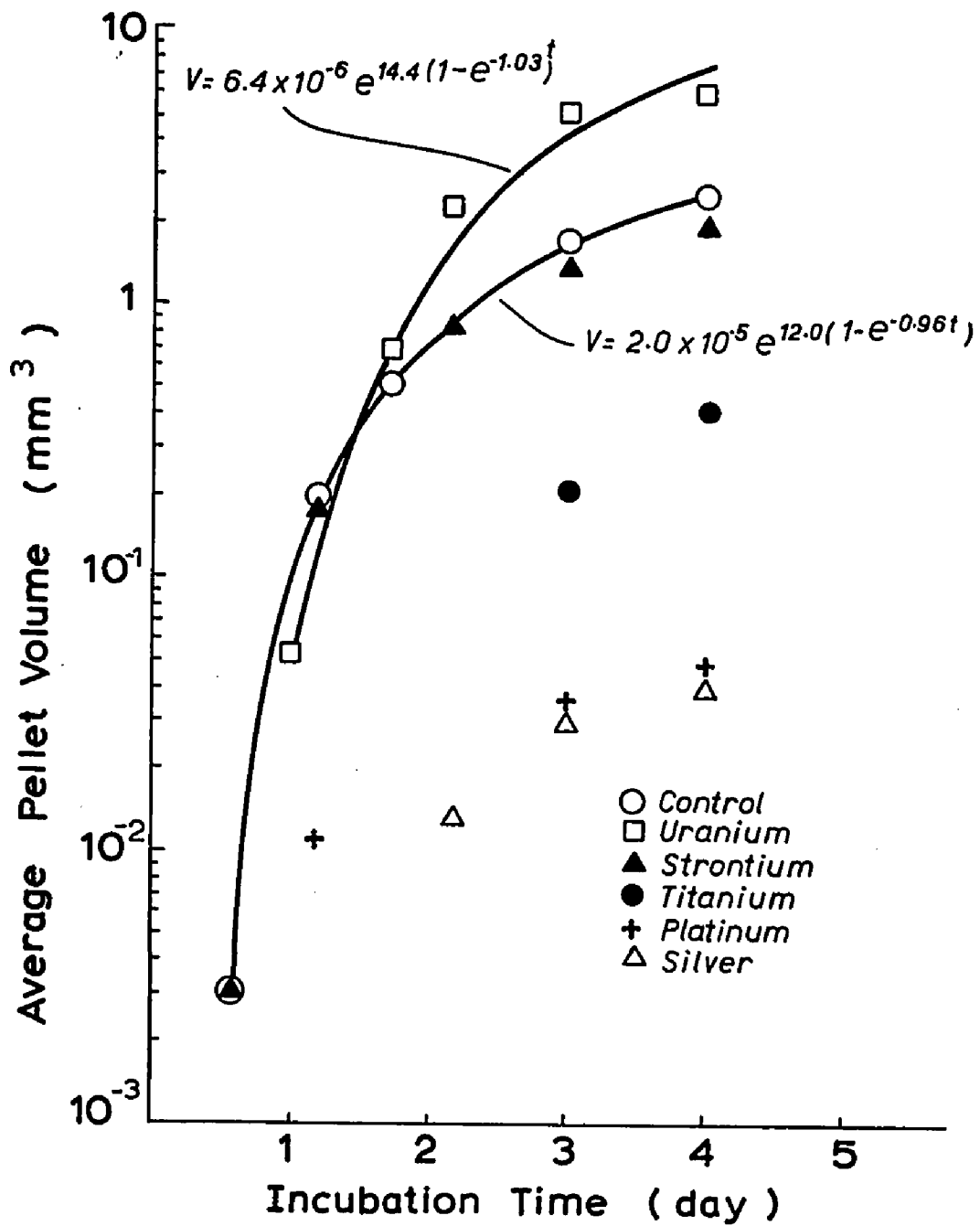
The exponential factor in Equation 6.7 indicates growth becomes slower as the organism increases in size. The cause of retardation in exponential growth is attributed to either an increase in generation time or loss in the proportion of growing cells, or a combination of both. During the pellet growth of fungi, it appears that a decrease in the proportion of actively growing cells is the major factor causing a retardation of growth. This decrease is attributed to a diffusion-limitation of growth substrates (see Section 5.3, Chapter 5).

In applying the Gompertzian function to the growth of a pellet, two curves represented respectively by the two Gompertzian equations are drawn to compare with the curves of pellet growth (Figure 18), they are shown in Figure 25. It is found, as would be expected, that the Gompertzian function describes pellet growth up to many thousand-fold increase in cellular volume. The experimental values of the dimensionless constant A/α for the two Gompertzian equations are in the range between 12 and 14 for culture C-1, which was grown in either glucose basal medium, or in the

basal medium plus 100 ml of uranium or strontium. From the point of diffusion-limitation of oxygen supply in the tumor, Burton (1966) developed a theory which predicts the value of A/α as being close to 10. The slightly higher values of A/α found in the present study indicated that diffusion-limitation of oxygen and nutrients may not be the only reason to cause the exponential retardation of growth, however these values are still close to the value predicted by the diffusion-limitation theory (Burton, 1966). Obviously in the present study of batch culture, the retardation of pellet growth due to accumulation of toxic products is inevitable. However, a continuous culture in which toxic products are constantly removed might give a value of A/α more close to 10.

Figure 25

Pellet size variation in Penicillium C-1 in the presence of either uranium, strontium, titanium, platinum, or silver. Data are used to compare to Gompertzian function particularly for uranium, strontium, and the control.



CHAPTER 7

CONCENTRATION FACTOR IN METAL UPTAKE BY

Penicillium C-1

Many factors are involved in metallic ion uptake by microbes. In studying the uptake of metallic ions by Penicillium C-1, the percentage of the metal removed from the medium must be compared with the environmental concentration of that ion and the total mass of cells. The concentration factor is thus defined as the ratio of the ion concentration absorbed or adsorbed by the cells to that present in the external environment. The following formula was developed to calculate the concentration factor (C.F.) in metal uptake by culture C-1:

$$\text{C.F.} = \frac{2 \times 10^5 \text{ (mg metal in mycelia/mg mycelial dry wt.)}}{\text{ppm metal in external environment}^*} \quad (7.1)$$

$$\text{where } 2 \times 10^5 = 0.2 \times 10^6$$

0.2 = ratio between dry and wet weight of
mycelia (assumed)

10^6 = conversion factor for ppm

* In the present study, external environment refers to the supernatant in the upper chamber of the dialysis flask.

7.1 Experimental

Twin-chambered dialysis flasks (Figure 6) modified after Gerhardt and Gallup (1963) were used. The lower chamber (280 ml) was filled with a glucose medium containing 100 ppm of uranium or strontium. The upper chamber contained 100 ml basal medium with 5% (v/v) inoculum of 5-day-old culture C-1. In order to reach an equilibrium with respect to diffusion of metal from lower to upper chamber, the dialysis flasks were left overnight prior to inoculation. It was assumed that the dialysis membrane (pore size 0.2μ) keeps salt crystals from coming in direct contact with the cells, and that only ionic forms of the metals exist in the culture chamber. After the required time of incubation at room temperature (25°C) on a rotary shaker at 200 rpm, the whole culture volume in the culture chamber was removed. Mycelia were separated from the supernatant for dry weight analysis. The concentration of uranium or strontium in the supernatant was also analyzed (for details of materials and methods, see Sections 3.2 and 3.3, Chapter 3).

The data were substituted into Equation 7.1 to calculate the concentration factors. The results are shown in Figures 26 and 27, respectively for uranium and strontium.

Figure 26

Concentration factor in uranium uptake by Penicillium C-1.

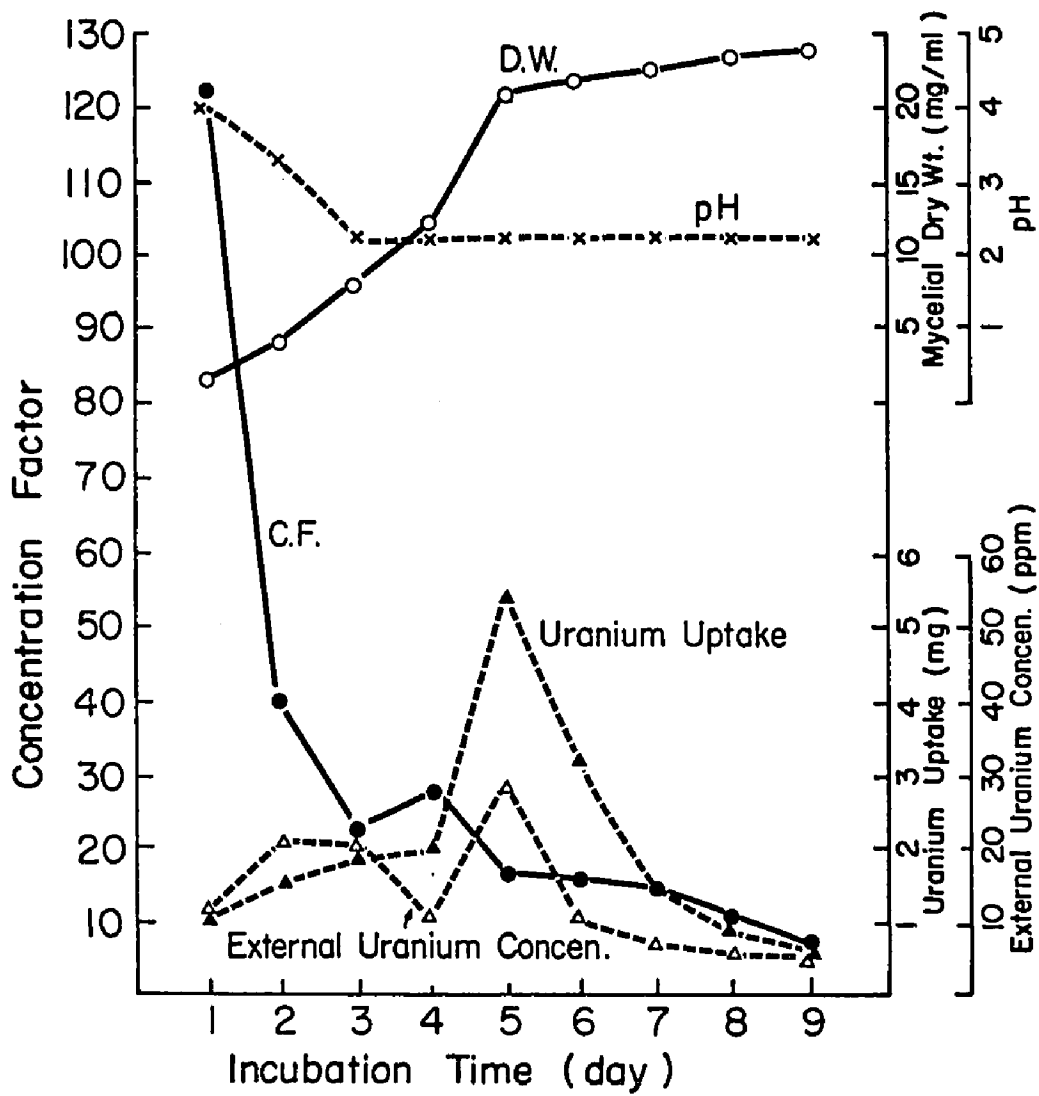
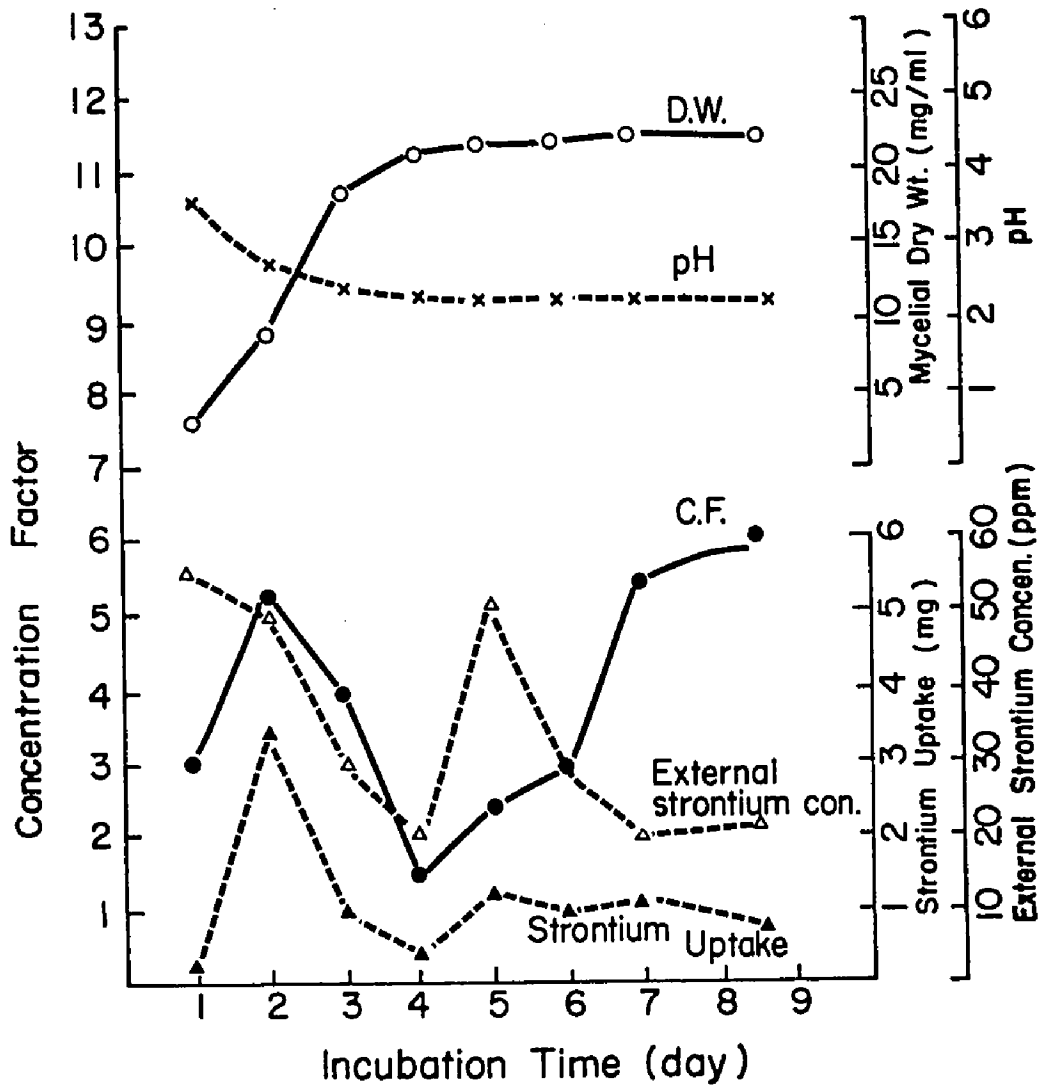


Figure 27

Concentration factor in strontium uptake by Penicillium

C-1.



7.2 Results and Discussion

In general the concentration factor for uranium uptake was much higher than for strontium. For uranium the concentration factor decreased from 122 to 8 as the incubation time increased from 1 to 9 days (Figure 26). For strontium the concentration factor varied from 1.5 to 6 during the 9-day period of incubation (Figure 27). The time effect of incubation on concentration factor was not significant for strontium as compared to uranium. The initial affinity between the cells and uranium was greater, but as the cells aged the affinity decreased steadily. One might offer a supposition that the higher initial affinity is contributed by the higher pH (4.0 at 1 day as compared with 2.3 after 3 days), but as seen in the upper part of the figure (Figure 26) the concentration factor still decreased after 3 days while the pH was at a constant of 2.3. Thus uranium removal would not appear to be highly dependent on pH during this period.

