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A pure culture of a soil organism coded M-32 was identified as Cellulomonas flavigena. This identification was carried out according to the routine procedures necessary to distinguish it from other related bacteria.

The establishment of a defined medium is probably the most significant finding of this research. Also important is the description of a cyclic cultural morphology found to be characteristic to C. flavigena.

A general description of the cellulolytic activity of C. flavigena was carried out in this study. Decomposition of a variety of cellulosic substrates suggests the presence of a complete cellulolytic system as described by any of the theoretical pathways for the degradation of native cellulose. The evidence presented in this thesis supports the constitutive nature of this enzyme system.

NUTRITIONAL INVESTIGATIONS

with

CELLULOMONAS FLAVIGENA

by

Paul Litton Fletcher, Jr.

A thesis submitted to

the Faculty of the Graduate School at

The University of North Carolina at Greensboro

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of the Requirements for the Degree

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Approved by

Bruce M Eberhart

APPROVAL SHEET

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

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September 6, 1966

Date of Examination

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I. STATEMENT OF THE PROBLEM

This study was initiated in an attempt to elucidate the characteristics of a bacterial culture capable of decomposing cellulose. The description of the biochemical and physical characteristics of the organism leading to its identification represents the first half of this thesis. The latter half consists of a diverse study of its nutritional requirements.

II. INTRODUCTION AND REVIEW OF THE LITERATURE

The study of cellulose is particularly interesting because this compound and its related polysaccharides represents the main repository carbon molecules in living systems (20). The degradation of cellulose is particularly interesting in that during the process it is converted to its basic chemical and biological unit, glucose.

As the human population of our planet increases, an adequate nutrition source must be maintained. The inability of monogastric animals to metabolize cellulosic carbon sources is due to a lack of enzymes necessary for their digestion. The facilitation of cellulose digestion might easily relieve nutritional deficiences in areas where conventional food stuffs are limited.

The availability of cellulose as a carbon source to animals can be effected in several ways. Merely to increase the mean of cellulose digestion in ruminants from 50% to 85% would represent an enormous increase in protein source available for human consumption. Such an increase could be effected by knowledge of the biochemistry of cellulose degradation.

Another area important in the study of cellulose degradation lies in the disposal of industrial cellulosic wastes which are among the most difficult to dissolve.

On the other hand, the prevention of cellulose decomposition is

equally important. Cowling (7) estimates that the loss of living timber in the United States at 2.0×10^{10} cubic feet per year. This does not take into account the rotting of harvested timber and cotton fabrics in areas such as the tropics where this problem is particularly acute. Relatively limited knowledge is available regarding the processes by which the semi-crystalline, highly insoluble cellulose is converted to glucose. Microorganisms are most responsible for causing this change. The industrial utilization of microorganisms thus far has been limited to the production of antibiotics and a few enzymes; however, the utilization of fermentation products is as old as the history of mankind. The application of cellulolytic microorgansims in industry depends on the findings of basic research in this field.

The bulk of cellulolytic organisms are to be found in soil. Here their activities decompose carbonaceous material from plant and animal sources that originated as gaseous carbon dioxide to be fixed in photosynthesis. This return of carbon to atmospheric carbon dioxide serves two purposes: first, by serving as an energy source for the soil microbes whose metabolic wastes are essential for plant nutrition; and, second, to become available again as carbon dioxide for photosynthesis. This is an important role of carbon in nature, without discussing many of its subleties that are also quite important.

A general review of the chemistry of cellulose and related materials can be found in Honeyman (15). A comprehensive review of microbial cellulose decomposition by Siu (31) is important, especially

when supplemented by the more recent reviews of Reese (28) and Gascoigne (12).

The soil microflora is a mixture of thousands of organisms whose interactions are as important as their individual activities. The study of cellulose decomposition by pure cultures is important in order to deduce at which points other systems could interact. Laboratory environmental control of pure cultures requires far less sophisticated equipment for obtaining repeatable data.

The isolation and identification of Cellulomonas flavigena from soil samples for this study represents a major portion of this thesis. The original description of this organism as Bacillus flavigena was by Kellerman and McBeth (18) who first described its ability to digest cellulose. Cellulolytic activity of C. flavigena was studied by Hammerstrom, et al., (14) with respect to the adaptability of its enzyme system. They described C. flavigena cellulase as constitutive; however, they were unable to demonstrate any activity without first concentrating culture fluid one-third to one-tenth of its original volume. Activity was demonstrated by this author without prior concentration according to the hydrocellulase assay of Flora (9) and the aryl-β-glucosidase assay of Eberhart (8).

Porter, et al., (27) described the action of trypsin on the cellulase activity of C. flavigena. They found trypsin had no effect on the rate of cellulose oxidation, but that glucose oxidation was enhanced when measured manometrically.