For both uranium and strontium, the external environmental concentrations varied from time to time. For either uranium or strontium, the sum of the metal in the mycelia and that equivalent to the external environmental concentration did not keep a constant value during the time course of incubation, although the total amount of either uranium or strontium added to the dialysis flask was 28 mg. The

variation in the external environmental concentration was presumed to be due to the increase in mycelial volume and the production of metabolites which interfered with the ionic activities of the metal across the dialysis membrane. For example, if one assumes all the uranium is in the ionic form, then the uranium in the upper chamber should be 7.4 mg prior to inoculation. After inoculation the total uranium (in the mycelia and supernatant) in the upper chamber was 2.2 mg at 1 day, and it increased to a maximum of 8 mg at 5 days. After 5 days it decreased steadily as the incubation proceeded. The large amount of unaccounted uranium in the upper chamber was presumed to be due to the formation of an organo-uranium complex and re-diffusion of the complex through the dialysis membrane to the lower chamber causing an over-all decrease in the ionic level of uranium in the upper chamber. In comparing uranium and strontium, there was a similarity in the variation of the external metal concentrations with the time of incubation. Generally the external concentration was higher at the beginning, but it decreased to a minimum at 4 days, and then increased to a maximum at 5 days. After 5 days the concentration decreased again gradually till the end of the incubation period. The total uranium or strontium uptake by the mycelia was proportional to the environmental concentration of that metal. The more uranium or strontium in the environment the more uptake by the mycelia.

CHAPTER 8

FACTORS AFFECTING URANIUM UPTAKE BY Penicillium C-1

8.1 Materials and Methods

The effect of pH, temperature, age of mycelia, concentration of uranium, and culture method on uranium uptake was investigated with Penicillium C-1. Except as otherwise stated, all studies were conducted in shake flasks (500 ml) containing 100 ml glucose medium (pH 5.0) plus 100 ppm uranium, and incubated at room temperature (25°C) on a rotary shaker (New Brunswick Scientific Co.) at 200 rpm. The inoculum (dry weight 8-9 mg/ml, pH 2.2) was generally prepared from 5-day-old culture. The mycelia were homogenized in a Waring blender for 30 seconds prior to inoculation. The seed culture was prepared by transfer of spores and hyphal tips. When temperature control was required, the flasks were incubated in a controlled environmental incubator-shaker (New Brunswick Scientific Co.). Prior to inoculation the flasks were set on the shaker at the required temperature for 30 minutes. For fermentor culture, a magnetic bottom-driven fermentor of 12 liters capacity was used (Section 4.1, Chapter 4). The level of uranium added to

the glucose medium in the fermentor was 100 ppm. Other culture conditions in the fermentor were the same as those described in Section 4.1 (Chapter 4). For additional materials and methods see Sections 3.2 and 3.3 (Chapter 3).

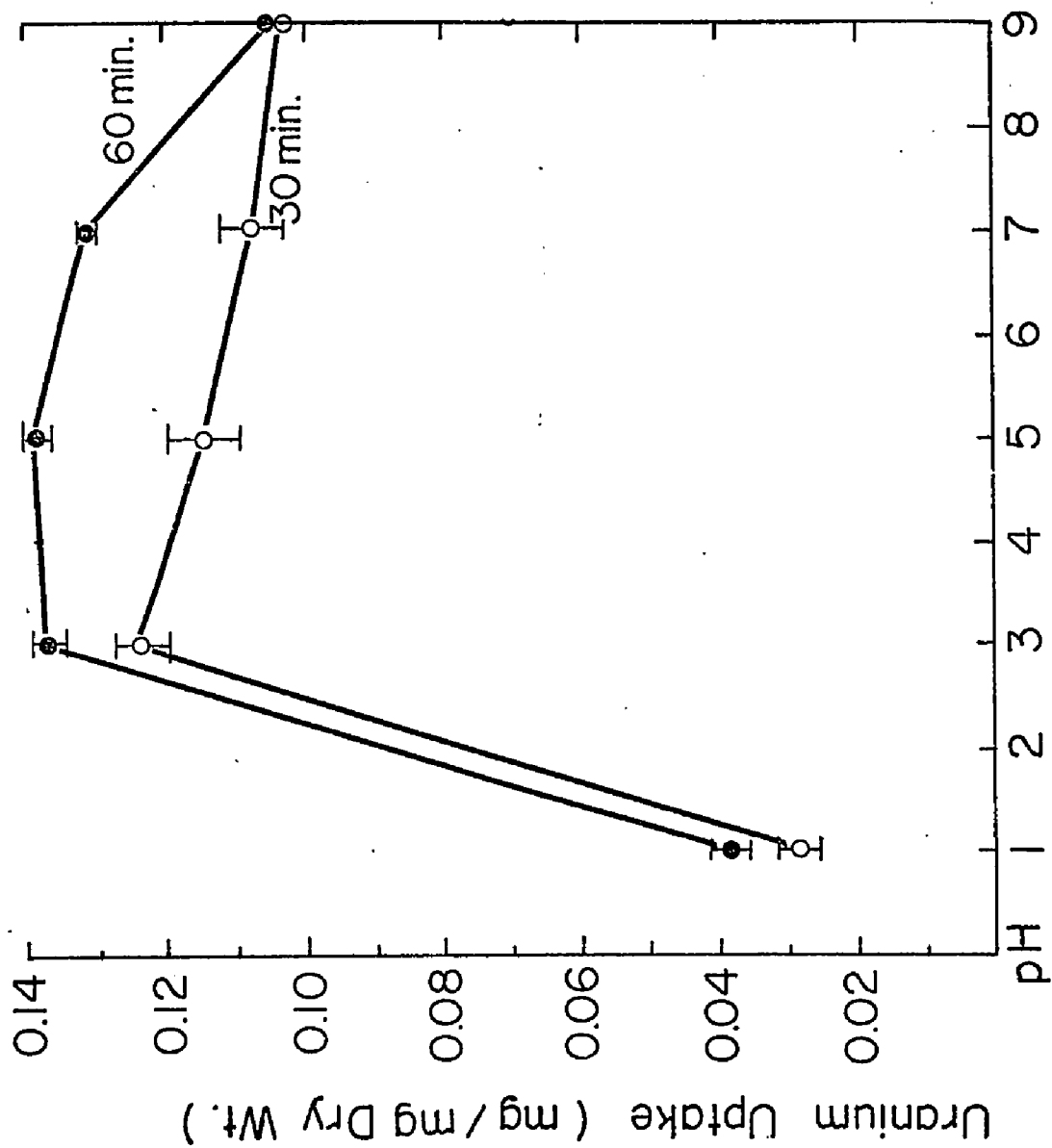
8.2 Results

pH: To test the pH effect on uptake of uranium by culture C-1, the initial pH of the medium was adjusted to 1.0, 3.0, 5.0, 7.0, and 9.0 with either hydrochloric acid or sodium hydroxide. To test flasks 10% (v/v) of inoculum from the 5-day-old culture was added. The uranium uptake obtained while using two incubation periods (30 minutes and 1 hour) is shown in Figure 28. The pH optimum for uranium uptake was approximately in the range between 3 and 5. The uptake decreased more rapidly on the acidic than on the basic side. When the pH decreased from 3 to 1, the uptake decreased from 0.14 to 0.04 mg/mg dry weight for the 60-minute period tested, whereas when the pH increased from 5 to 7, and from 7 to 9, there was only a slight decrease in uranium uptake.

Temperature: A 10% (v/v) inoculum of 5-day-old culture was added to each flask. Uranium uptake was measured at the end of 5 minutes, 20 minutes and 2 hours of incubation at various temperatures ranging from 5 to 55°C.

Figure 28

Effect of pH on uranium uptake by Penicillium C-1. The culture was incubated 30 and 60 minutes in shake flasks containing glucose medium plus 100 ppm uranium. The data points represent the average of duplicate experiments.



The results are shown in Figure 29. The maximum uranium uptake during the periods of 20-120 minutes was 0.16-0.17 mg/mg dry weight. The optimum temperature for uranium uptake was in the range of 15 to 25°C.

Age of mycelia: To investigate the relationship between mycelial age and uranium uptake, mycelia were removed from 5 and 15 days shake flask cultures. To test flasks containing uranium (in the presence of exogeneous energy source) were added 10% (v/v) inocula from 5 and 15-day-old mycelia. Uranium removal was followed over a 2-hour-period. The results are shown in Figure 30. Uranium uptake in both instances gave a similar pattern, with the younger mycelia being more active. Upon addition of inoculum, there was almost an immediate uptake of uranium by the mycelia. The uptake of uranium was quite high during the first hour. After 1 hour, a slight release of uranium was observed. On examining the time sequence of uranium uptake by the younger mycelia, the uptake curve can be divided into four descriptive phases, i.e., I, II, III, and IV (Figure 30). In Phase I, there was a rapid binding of uranium by the mycelia. After the rapid binding phase (which was probably physico-chemical) a still relatively fast uptake of uranium was observed in Phase II during the following 20-minute period. During this period the rate of

Figure 29

Effect of temperature on uranium uptake by Penicillium C-1. The culture was incubated 5 minutes, 20 minutes and 2 hours in shake flasks containing glucose medium plus 100 ppm uranium. Prior to inoculation the flasks were pre-adjusted on the shaker at the required temperature for 30 minutes. The data points represent the average of duplicate experiments.

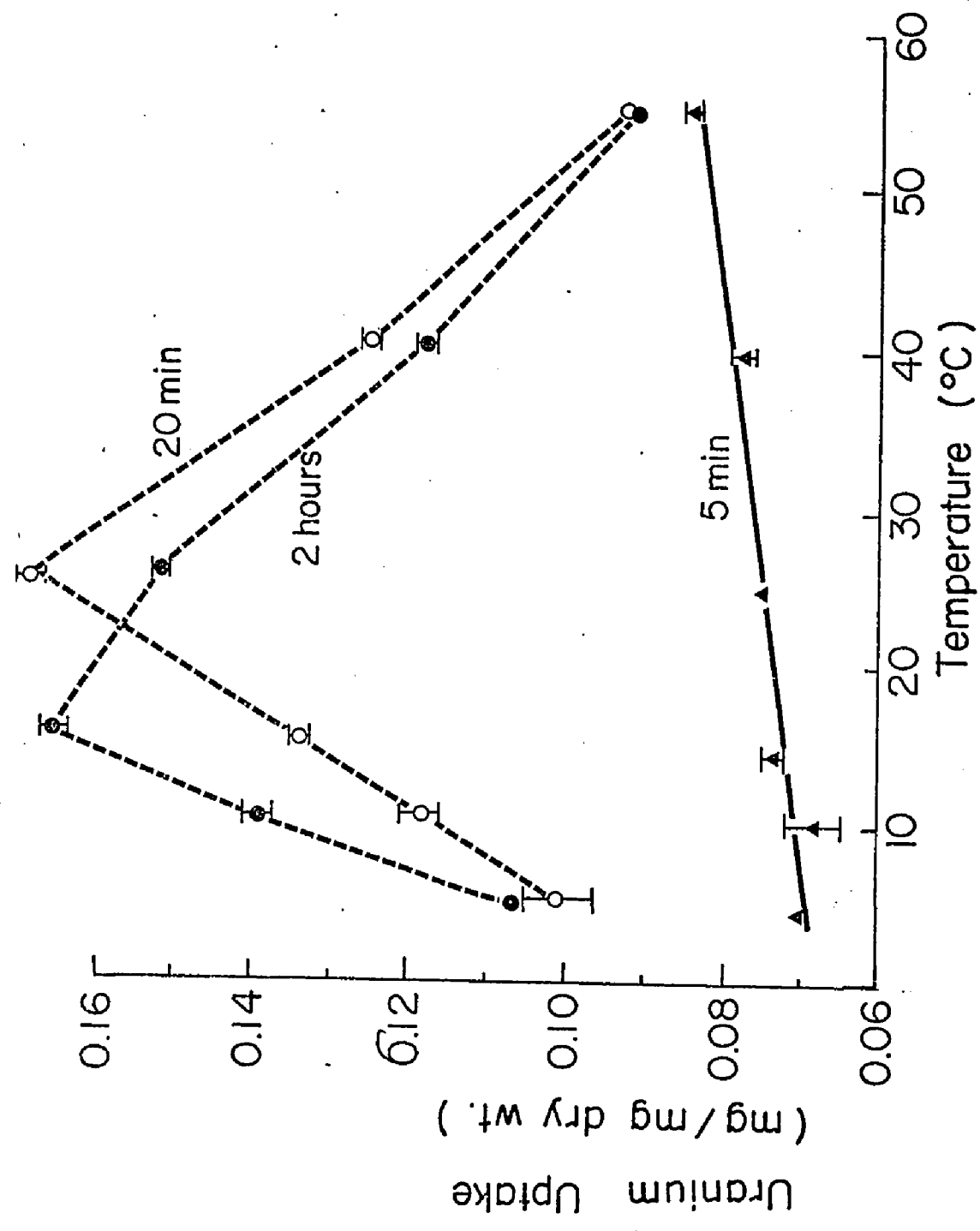
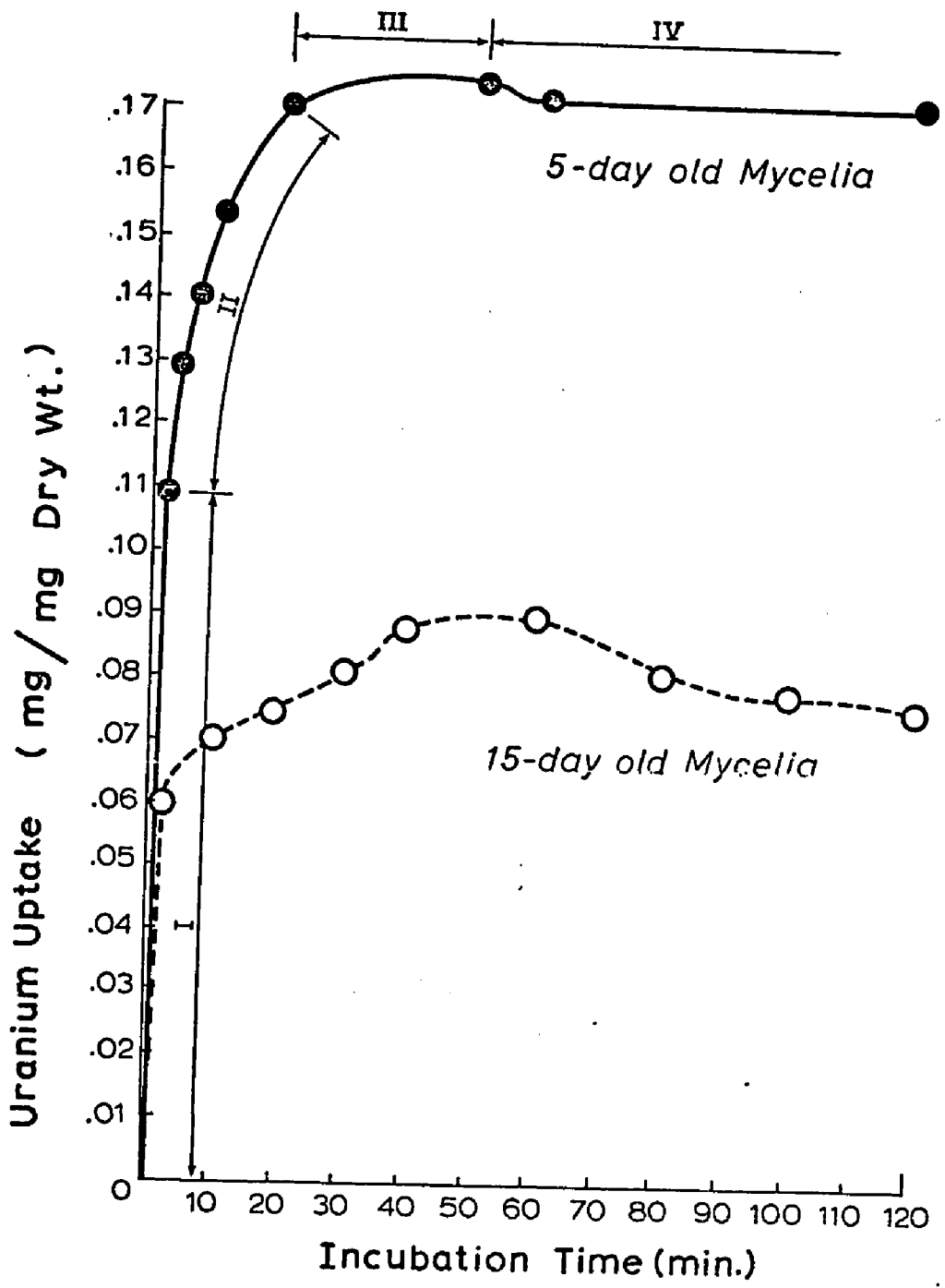


Figure 30

Effect of mycelial age on uranium uptake by Penicillium C-1.
The culture was incubated in shake flask containing glucose
medium plus 100 ppm uranium.



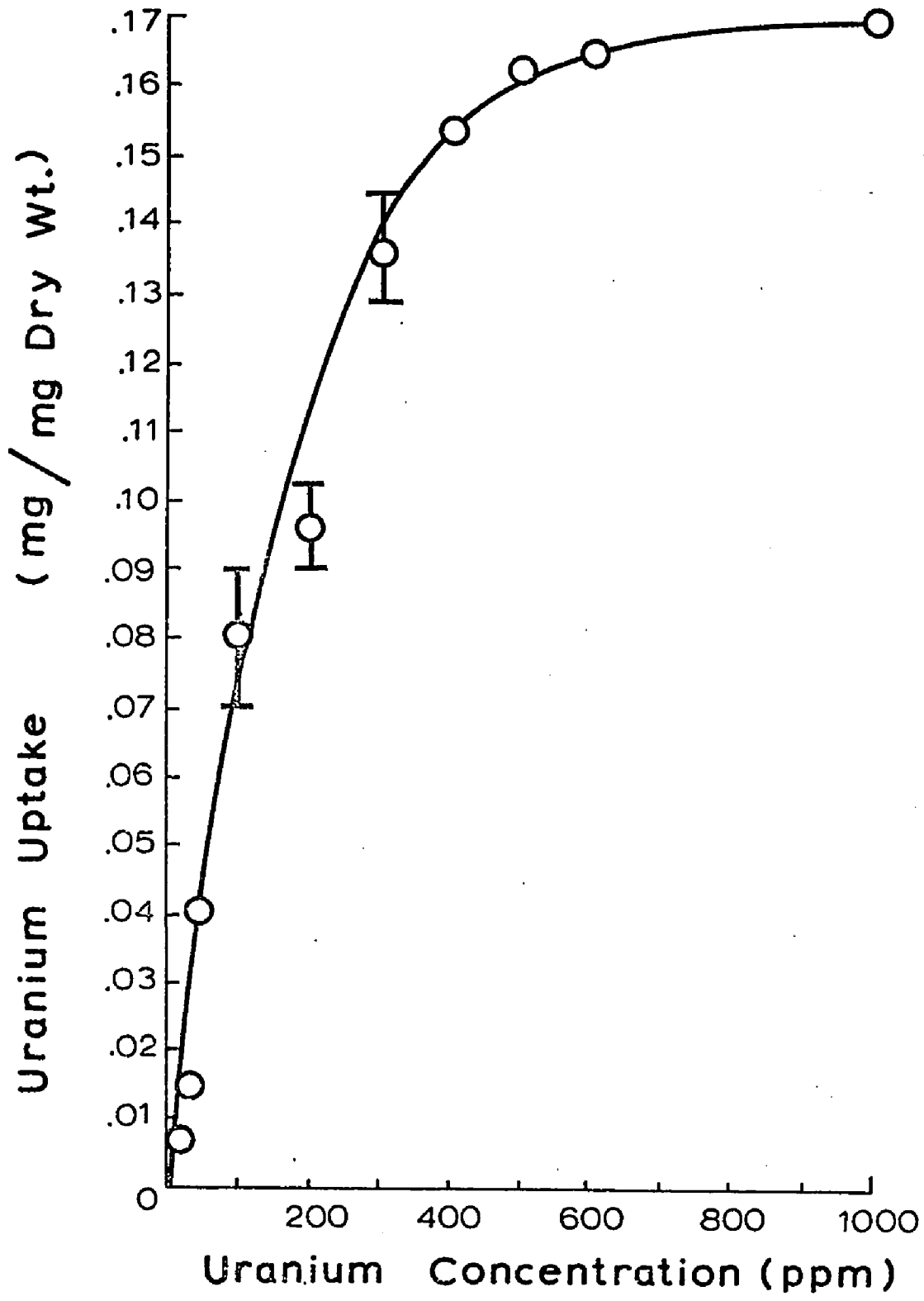
uptake was about 3 $\mu\text{g}/\text{mg}$ dry wt./min. Very little change was observed in Phase III which lasted from 20 to 50 minutes. Finally, in Phase IV, occurring after 50 minutes of incubation time, a slight release of uranium followed. The rate of release was about 0.3 $\mu\text{g}/\text{mg}$ dry wt./min.

Uranium concentration: To study the relationship between concentration of uranium and uranium uptake, uranium was added at 10, 30, 50, 100, 200, 300, 400, 500, 600, and 1,000 ppm to test flasks. A 10% (v/v) inoculum of 5-day-old culture was added. The results for uranium uptake at the end of 5-minute test period can be seen to be a function of the uranium concentration. Over a range of 10 to 300 ppm, uranium uptake per unit weight of mycelia was nearly linear. Above 300 ppm, the uranium removal from the medium became limiting and approached 0.17 mg/mg dry wt. The uptake as a function of the initial concentration of uranium showed a saturation trend as the concentration increased to 1,000 ppm (Figure 31).

Methods of culture: Both batch and continuous culture were employed to investigate the variation of uranium uptake with time in culture C-1. A 5% (v/v) inoculum of 5-day-old culture was used. In batch tests, the culture was grown in both shake flasks and fermentors. The uranium uptake

Figure 31

Effect of uranium concentration on uranium uptake by Penicillium C-1. The culture was incubated 5 minutes in shake flask containing glucose medium plus uranium at various concentrations (10, 30, 50, 100, 200, 300, 400, 500, 600, and 1,000 ppm). The data points represent the average of duplicate experiments.



obtained at various times in the course of cultivation is shown in Figure 32. In the upper part of this figure are the mycelial dry weights measured at particular time intervals (uranium uptake is shown in the lower part of the figure). For comparison, an identical experiment was done with culture C-2, the results are shown in the same figure (Figure 32). At an initial level of 100 ppm uranium, the uptake of uranium by the mycelia of C-1 was as high as 70-80% during the first week. As the cells aged, the uranium was gradually released. In contrast to C-1, the uptake in C-2 was lower at the beginning (30-40%), but the percentage of uptake increased proportionally as the dry weight increased. During continuous cultivation, culture C-1 was grown at first batch-wise in the fermentor for 6 days and then the fermentor was switched to continuous operation. The inflow consisted of glucose basal medium and uranium at 100 ppm. The culture volume was maintained at 6 liters. The dilution rate was increased stepwise from 0.06 to 0.5, and then decreased to zero at 14 days. The changes in uranium uptake as well as in mycelial dry weight with time of cultivation are shown in Figure 33. As seen in this figure, the initial uptake was as high as 95%, but decreased gradually to a minimum of 67% at 7 days, the second day after the fermentor switched from batch to continuous operation. After 8 days the uptake increased to

91%, and then fluctuated between about 75 to 85% during the continuous cultivation period (Figure 33). During the continuous culture experiments, temperature shock was applied to culture C-1, and the variation of uranium uptake with time as affected by temperature was examined. The experiment was done in a fermentor under the same conditions as before, except that a pulse change in temperature was applied at various times. The culture was started on a batch basis with continuous operation started at 2 days. The dilution rate was increased stepwise to a maximum of 0.3 at 9 days, and then levelled off (Figure 34a). The temperature was perturbed from initial steady state (25°C) by pulse changes at various times in the course of fermentation (Figure 34a). The resulting response in uranium uptake as well as in growth of mycelial dry weight are shown in Figures 34c and 34b. As seen in these figures, uranium uptake immediately after each time of pulse changes decreased rapidly, whereas the growth in dry weight was not significantly affected. The highest temperature (46°C) as a pulse change was conducted at 12 days (Figure 34a), and after this the uranium uptake decreased toward a final value of 50% at 14 days when the fermentor was stopped (Figure 34c).

Figure 32

Comparison of uranium uptake by Penicillium C-1 in shake flask and in fermentor culture. The culture was grown on glucose medium containing 100 ppm uranium over periods of 21 days (shake flask) and 12 days (fermentor). The figure also shows the results obtained under the same culture condition with culture C-2.

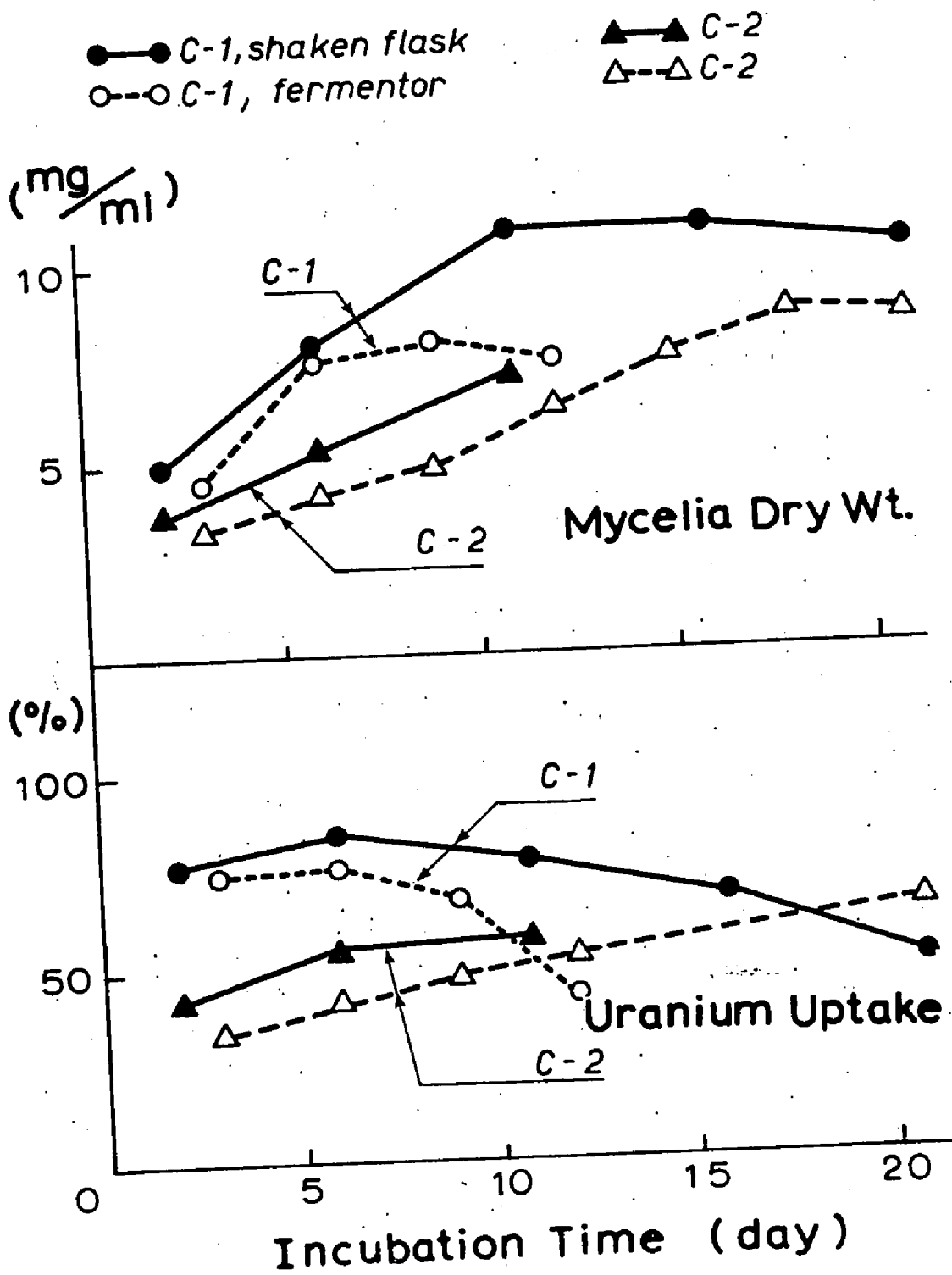


Figure 33

Uranium uptake by Penicillium C-1 in a continuous fermentor culture. The fermentor contained glucose medium plus 100 ppm uranium.

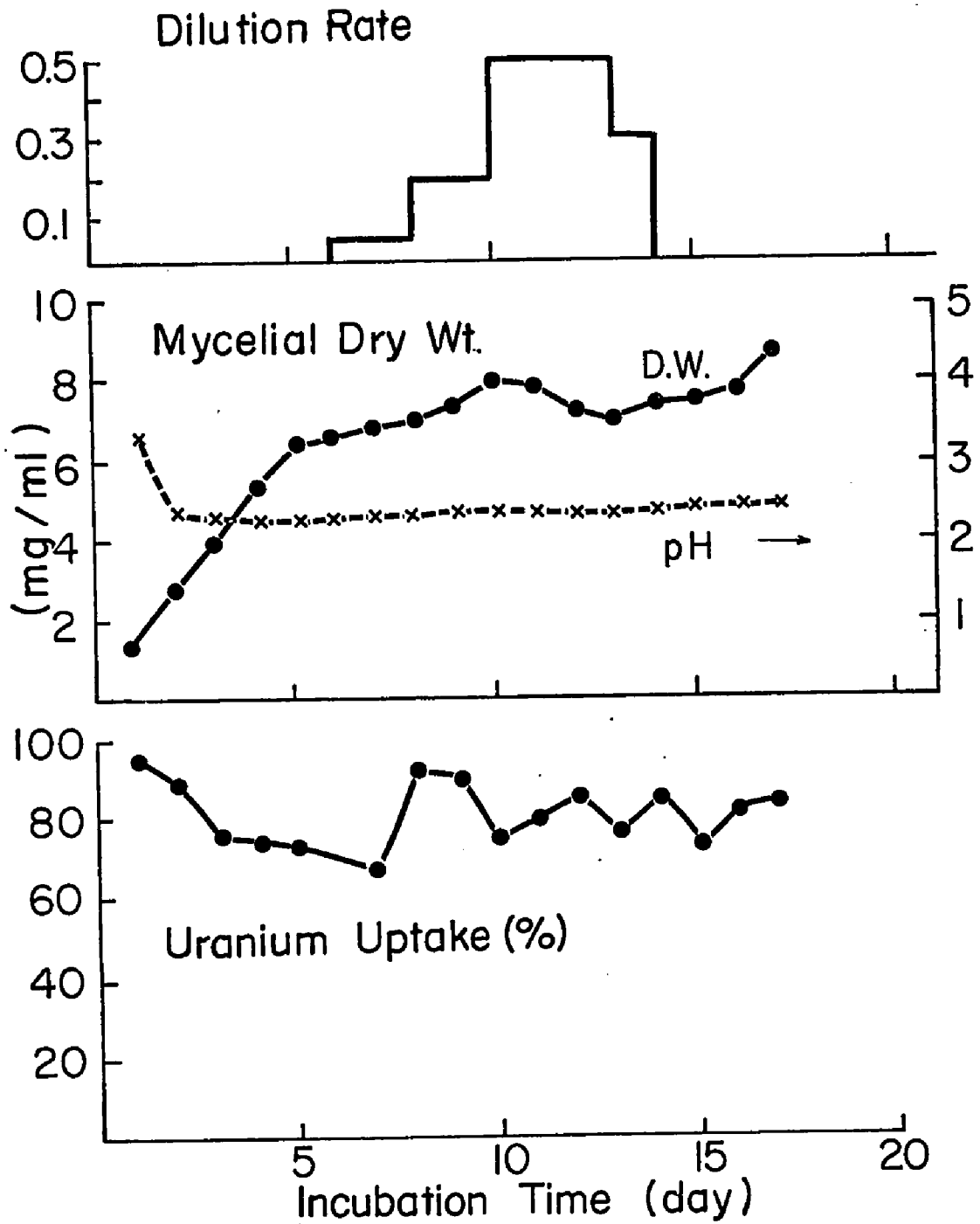
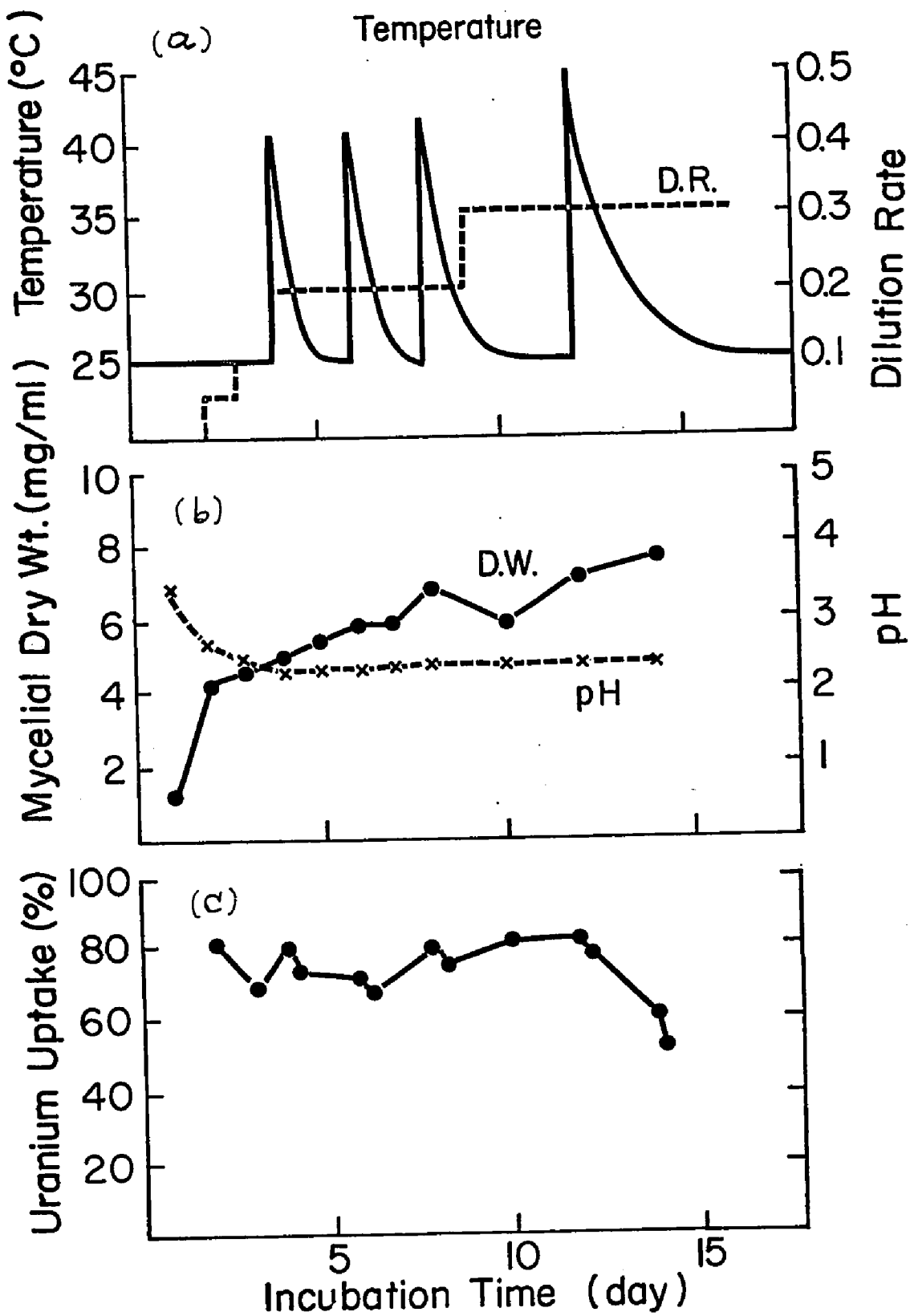


Figure 34

Effect of temperature shock on uranium uptake by Penicillium C-1 in continuous fermentor culture. The fermentor contained glucose medium plus 100 ppm uranium. The temperature was perturbed from initial steady state (25°C) by pulse changes at the times specified in the course of fermentation.



8.3 Discussion

The uranium uptake by Penicillium C-1 was affected by many factors such as pH, temperature, age of mycelia, concentration of uranium, etc. Among these factors, the relationship of temperature and age of mycelia to uranium uptake appeared to be most associated with physiological conditions of cells. The optimum temperature for uranium uptake in 20-120 minutes period was between 15 and 25°C. The effect of temperature on uranium uptake was also examined by applying temperature shocks (pulse changes in temperature from 25°C to about 40 and 45°C) to a continuous culture of Penicillium C-1. The response in uranium uptake showed that there was a rapid decrease in uranium uptake immediately after each temperature shock (Figure 34).

As for the factor of age of mycelia on uranium uptake, young mycelia from a 5-day-old culture were about twice as effective in uranium uptake as mycelia from a 15-day-old culture (Figure 30). The effect of mycelial age on uranium uptake is also evident from the experimental results obtained from batch culture of Penicillium C-1 on a glucose medium containing 100 ppm uranium in either shake flasks or fermentors (Figure 32). The uranium uptake as expressed on the basis of percentage of uranium removed by the mycelia from the medium decreased as the culture aged. For example in culture in fermentor, about 75% of the initial uranium

was removed from the medium at 6 days, whereas at 12 days it decreased to about 40%, although the mycelial dry weights at these two time intervals were nearly equal. In contrast to batch culture, the percentage of uranium removed in the time course of continuous culture never dropped below 60%. From 8 days to the end of cultivation (17 days), the percentage of uranium removed varied between about 75 to 85%. It is believed that during the continuous culture, almost constant physiological conditions were maintained. Therefore, uranium uptake did not definitely decrease as the cultivation proceeded.

The effect of pH on uranium uptake is also noteworthy. When the pH decreased from the optimum value of 3 to 1, there was a rapid decrease in uranium uptake from 0.14 to 0.04 mg/mg dry weight for the incubation periods tested (Figure 28). The rapid decrease in uranium uptake in the lower pH range below pH 3 was probably due to the competition between the ions of hydrogen and uranium on the adsorption sites of the cell surface and to physiological damage to the cell. When the pH increased from 5 to 7, and from 7 to 9, there was a steady, but a slight decrease in the uptake. When comparing uranium uptake (Figure 28) with growth (Figure 7a) as affected by pH (separate experiments), there was a similar pattern in the curves between growth and uranium uptake. The similarity between uranium uptake

and growth as affected by pH also suggested that at least part of the uranium taken up by the mycelia was growth associated.

CHAPTER 9
MECHANISM OF URANIUM UPTAKE

BY Penicillium C-1

9.1 General Consideration

Generally the methods of uptake of substances from the environment by the cells of microbes can be classified into the following categories.

Phagocytosis and Pinocytosis: These methods do not occur in the ordinary bacteria, fungi, and algae; but occur in amoeboid organisms which extend pseudopodia to surround and engulf extracellular substances. When the ingested substances are solid it is called phagocytosis; when they are fluids of microscopically visible droplets, the process is called pinocytosis.

Flocculation: This might be an important method in metal uptake by floc forming bacteria. Some of the floc-formers have a characteristic growth habitat known as zoogloal or gelatinous matrix in which cells are embedded. Zoogloea-producing bacteria such as the genus Zoogloea can

concentrate high levels of metallic ions of copper, cobalt, iron, and zinc in the zoogloal matrix (Friedman and Dugan, 1968). The organisms settle out of suspension affording a convenient means of separating ions. Since the electric charges of the cell surface are negative, a possible mechanism of floc formation was suggested: negative charged surface of adjacent cells are bridged by ionic bonds intermediately by cations (Tezuka, 1969).

Adsorptive uptake: This can be further classified into (a) direct adsorption of preformed metal containing particles, and (b) adsorptive uptake of metallic ions. Adsorption to cell surface is an important function of sheathed bacteria such as Chlamydoxiales and certain flagellates. Chemical composition of their cell surface is supposed to have a unique affinity for iron and manganese which become fixed at the cell surface in the form of hydroxide or salt (cited from Silverman and Ehrlich, 1964).

Transport across cell membrane: This may be either by diffusion or by active transport. Generally diffusion refers to transport along concentration gradient, whereas active transport is against the gradient and energy must be expended. If the diffusion is faster than would be expected and can be inhibited completely by certain compounds, it is then called facilitated diffusion.

Both adsorptive uptake and cell membrane transport of metallic ions appeared to be of same importance in the method of uptake. They may occur at the same site on the cell surface. For example, cells of baker's yeast suspended in a dilute solution of uranyl nitrate rapidly took up uranium. The rapid phase was less than two minutes followed by a very slow continuing phase of at least 3 hours. It was suggested that the rapid phase is associated with the formation of a complex with functional groups on the cell surface, and that the slow continued phase is possibly associated with the transport across the cell membrane (Rothstein and Larrabee, 1948).

Adsorption site on the surface of the cell can serve as a reservoir of ions which penetrate into the cell thereafter. The factors which govern the exchange of cations across cell surface are not exclusively metabolic, there exists a dynamic equilibrium between the medium and the cell. Rather, the majority of the ions taken up is on or near the cell surface in a readily exchangeable form. For example, the calcium content of the cells of Chlorella pyrenoidosa in potassium-free solutions undergoes a slight decrease, but when the cells are washed in pure solutions of potassium chloride they undergo a significant loss of calcium while the potassium content within the cells increases (Scott, 1944). In the study of strontium uptake by

Chlorella pyrenoidosa (Austin et al., 1967), it was indicated that the sorption of strontium was rapid and not limited to metabolic processes. Most of the strontium was located near the cell surface and was readily exchangeable.

9.2 Hypotheses Concerning the Mechanism of Uranium Uptake By Penicillium C-1

Two possible mechanisms, cell surface adsorption and biological involvement, are to be considered. Since the gross uptake of uranium was so great (on mycelial dry weight basis, the uptake within one hour could be as much as 0.17 mg/mg dry weight), and the proportion of the uranium in the initial rapid equilibrium with the mycelia was so high (the fraction of the initial uptake within 2 minutes could be as high as 65% of the gross uptake during the one hour period, Figure 30), therefore,

1st hypothesis: Surface adsorption is the principal mechanism of uptake of uranium by Penicillium C-1

Next, in view of the fact that (a) uranium was involved with increased pellet size and growth (Figure 18), (b) uranium uptake was temperature dependent (Figure 29), and (c) younger mycelia was more effective in uranium uptake

than old mycelia (Figure 30), therefore,

2nd hypothesis: A small fraction of the uranium is biologically involved.

9.3 Experimental Studies

In order to test the hypotheses (Section 9.2) concerning the uranium uptake by Penicillium C-1, various experiments were conducted. Mycelia used in these experiments were harvested from a culture vessel which was reconstructed from a 5-liter beaker. This vessel containing 2 liters glucose basal medium was inoculated directly by transfer of the total spores and hyphae from a slant culture (slant area about 1.5 cm x 5 cm), and cultured by vigorous aeration at room temperature (25°C). The vigorous aeration also provided mixing, thus a complete dispersed form of mycelia was obtained. The mycelia were washed twice with distilled water, and suspended in distilled water before being utilized. The uranium used was in the form of $\text{UO}_2\text{SO}_4 \cdot 3\text{H}_2\text{O}$. Except otherwise stated the experiments were completed at room temperature (25°C). For additional materials and methods see Section 3.3 (Chapter 3). Details of these studies are described separately in the following.

a) Application of Freundlich Equation to Uranium Uptake
by Penicillium C-1

The Freundlich equation (Freundlich, 1926) has been widely used in physico-chemical adsorption to express the relationship between the amount of substance adsorbed per unit mass of adsorbent and the concentration of adsorbate in liquid. The Freundlich equation is expressed as follows:

$$X/Y = \kappa C'^{\nu} \quad (9.1)$$

where X = mass of substance adsorbed

Y = mass of adsorbent

C' = concentration of the substance in liquid

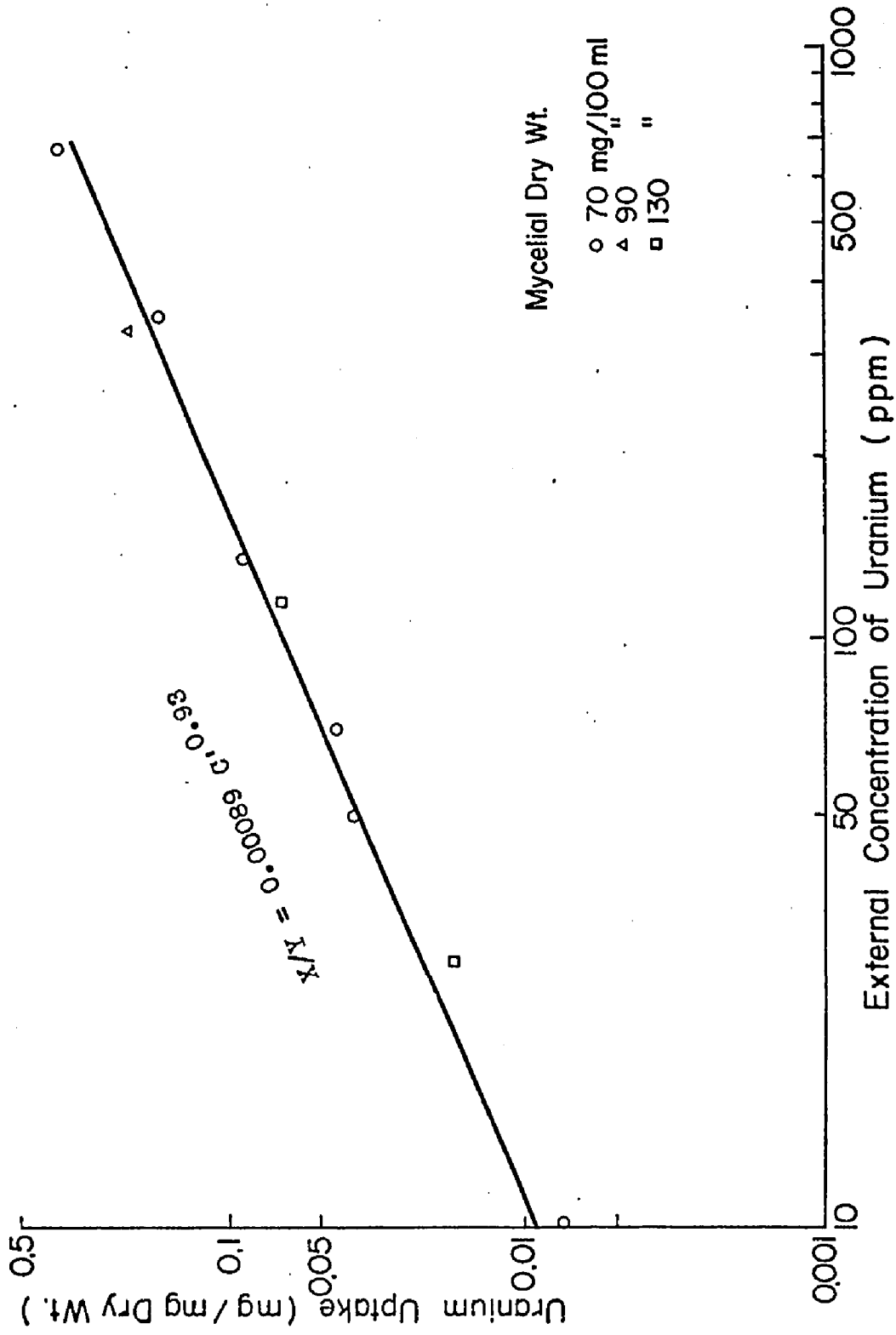
κ, ν = constants

In uranium uptake by culture C-1, the uptake may be viewed as a physico-chemical adsorption in which uranium is the adsorbate and biomass the adsorbent. To test this hypothesis, uranium was added to test flasks to give final concentrations ranging from 10 to 1,000 ppm. Each flask (500 ml) contained 100 ml of 1% (w/v) glucose solution, to which 5-day-old mycelia were added at different levels in terms of dry weight. The flasks were shaken at 200 rpm for 5 minutes on a rotary shaker (New Brunswick Scientific Co.). The uranium uptake obtained with various external

environmental concentrations of uranium was plotted on a log-log graph as shown in Figure 41. For these experimental points a line of regression was drawn, which is expressed by equations: $\log(X/Y) = -3.05 + 0.93 \log C'$, or $X/Y = 0.00089C'^{0.93}$. To see how the Freundlich equation fits the experimental data, the coefficient of correlation was calculated which gives 0.998 (For calculations see Appendix II). Thus the relationship between the amount of uranium uptake per unit mycelial dry weight and the external concentration of uranium, as expressed by the Freundlich equation $X/Y = 0.00089C'^{0.93}$ is highly significant. As previously stated the Freundlich equation has been extensively used to describe the adsorption by non-biological systems. In the present studies, the Freundlich equation also describes the uranium uptake by Penicillium C-1 during a 5-minutes exposure period of mycelia to uranium. The mycelia took up uranium as a direct response to the external concentration of uranium. It is seen that during a short period such as 5 minutes, the uptake of uranium by the mycelia of Penicillium C-1 was exclusively a phenomenon of surface adsorption.

Figure 35

Relationship between uranium uptake by Penicillium C-1 and the external environmental concentration of uranium as expressed by the Freundlich equation. The culture was incubated 5 minutes in shake flasks containing 1% (w/v) glucose plus uranium at concentrations ranging from 10 to 1,000 ppm.



b) Comparison of the Uranium Uptake by Penicillium C-1
Between Live and "Dead" Mycelia

For further tests of the hypothesis concerning the adsorptive uptake of uranium by Penicillium C-1, the mycelia were treated by various chemical or physical methods to kill the cells before exposure to uranium. Each test flask (500 ml) contained 100 ml of 1% (w/v) glucose plus 100 ppm uranium, to which 5-day-old mycelia which had undergone treatment were added. For the control, an identical experiment was completed with the untreated live mycelia. The flasks were shaken at 200 rpm for 5 minutes on a rotary shaker (New Brunswick Scientific Co.). The uranium uptake obtained with mycelia which had undergone various treatments are shown in Table 4. From the table it is seen that "dead" cells took up uranium as much as live cells, although there were differences in uranium uptake per unit mycelial dry weight in the types of treatments. These differences are presumed to be attributed to changes in cell morphology or modifications in chemical characteristics of cells. For instance in the case of heat treatment (100°C, 5 minutes) of mycelia, very high values of uranium uptake (0.162 mg/mg dry weight) were obtained, this was attributed to protein coagulation of cells as seen in the increased viscosity of the culture at a temperature of 100°C. In view of the fact that dead mycelia can take uranium up as well as live

TABLE 4. COMPARISON OF URANIUM UPTAKE BY *Penicillium C-1* BETWEEN LIVE AND "DEAD" MYCELIA. THE CULTURE WAS INCUBATED 5 MINUTES IN SHAKE FLASKS CONTAINING 1%(w/v) GLUCOSE AND 100 ppm URANIUM.

EXPERIMENT	TREATMENT OF MYCELIA BEFORE ADDED TO MEDIUM	MEDIUM COMPOSITION	URANIUM UPTAKE (mg/mg D.W.)
1 (Mycelial Dry Wt. 85mg/100ml)		1% glucose 100 ppm U	0.096±0.008 †
		1% glucose 100ppm U 0.5mM NaN ₃	0.107±0.004
		1% glucose 100ppm U 30mM NaN ₃	0.094±0.015
		1% glucose 100ppm U 0.05mM DNP	0.104±0.009
2* (28mg/100ml)	Washed with absolute methanol 5 minutes	1% glucose 100 ppm U	0.088±0.010
3* (120mg/100ml)	Waring blended 5 minutes	1% glucose 100 pp U	0.055±0.008
4* (80mg/100ml)	Heat treated 100°C, 5 minutes	1% glucose 100 ppm U	0.162±0.005

* The mycelia in these experiments were considered to be dead.

† Standard deviation of duplicate values.

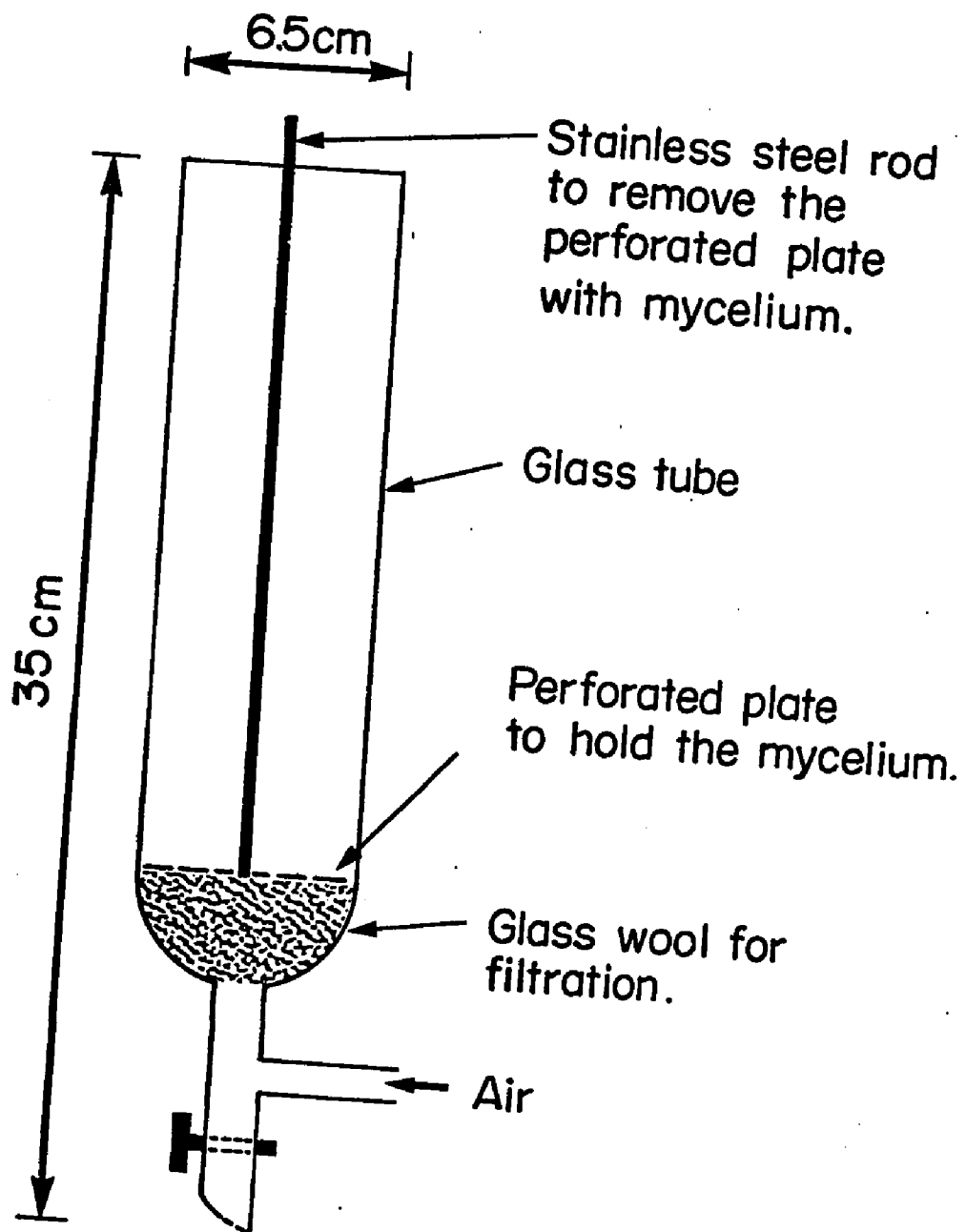
mycelia, it is concluded that uranium uptake by Penicillium C-1 is largely a phenomenon of surface adsorption.

c) Effect of Washing on Uranium Desorption From Penicillium C-1

Washing the mycelia of Penicillium C-1 with distilled water did not leach out uranium; but washing with solutions of EDTA, phosphate, phosphoric acid, and other chemicals, the uranium could be removed. Experiments were completed in a tube type reactor which contained mycelia plus either uranium or other chemicals. The tube reactor is shown in Figure 36. The bottom of the tube contained glass wool to hold and separate the "mycelia-liquid mixture". Two constricted openings are located at the bottom, the one is fitted with a stop-cock, while the other is a port for aeration. During the reaction, the cock was closed and air was introduced. The air was also used to provide mixing. The 5-day-old mycelia were first reacted with 200 ml of 1% (w/v) glucose plus 100 ppm uranium. After 30 minutes the cock was opened, and the effluent was collected for analysis of uranium removal. The tube was then refilled with same volume of solution of the above mentioned chemicals, and the mycelia were washed (cock closed and air introduced into the reactor) for 5 minutes to test for the desorption of uranium. The cock was opened again and the

Figure 36

Tube type reactor used for experiment on desorption of uranium from Penicillium C-1.



liquid was withdrawn for analysis of uranium desorbed by the chemicals. The experimental conditions and the results obtained with the various washings are shown in Table 5.

The uranium taken up by the mycelia was not removed by distilled water; but with 0.01% EDTA or K_2HPO_4 the uranium was leached out from the mycelia at levels of 20 and 44% respectively during 5-minutes exposure period. With phosphoric acid, the percentage removal was proportional to the acid concentration. At concentration of 0.01%, H_3PO_4 removed 27% of the sorbed uranium, when the concentration of acid was increased to 0.1% the uranium was removed 29%.

EDTA is known as a chelating agent for metals in general, whereas phosphate is one of the chemical compounds known to form a uranyl phosphate complex with uranium (e.g., Rothstein, 1958). The fact that both EDTA and phosphate could remove about 20 to 50% of the uranium rapidly from the mycelia, suggested that at least a certain fraction of the uranium was adsorbed on the cell surface.

d) Effect of Energy Source on Uranium Uptake by
Penicillium C-1

To test flasks (1-liter) containing 500 ml of 100 ppm uranium solution, either glucose or ethanol was added to a final concentration of 1%. For the control, the flasks contained uranium solutions only. To each flask, 5-day-old

Table 5

Effect of washing with chemicals on uranium desorption from Penicillium C-1. The culture, pre-incubated 30 minutes in a tube type reactor (Figure 42) containing 1% (w/v) glucose and 100 ppm uranium, was washed 5 minutes with a specific chemical.

WASHING LIQUID	URANIUM REMOVED BY MYCELIUM (mg/200ml)	URANIUM RELEASED TO THE LIQUID (mg/200ml)	URANIUM RELEASED FROM MYCELIUM TO THE LIQUID (%)
Distilled* water	9.4	0	0
0.01% EDTA*	9.5	1.9	20
0.01% K ₂ HPO ₄ *	9.7	4.3	44
0.01% H ₃ PO ₄ *	10.0	2.7	27
0.1% H ₃ PO ₄ *	9.7	2.8	29
1.0% K ₂ HPO ₄ *	9.0	4.5	50
1.0% NaCl †	14.0	3.5	25
1.0% NaNO ₃ †	17.4	2.8	16

* Mycelial Dry Weight ----- 300mg / 200ml

† " " " ----- 2500mg / 200ml

mycelia (dry weight 120-130 mg/500 ml) were added, which were then incubated on a rotary shaker (New Brunswick Scientific Co.) at 200 rpm for 2 hours. The uranium uptake obtained as a function of incubation time is shown in Figure 37.

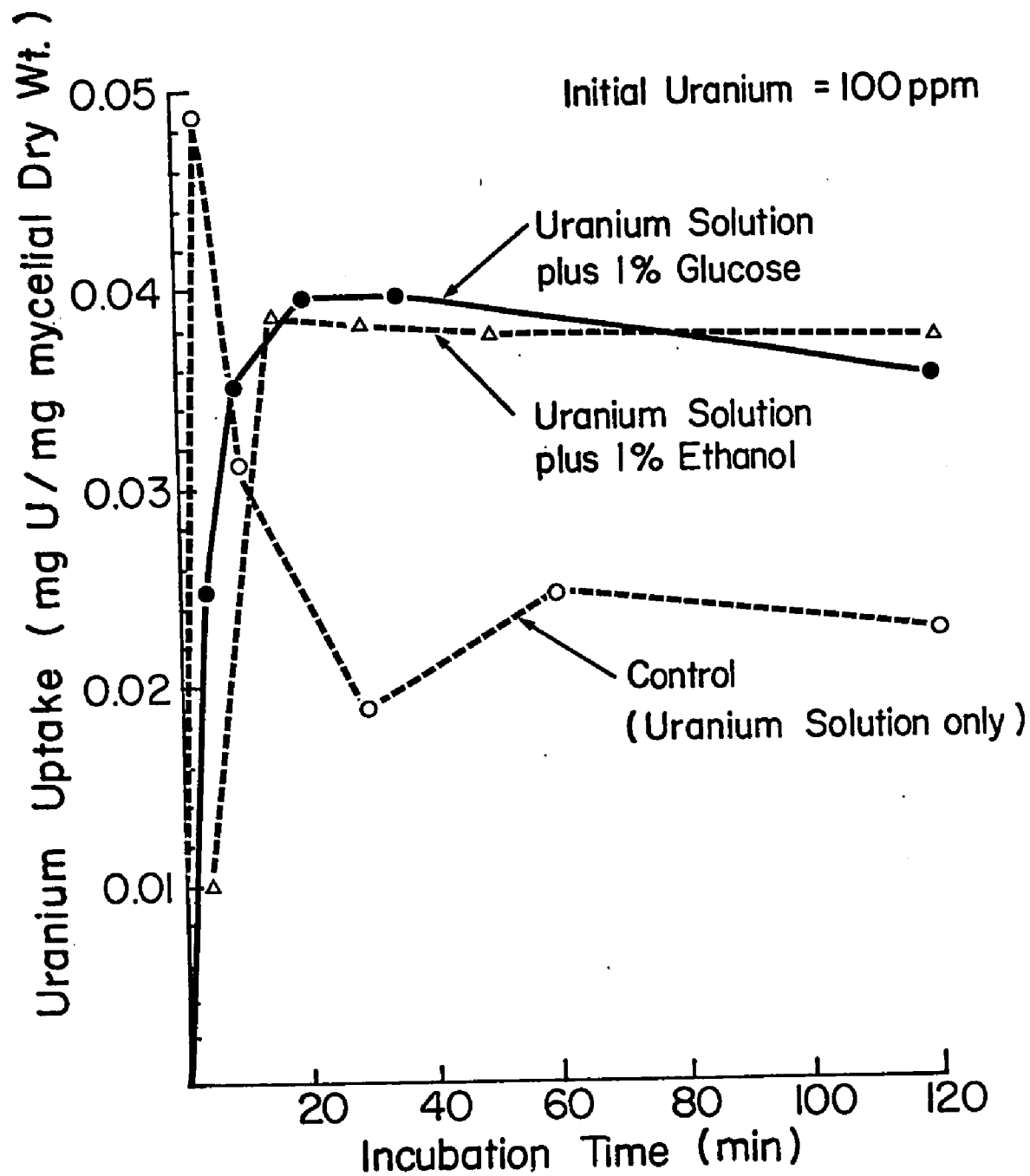
In the presence of uranium at 100 ppm, the addition of glucose or ethanol as a sole source of carbon to the mycelia stabilized the uranium uptake. The stabilization was seen after a period of 10 minutes from the start of incubation. For instance at 1 hour, the uranium uptake increased from 0.025 to about 0.038 mg/mg mycelial dry weight with the addition of either carbon source.

e) Effect of Metabolic Inhibitors on Uranium Uptake by Penicillium C-1

Some common metabolic inhibitors, such as 2,4-dinitrophenol (DNP), and sodium azide (NaN_3), were used to test their effects on uranium uptake by Penicillium C-1. To test flasks (1-liter) containing 500 ml of 1% glucose solution plus 0.1 ppm uranium, 0.1 mM DNP or 30 mM NaN_3 was added. For the control, the flasks contained only glucose plus uranium. To each flask, 5-day-old mycelia (dry weight 1,100 - 1,300 mg/500 ml) were added, which was then incubated on a rotary shaker (New Brunswick Scientific Co.) at 200 rpm for 3 hours. The uranium uptake obtained during

Figure 37

Effect of energy source on uranium uptake by Penicillium C-1. The culture was incubated in shake flasks containing 100 ppm uranium plus either glucose or ethanol as a source of energy. For the control the flask contained only 100 ppm uranium. The data points represent the average of duplicate experiments.



addition of 30 mM NaN_3 was quite different from that of the control (Figure 38), whereas with 0.1 mM DNP there was no significant difference (not shown in the figure). In the case of addition of 30 mM NaN_3 , the initial uptake within the 10-minute period (0.027 $\mu\text{g}/\text{mg}$ dry weight) was higher than that of the control (0.018 $\mu\text{g}/\text{mg}$ dry weight). However, at 30 minutes there was a rapid decrease in the uptake whereas in the control the uptake increased steadily to 0.035 $\mu\text{g}/\text{mg}$ dry weight in 80 minutes. After 80 minutes, the uptake with NaN_3 increased again to 0.022 $\mu\text{g}/\text{mg}$ dry weight at 110 minutes, and then proceeded to follow the curve of uranium uptake as observed in the control.

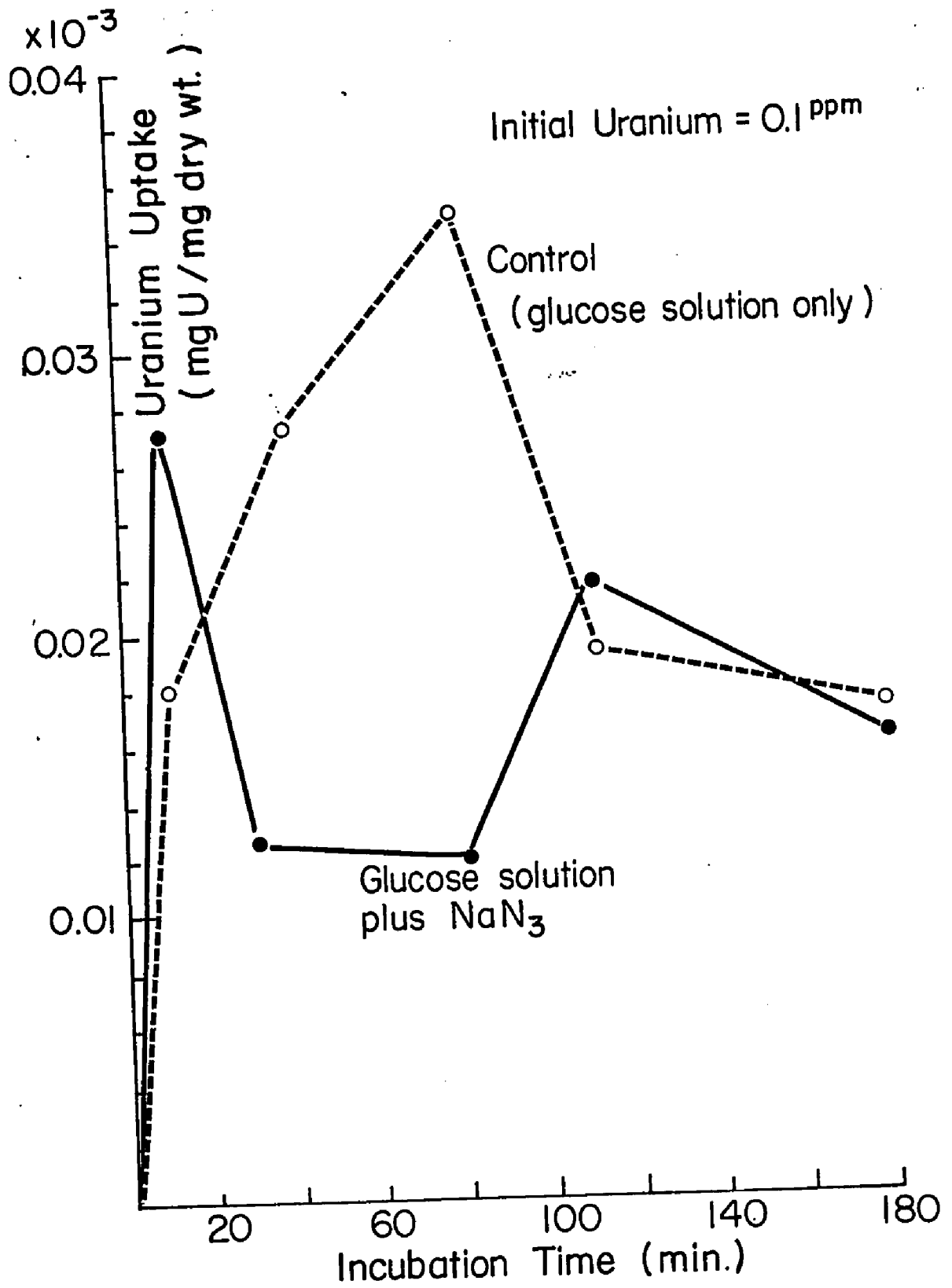
Both DNP and NaN_3 are electron transport inhibitors of oxidative phosphorylation. The depression of uranium uptake by addition of 30 mM NaN_3 suggested that NaN_3 did impair the metabolism involved in uranium uptake.

f) Effect of Uranium on Oxygen Uptake by *Penicillium* C-1

Manometric measurement: For measurement of oxygen uptake, mycelia of 3 and 5-day of age were suspended in distilled water and added to the main compartments of the Warburg flasks. To the main compartment, uranium was added at either 10 or 100 ppm. The substrate, either glucose or ethanol, was added to the side arm of the flask. The total working volume of each flask was 3.0 ml. To absorb carbon

Figure 38

Effect of the metabolic inhibitor, sodium azide, on uranium uptake by Penicillium C-1 in shake flask culture. The flask containing 1% (w/v) glucose and 0.1 ppm uranium was added with 30 mN NaN_3 as a metabolic inhibitor. The data points represent the average of duplicate experiments.



dioxide, 0.2 ml of 20% KOH and a strip of filter paper was added to the center well. In the control flasks, identical materials except uranium were added. The experiments were done with a Gilson differential respirometer at 25°C and a shaking rate of 120 oscillations/minutes, over a period ranging between 1 and 4 hours.

The effect of uranium on oxygen uptake by Penicillium C-1 is shown in Figures 39-41. Depending on the type of substrate, and the concentration of uranium, the effects of uranium on oxygen uptake were different. In most experiments there was no strong evidence that uranium did stimulate oxygen uptake by Penicillium C-1. However, in one instance, a preparation containing 5-day-old mycelia held one day in distilled water before experiment, the oxygen uptake started rapidly after a lag phase of 30 minutes in the glucose solution containing 10 ppm uranium (Figure 40b). At 100-minutes the total oxygen consumption in the presence of uranium exceeded that without uranium. At the end of 4-hour incubation, the total oxygen uptake in the presence of uranium was 31 μ l/mg dry weight, whereas in the absence of uranium it was 23 μ l/mg dry weight.

Between the two levels of uranium concentration, generally oxygen uptake was greater at 100 ppm than at 10 ppm uranium. For example, when ethanol was utilized, the total oxygen uptake over an incubation period of 4 hours

Figure 39

Effect of uranium on oxygen uptake by Penicillium C-1 in Warburg flask. Uranium was added at either 10 or 100 ppm to the flask containing mycelia (3-day-old) and substrate (glucose). The endogenous respiration was not subtracted from the respiration in calculating the oxygen uptake.

3-day-old Mycelia

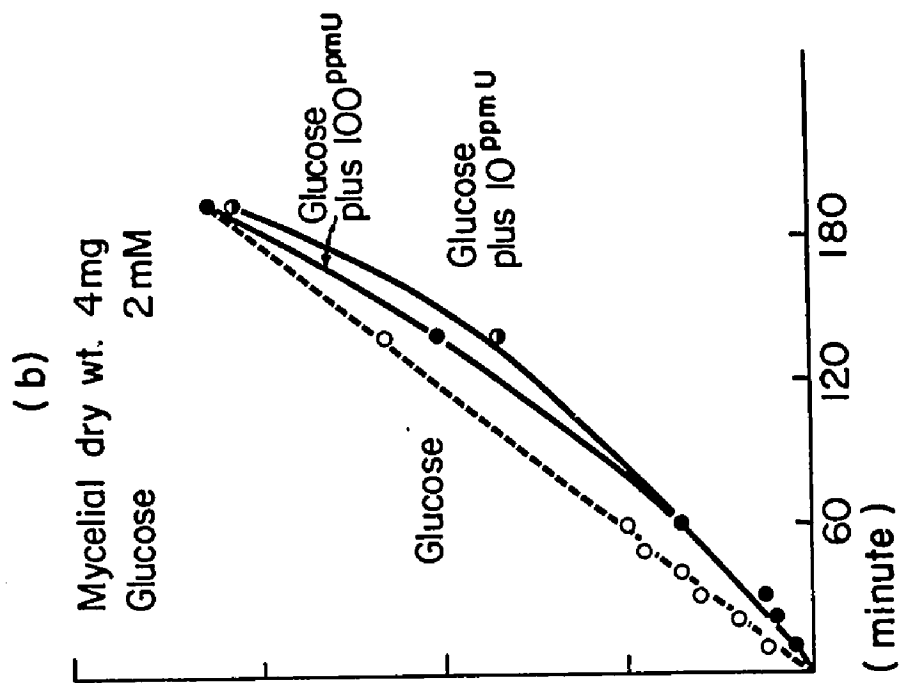
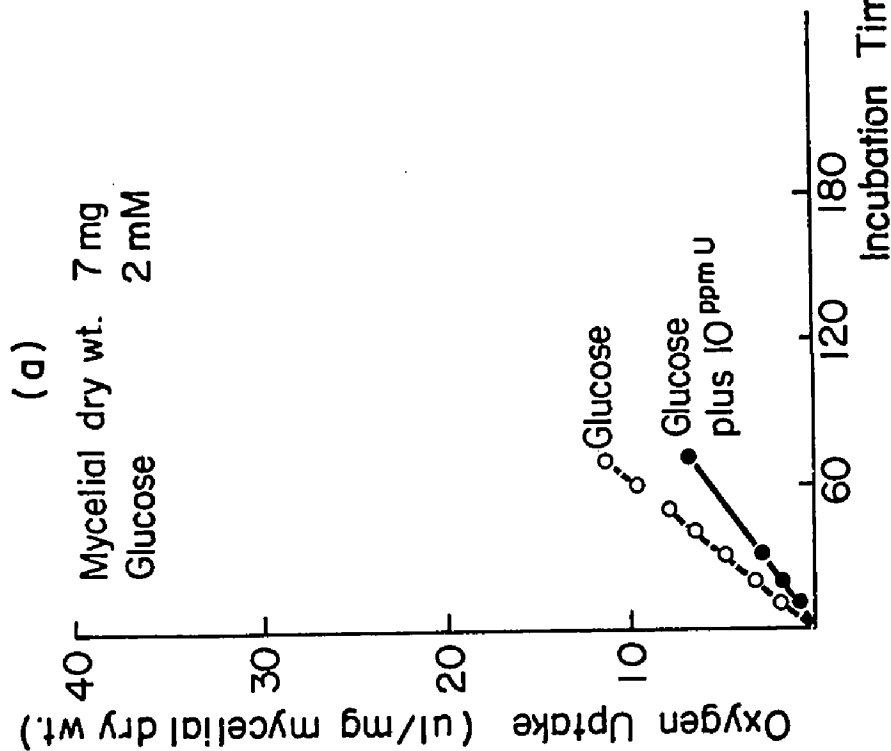
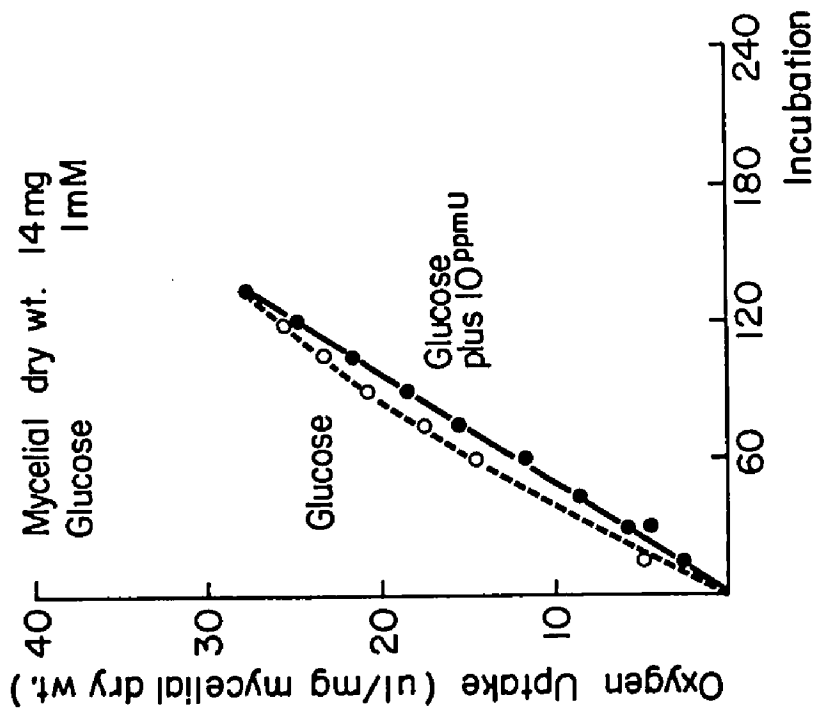


Figure 40

Effect of uranium on oxygen uptake by Penicillium C-1 in Warburg flask. Uranium was added at 10 ppm to the flask containing mycelia (5-day-old) and substrate (glucose). The endogenous respiration was not subtracted from the respiration in calculating the oxygen uptake.

5-day-old Mycelia

(a)



(b)

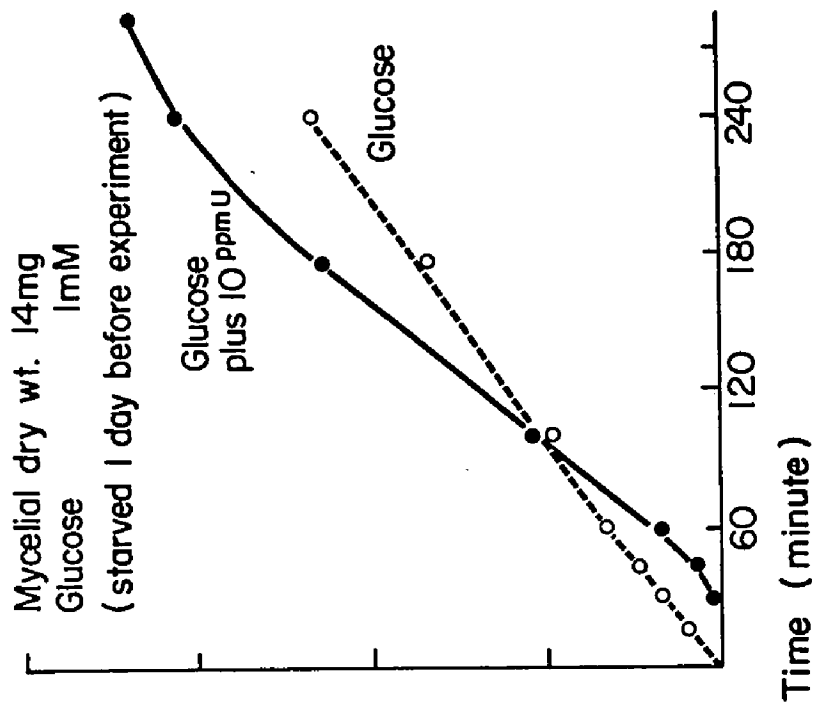
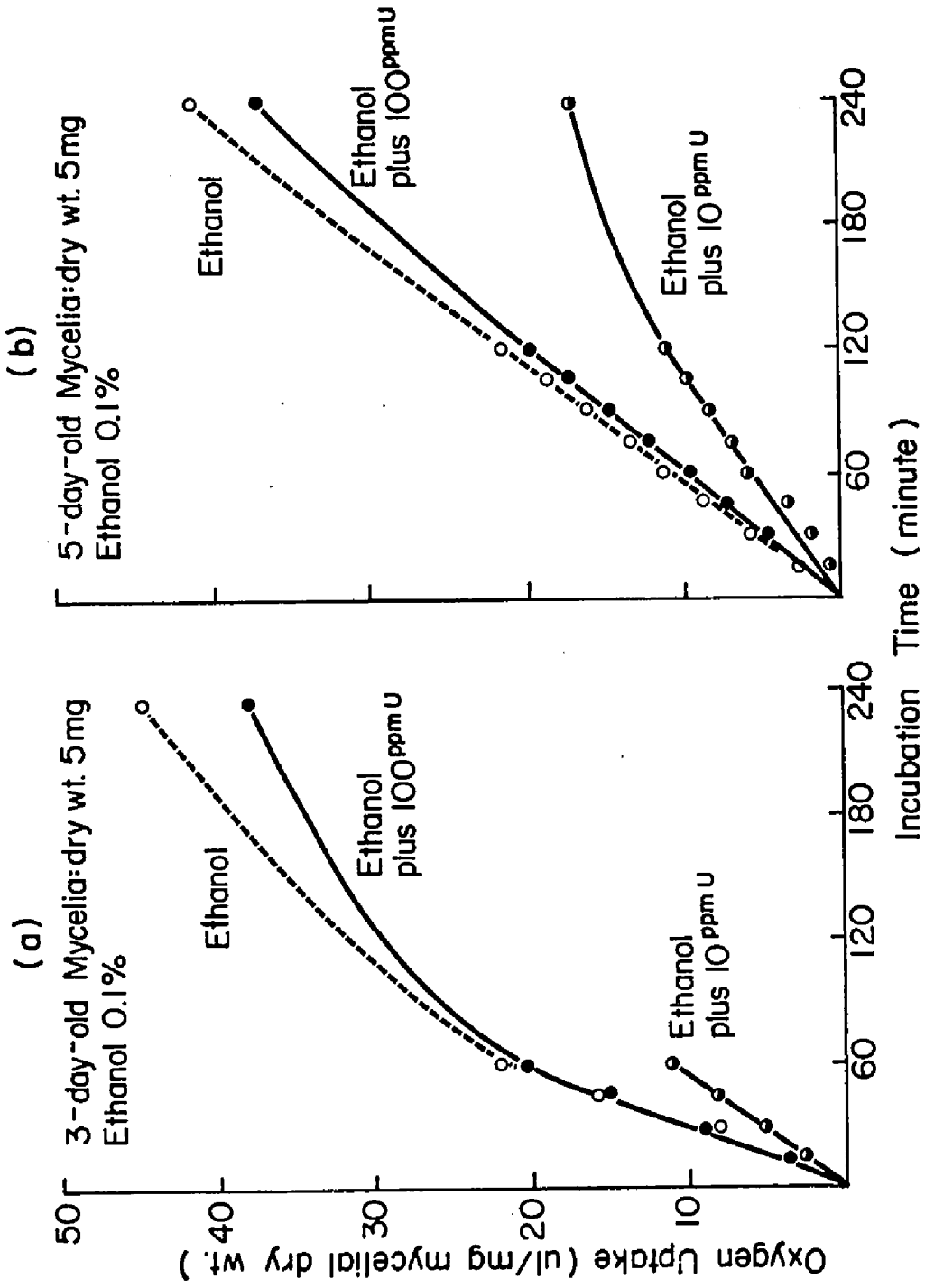


Figure 41

Effect of uranium on oxygen uptake by Penicillium C-1 in Warburg flask. Uranium was added at either 10 or 100 ppm to the flask containing mycelia (a, 3-day-old; b, 5-day-old) and substrate (ethanol). The endogenous respiration was not subtracted from the respiration in calculating the oxygen uptake.



was 37 $\mu\text{l}/\text{mg}$ dry weight with 100 ppm uranium, whereas with 10 ppm uranium it was 17 $\mu\text{l}/\text{mg}$ dry weight (Figure 41).

Since the activation of most metabolic processes is accompanied by increased respiration, the increased oxygen uptake in the presence of 100 ppm uranium as compared with 10 ppm uranium suggested that higher concentrations of uranium (100 ppm) were more stimulative to Penicillium C-1 in the liquid medium containing a source of carbon than lower concentration of uranium. It is suggested that the formation of different kinds of complex ions of uranium (e.g. U^{4+} , U^{6+} , etc.) was affected by the concentrations of uranium in the culture medium, which in turn influenced the cells of Penicillium C-1.

In the studies of fungistatic action, a higher concentration of metal occasionally permitted the growth, while a lower concentration had a fungicidal effect. This phenomenon was referred to as the "paradoxical effect" (Muroma, 1958). This phenomenon was used to describe the effect of rare earth metals (cerium, europium, lanthanum, neodymium, praseodymium, samarium, scandium, ytterbium, and yttrium) when studied with certain bacteria (Muroma, 1958).

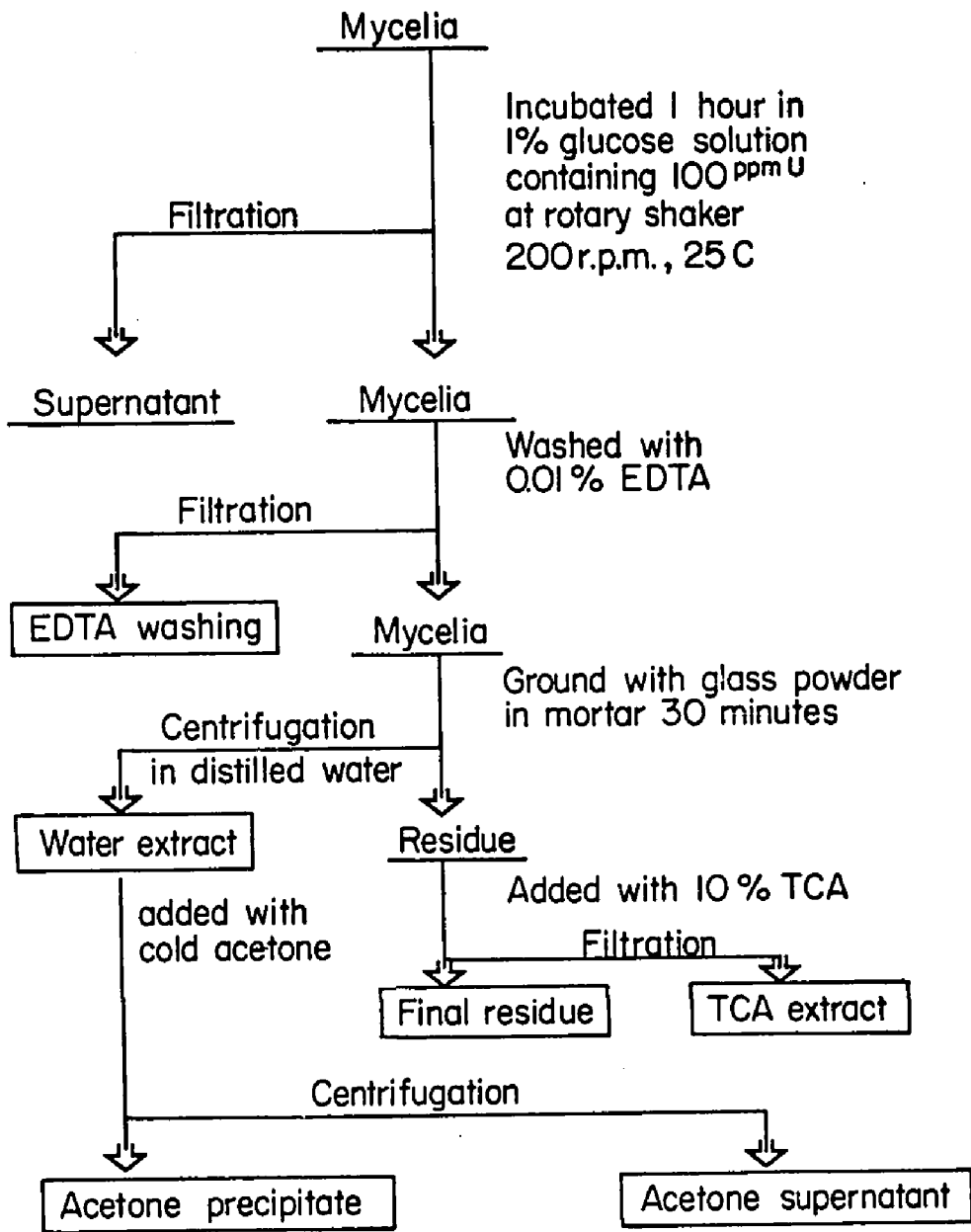
g) Distribution of Uranium in Mycelial Fractions of
Penicillium C-1

Preparation of mycelial fractions: Mycelia previously incubated 1 hour in 1% (w/v) glucose solution containing 100 ppm uranium were washed with 0.01% EDTA, ground with twice its dry weight of glass powder in mortar for 15 minutes, and centrifuged at 10,000 rpm in a Sorvall centrifuge (RC 2B, GSA rotor) for 30 minutes in distilled water (water extract). The residue was then extracted with equal volume of 10% (w/v) cold trichloroacetic acid (TCA extract). To determine the nature of uranium in the water extract fraction, three volumes of cold acetone were added to the fraction of water extract and left overnight in refrigerator. The precipitate was separated by centrifugation from the supernatant. The experimental procedure is summarized in Figure 42.

Determination of protein: The protein content of the mycelial extracts was determined colorimetrically by the reaction with Folin's reagent with bovine serum albumin as a standard (Bailey, 1967). To determine the protein content in solid materials, the organic nitrogen content was analyzed by the modified Kjeldahl digestion method (Furman, 1962), and then the corresponding protein was estimated by multiplying with 6.25.

Figure 42

Experimental procedure for preparation of mycelial fractions.
Mycelia were previously incubated 1 hour in glucose solution
containing 100 ppm uranium.



The distribution of uranium in mycelial fractions is shown in Table 6. The protein content of each fraction is also presented in this table. It is noted that more than half (63.7%) of the uranium taken up by the mycelia was found in the fraction of TCA extract, which contained about half (50.6%) of the total protein of the mycelia. In the fraction of the final residue after TCA extract, the uranium content was 21.5%, whereas the content of protein was 28.8%. Combining these two fractions, the total uranium and the total protein were about 85 and 80% respectively, suggesting that the uranium was very strongly combined with the protein of the mycelia. In the fraction of water extract, the uranium content was 12.8% whereas the protein in this fraction was 20.6%. When acetone was added to the fraction of water extract, about two-thirds of the protein in this fraction was precipitated, but only 16% of the uranium in this fraction was in the precipitate. The protein nature of the water extract was probably most involved in the cell surface enzyme, but this type of protein was less strongly combined with the uranium when compared with the protein in the fractions of TCA extract and final residue.

TABLE 6. DISTRIBUTION OF URANIUM IN MYCELIAL FRACTIONS OF *Penicillium* C-1.

FRACTION	URANIUM (mg)	URANIUM (%)	PROTEIN (mg)	PROTEIN (%)	URANIUM PROTEIN
EDTA washing	2.2	1.9	0	0	
Water extract	14.7	12.8	530	20.6	0.028
Acetone sup.	(12.9)	(11.3)	(170)	(6.6)	0.076
Acetone ppt	(2.5)	(2.2)	(360)	(14.0)	0.007
TCA extract	73.0	63.7	1300	50.6	0.056
Final residue	24.6	21.5	740	28.8	0.033
TOTAL	114.5	100.0	2570	100.0	0.045

Total mycelial dry weight = 6300 mg

h) Association Between Uranium and Enzyme Preparation of Penicillium C-1

Preparation of crude enzyme: For the preparation of crude enzyme from Penicillium C-1, mycelia were disrupted by grinding with glass powder in mortar, and centrifuged to obtain a cell-free water extract (see Section 9.3g). The extract was subjected to further precipitation by addition of twice volume of propyl alcohol (Pazur, 1966). The precipitate was collected by centrifugation. The precipitate was dissolved in water and assayed for glucose oxidase activity and uranium content. Uranium content was determined on the cell-free water extract.

Assay of glucose oxidase activity: Glucose oxidase activity was measured by determining the rate of oxygen uptake during the oxidation of glucose by the precipitated cell-free extract in a Warburg flask with a Gilson differential respirometer (see Section 9.3f). One unit of glucose oxidase activity is the quantity of enzyme which causes the uptake of 11.2 μ liters of oxygen per minute in the Warburg manometer at 30°C and atmospheric pressure with a substrate concentration of 0.16 M glucose in 0.1 M potassium phosphate buffer of pH 5.9 containing excess oxygen and catalase (Pazur, 1966). Specific activity is expressed as units of enzyme activity per milligram of protein. The protein in

the enzyme preparation was determined colorimetrically (Bailey, 1967).

To study the effect of uranium on glucose oxidase activity of the precipitated cell-free extract, 100 ppm uranium was added to the Warburg flask containing glucose and the precipitated cell-free extract. The glucose oxidase activity was measured in terms of oxygen uptake at 25°C with a Gilson differential respirometer (see Section 9.3f).

The glucose oxidase activity and the uranium content of the precipitated cell-free extract are shown in Table 7. The precipitated cell-free extract, containing 61 mg protein, gave a total glucose oxidase activity of 2.3 units, whereas the uranium content was negligible. The uranium content in the precipitated cell-free extract was 0.06 mg, whereas in the initial water extract it was 11 mg. This was equivalent to only 0.5% of the uranium existing in the original extract. This indicated that there were two possible effects of uranium as it relates to glucose oxidase activity. The first effect is that uranium does not form a complex with the enzyme, while the second effect suggest the association is weak and possibly exists in the form of metal enzyme complex. The interrelationship of metal and enzyme could be of two types, the first as a metalloenzyme and the second in the form of a metal enzyme complex. In contrast to metalloenzymes, metal enzyme complexes are in the form of loose association between the metal and protein of the

TABLE 7. ENZYMATIC ACTIVITY AND URANIUM CONTENT
IN CELL-FREE EXTRACT OF *Penicillium C-1*.

Glucose oxidase activity	0.18 $\frac{\text{UNITS}}{\text{ml}}$	Total activity (glucose oxidase)	2.3 UNITS	Sp. activity (glucose oxidase)
Protein	4.7 $\frac{\text{mg}}{\text{ml}}$	Total protein	61 mg	0.04 $\frac{\text{UNIT}}{\text{mg}}$
Uranium	5 ppm	Total uranium	0.06 mg	$\frac{\text{Uranium}}{\text{protein}} =$ 0.001

ENZYME PRECIPITATE 13ml

enzyme, therefore the metal tends to be lost in purification and there appears to be no dependence on metal for activity (Metal as constituents of enzymes, Nutrition Rev. 29: 97-100, 1971).

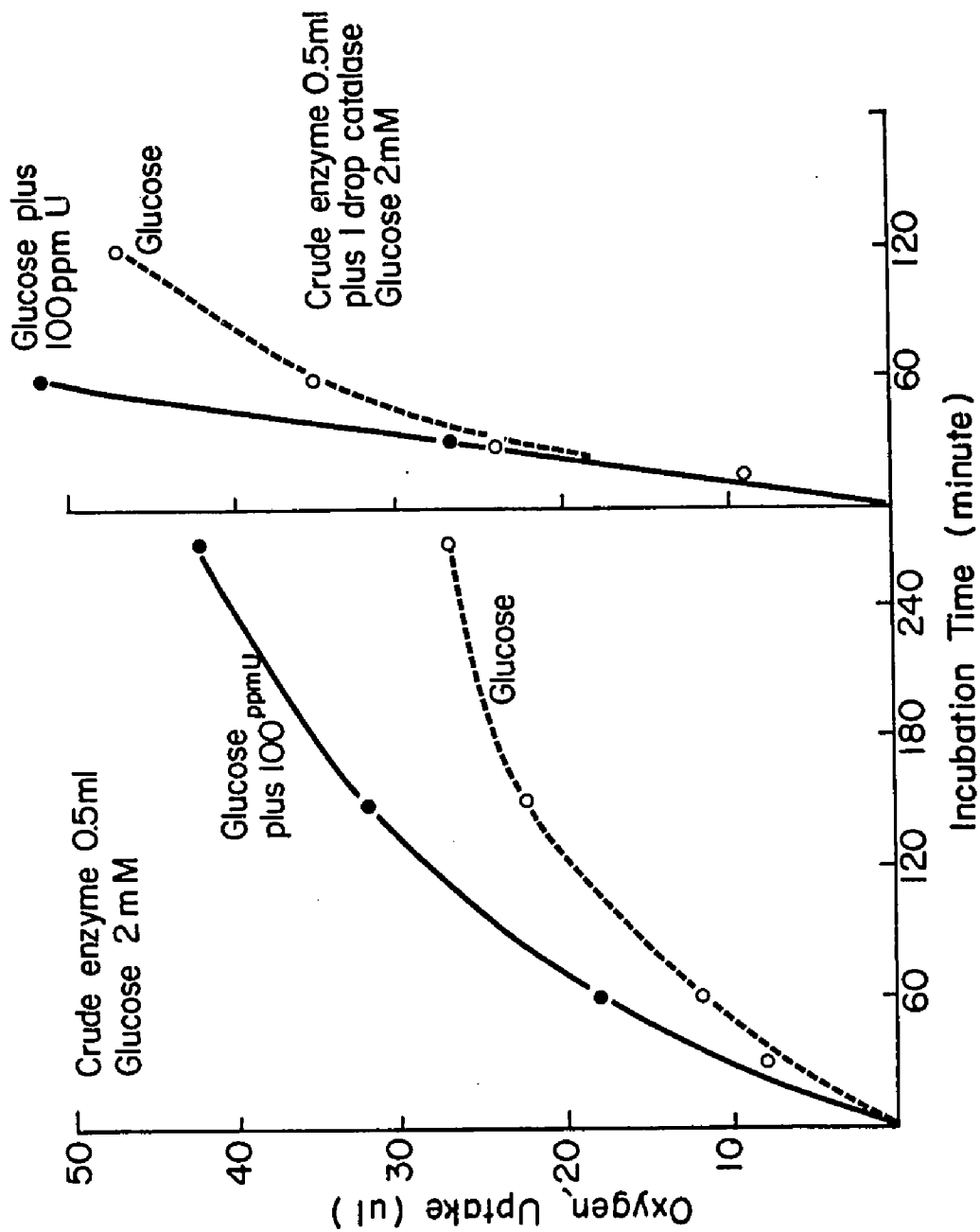
Nevertheless, a further experiment was made to study the effect of uranium on the glucose oxidation of the cell-free extracts. The precipitated cell-free extract was prepared as in the preceding experiment (Figure 42). 0.5 ml of the precipitated extract was added with uranium along with glucose. The glucose oxidation of the cell-free extract was tested in terms of oxygen uptake. The results are shown in Figure 43. A higher glucose oxidation was observed in the presence of uranium than in the absence.

9.4 Summary

The uptake of uranium by mycelia of Penicillium C-1 involves both adsorptive and biological reactions. The initial uptake was rapid, followed by a period of continuous uptake of uranium which lasts 30-60 minutes (Figures 37 and 38). From observations made the first 5 minutes of the initial uptake of uranium by Penicillium C-1, it was indicated that the initial uptake was largely a phenomenon of surface adsorption. The important evidences of adsorptive uptake are: (1) the uranium uptake during the first 5 minutes can be described by the Freundlich equation, which

Figure 43

Effect of uranium on oxygen uptake by cell-free extract (crude enzyme mixture) of Penicillium C-1 in Warburg flask. Uranium was added at 100 ppm to the flask containing the cell-free extract and substrate (glucose).



has been widely used in physico-chemical adsorption to express the relationship between the amount of substance adsorbed per unit mass of adsorbent and the concentration of adsorbate in liquid (Figure 35); (2) dead mycelia can take up uranium as well as live mycelia (Table 4); (3) uranium in the mycelia can be leached out by washing with EDTA, phosphate, phosphoric acid, and other chemicals (Table 5); (4) uranium uptake was not limited by metabolic processes, e.g., mycelial suspensions in solutions of uranium without addition of energy sources still can take up uranium (Figures 37 and 38). Of the total uranium uptake, the initial uptake during the first 5 minutes accounted for about at least 50%. This value was estimated from Tables 4 and 5, and from Figures 36-38.

Uranium uptake by the mycelia of Penicillium C-1 was not exclusively by physico-chemical adsorption. As previously mentioned, the mycelia formed larger pellets in the presence of uranium than in the absence (Chapter 5). In addition the uranium uptake was temperature dependent and the younger mycelia removed more uranium than the older mycelia (Chapter 8). These effects suggests biological involvement in uranium removal (Chapters 5 and 8). More direct evidences supporting biological involvement are: (a) over a certain period during the incubation, the uranium uptake was higher in the presence of a carbon

source than in the absence (Figure 37); (b) metabolic inhibitors such as sodium azide depressed uranium uptake (Figure 38); (c) in certain media (e.g., ethanol) the growth of Penicillium C-1 was stimulated in the presence of uranium as compared with in the absence (Zajic and Chiu, 1972a).

In studying the association between uranium and precipitated cell-free extracts (acetone precipitate and propyl alcohol precipitate), there was no evidence indicating that uranium was a necessary component of crude enzyme mixture (Tables 6 and 7). However, in the presence of uranium, glucose oxidation by a crude enzyme preparation was stimulated (Figure 43). These data give evidence that uranium uptake by Penicillium C-1 is involved in certain biological reactions, some of which may be enzyme based.

CHAPTER 10

CONCLUSION

One of the fundamental phenomena in microbes is their ability to take up specific metals. Of these metals some are metabolically required, while others are only accumulated without known biological functions. Compared with the common metals, e.g. potassium, calcium, etc., which are usually found in the microbial cells, most heavy metals are toxic to microbes. Data presented herein indicate that certain heavy metals, such as uranium and strontium, were not only non-toxic to particular microbes such as Penicillium cultures C-1 and C-2, but also these metals could be taken up by the cells. Both Penicillium cultures showed significant growth in a glucose basal medium or when grown on the same medium containing uranium and/or strontium at concentration of 100 ppm. These cultures were isolated in a methanol enrichment system containing a complex mixture of metal salts, i.e., uranium sulfate, strontium chloride, silver sulfate, titanium tetrachloride, and platinum chloride.

Experimental studies on the uptake of these metals by the Penicillium cultures indicated that they were able

to take up about 70% of the uranium, when the uranium concentration in the medium was at 100 ppm. Of the two cultures more complete studies have been made with culture C-1 particularly as it relates to uranium. The uranium uptake by Penicillium C-1 was affected by many factors such as pH, temperature, age of mycelia, external concentration of uranium, etc. Among these factors, the relationship of temperature and age of mycelia to uranium uptake appeared to be most associated with the physiological conditions of the cells (Section 8.3, Chapter 8). The uranium removed from the medium is either adsorbed or absorbed, or a combination of both. Further studies with Penicillium C-1 as it relates to uranium indicated that the uranium uptake involved adsorptive uptake as well as biological function. Of the total uranium uptake, the rapid adsorptive uptake during the first 5 minutes accounted for about at least 50% (Chapter 9). The uranium uptake by the mycelia of Penicillium C-1 was not exclusively physico-chemical adsorption. It is concluded that the uranium uptake is also growth associated, and at least part of the uranium taken up formed some type of complex with the membrane structure, possibly in the form of the uranium enzyme complex (Chapter 9).

The industrial importance of this development as it relates to uranium, and possibly, to other metals of

limited supply, is quite obvious. Both uranium and strontium are very abundant in radioactive wastes from mine water or from by-products in fissionable reactions. In ocean water, strontium is relatively rich. The ocean water contains about 13 ppm strontium, whereas estimates for uranium are 0.001 ppm. Marine sediments which are high in microflora are almost a thousandfold richer in uranium. The use of microbes to recover industrially important metals provides a new process approach to metal beneficiation. The design parameters may well have to be considered in terms of findings such as those reported herein. In conclusion, it is found that this study contributes toward the understanding of the basic characteristics involved in the recovery of heavy metals by microbiological processes.

APPENDIX I

DERIVATION OF THE EQUATION IN FIGURE 20

According to diffusion phenomena, the equation of balance for material transport across dr , a thin layer of spherical element, will be:

$$\begin{aligned}
 & \underbrace{D(4\pi r^2)(dC/dr)}_{\text{inflow}} - \underbrace{[D(4\pi r^2)(dC/dr) - \frac{d}{dr} D(4\pi r^2)(dC/dr)dr]}_{\text{outflow}} \\
 & \qquad \qquad \qquad = (4\pi r^2)(dr)Q \qquad (1) \\
 & \qquad \qquad \qquad \underbrace{\hspace{10em}}_{\text{consumption}}
 \end{aligned}$$

i.e.,

$$\frac{d}{dr} (r^2 \frac{dC}{dr}) = \frac{\rho Q}{D} r^2 \qquad (2)$$

where D = diffusion coefficient; $[\frac{L^2}{T}]$

r = radius coordinate; $[L]$

C = concentration of growth limiting substance; $[\frac{M}{L^3}]$

ρ = density of pellet; $[\frac{M}{L^3}]$

Q = metabolic demand rate per unit weight of pellet; $[\frac{M}{M \cdot T}]$

Integration of Equation (2) gives:

$$r^2 \frac{dC}{dr} = \frac{1}{3} \frac{\rho Q}{D} r^3 + K \quad (3)$$

where $K = \text{constant}$

The constant K is given by boundary condition $dC/dr = 0$ when $r = r_0$, where r_0 is the radius of non-living central core of pellet. Then,

$$K = - \frac{1}{3} \frac{\rho Q}{D} r_0^3 \quad (4)$$

Substitution of Equation (4) into Equation (3):

$$\frac{dC}{dr} = \frac{1}{3} \frac{\rho Q}{D} \left(r - \frac{r_0^3}{r^2} \right) \quad (5)$$

Integration of Equation (5):

$$C = \frac{1}{3} \frac{\rho Q}{D} \left(\frac{r^2}{2} + \frac{r_0^3}{r} \right) + K' \quad (6)$$

K' is given by another boundary condition: $C = 0$, when $r = r_0$. Then,

$$K' = \frac{1}{2} \frac{\rho Q}{D} r_0^2 \quad (7)$$

Substitution of Equation (7) into Equation (6):

$$C = \frac{1}{3} \frac{\rho Q}{D} \left(\frac{r^2}{2} - \frac{3r_o^2}{2} + \frac{r_o^3}{r} \right) \quad (8)$$

Among growth limiting substances oxygen may be selected as a controlling factor for aerobic organism. Therefore, in Equation (8) the case for oxygen can be developed, i.e., C represents oxygen concentration, and Q , oxygen demand, and assume that $C = C_{ps}$, which is the oxygen concentration at pellet surface, then $r = R$ gives the maximum size attainable in pellet growth. Substitution of $C = C_{ps}$ and $r = R$ into Equation (8) gives:

$$C_{ps} = \frac{1}{3} \frac{\rho Q}{D} \left(\frac{R^2}{2} - \frac{3r_o^2}{2} + \frac{r_o^3}{R} \right) \quad (9)$$

On the other hand, taking the whole pellet as a basis, the rate of oxygen transfer from liquid medium to cells is equal to the rate of oxygen consumption by the cells. The equation for this is:

$$k(r\pi R^2)(\bar{C} - C_{ps}) = \frac{4}{3} (R^3 - r_o^3)\rho Q \quad (10)$$

where \bar{C} = oxygen concentration in the bulky liquid; $\left[\frac{M}{L^3}\right]$

k = liquid film coefficient of oxygen
transfer from liquid medium to
cells; [$\frac{L}{T}$]

Re-arranging Equation (10):

$$C_{ps} = \bar{C} - \frac{\rho Q}{3k} \left(R - \frac{r_o^3}{R^2} \right) \quad (11)$$

From Equations (9) and (11):

$$\bar{C} = \frac{\rho Q}{3} \frac{1}{D} \left(\frac{R^2}{2} - \frac{3r_o^2}{2} + \frac{r_o^3}{R} \right) + \frac{1}{k} \left(R - \frac{r_o^3}{R^2} \right)$$

This is the equation appeared in Figure 17.

APPENDIX 2

CALCULATION OF THE NUMERICAL VALUES IN THE
FREUNDLICH EQUATION (SECTION 9.3a, CHAPTER 9)

From the 9 sets of experimental data (X/Y vs. C' ;
 X/Y represents mg uranium uptake per mg mycelial dry weight,
and C' the external environmental concentration of uranium
in ppm. See Figure 41), i.e.,

C' :	10	28	49	69	110	130	320	330	670
X/Y :	.007	.018	.038	.042	.066	.086	.21	.18	.38

the calculation is proceeded as follows (Bryant, 1960):

x [= $\log C'$]	y [= $\log(X/Y)$]	xy	x^2	y^2
1.00	- 2.12	- 2.12	1.00	4.49
1.45	- 1.74	- 2.52	2.10	3.03
1.69	- 1.42	- 2.40	2.86	2.02
1.84	- 1.38	- 2.54	3.39	1.90
2.04	- 1.18	- 2.41	4.16	1.39
2.11	- 1.07	- 2.26	4.45	1.14
2.51	- 0.68	- 1.71	6.30	0.46
2.52	- 0.74	- 1.86	6.35	0.55
2.83	- 0.42	- 1.19	8.01	0.18
<hr/>	<hr/>	<hr/>	<hr/>	<hr/>
17.99	-10.75	-19.01	38.62	15.16
(Σx)	(Σy)	(Σxy)	(Σx^2)	(Σy^2)

$$G_{xx} = 9\Sigma x^2 - (\Sigma x)^2 = 9(38.62) - (17.99)^2 = 23.94$$

$$G_{yy} = 9\Sigma y^2 - (\Sigma y)^2 = 9(15.16) - (-10.75)^2 = 20.88$$

$$\begin{aligned} G_{xy} &= 9\Sigma xy - (\Sigma x)(\Sigma y) \\ &= 9(-19.01) - (17.99)(-10.75) = 22.30 \end{aligned}$$

$$b = G_{xy}/G_{xx} = 22.30/23.94 = 0.93$$

$$\begin{aligned} a &= (\Sigma y - b\Sigma x)/9 = [-10.75 - (0.93)(17.99)]/9 \\ &= -3.05 \end{aligned}$$

$$y = a + bx = -3.05 + 0.93x$$

i.e.,

$$\log(X/Y) = -3.05 + 0.93x$$

or,

$$X/Y = 0.00089C'^{0.93}$$

This is the Freundlich equation which expresses the relationship between X/Y and C' as shown in Figure 41.

The correlation coefficient is

$$r' = G_{xy} / \sqrt{G_{xx}G_{yy}} = 22.30 / \sqrt{(23.94)(20.88)} = 0.998$$

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