Probably the best nutritional study of C. flavigena is by

Garrison and Harris (11). Six species of Cellulomonas were investigated in this study. Their work describes growth optima, lysozyme susceptibility, nitrogen metabolism, dehydrogenase activity, and respiratory activities of C. flavigena on several carbohydrates and Krebs cycle intermediates. C. flavigena was found to digest diverse proteins, oxidize several carbohydrates, and showed evidence for the presence of oxidative metabolism via the Krebs cycle.

III. METHODS AND MATERIALS

Introduction

This work represents the systematic identification of an organism isolated from fresh bovine feces by Frank Hulcher at V. P. I. in August 1954. Hulcher was screening for organisms possessing a cellulolytic system of enzymes and isolated many cellulase producers of varying activity. His doctoral dissertation concerned the nutritional characteristics of one of these organisms--Cellvibrio gilvus. The remaining organisms were coded and stored as lyophilized cultures. This culture was coded M-32.

Bacterial Maintenance

Culture M-32 was found to grow easily with no apparent loss of its original characteristics when maintained on T-SOY medium (Difco), liquid or solid. Stock cultures were stored at 4 C on T-SOY slants once growth appeared. Transfers were made at one month intervals. Cells subcultured from these refrigerated slants produced growth in adequate media within 26 hours after transfer within the limits of the optimal growth temperatures.

Defined Media

Several attempts have been made in order to develop a chemically defined growth medium for M-32. In isolation, original cultures were grown and purified by Hulcher (16) in his work with Cellvibrio gilvus (sp. n). A modification of the Fuller and Norman medium (10) was used. A modification of Dubos medium (16) was used in nutritional experiments. Cellulose was substituted for carbohydrate in fermentation experiments. Casein hydrolysate was omitted in amino acid assay. Vitamins included are those listed in the Media section (Appendix I). T-SOY (Difco) was used as an all-purpose maintenance medium, in plates, slants, and broth.

Lyophilization

In addition to transfer and refrigeration, M-32 cultures were stored by the freeze-drying technique. Lyophilization was carried out according to the method outlined by the American Type Culture Collection (ATCC) (26). Cultures maintained in this manner have remained viable for 11 years even when stored at room temperature. After lyophilization mutants and variant cultures obtained from this work are stored at -20 C. Reconstitution is effected by the addition of 0.5 ml sterile T-SOY broth to the inner shell vial aseptically. This suspension is then used for inoculum.

Media

During the course of this investigation several different media were used. The various media were used as specific needs for certain experiments, especially in the development of a chemically defined medium. A list of the different media used in this study is included in Appendix I.

Nutritional Experiments

Carbohydrates. The techniques used to determine which carbohydrates are metabolized were quite varied. During the identification procedures the traditional 0.5% carbohydrates in Phenol Red Broth (Difco) and agar tubes were used to designate pH changes.

In determining the utilization of various carbohydrates two additional techniques were used: addition of 10% solutions to Sugar-free Basal with a one per cent final concentration and an auxanographic method in which Sugar-free Basal agar was seeded with M-32 washed cells, four different carbohydrates were added on each plate. Compound utilization was determined by changes in optical density in the case of the broth tubes. Details of this experiment appear in the next section. The auxanographic method was scored according to growth responses of cells adjacent to each single carbohydrate. Details appear in the next section.

Amino Acids. Two different assays were used to determine the amino acid requirements of M-32. Twenty ml quantities of amino acids neutralized with 1.0M phosphate buffer at a level of 5 mg / ml were stored frozen until added to the assay medium before autoclaving. The first assay involved nine pools of four or five amino acids at a level of 0.4 mg / ml each in the Amino Acid Assay Medium. A final assay determined the growth of M-32 on amino acids in individual tubes. Growth was measured by changes in optical density. Most of these amino acids were reagent grade, stored under refrigeration. Details of this experiment appear in the Results and Discussion section.

Substrates

Hydrocellulose. The hydrocellulose used in both enzymatic assays and as a carbohydrate for M-32 was prepared as modified from the procedure of Hungate (18). Purified cotton linters were packed into a flask containing 270 ml of concentrated HCl diluted to 300 ml (10.8 N) so that all free liquid was taken up. This remained for 24 hours at room temperature with intermittent agitation. The resulting slurry of hydrocellulose was poured into a large excess of water, washed several times by decantation, and neutralized with $NaHCO_3$. Exhaustive dialysis against a slow flow of distilled water was performed until the contents of the dialysis bag were negative when tested for the presence of chloride ion. A one minute treatment in a Waring blender at high speed yielded the well-dispersed stock hydrocellulose suspension which was stored under refrigeration. The concentration of the hydrocellulose in the stock suspension was determined from the dry weight of aliquots.

When diluted in distilled water the hydrocellulose formed a uniform turbid suspension of finely divided particles which settled out of suspension over a matter of hours. However, in the presence of electrolytes such as the assay buffer, the particles had a tendency to flocculate and settle from suspension much more rapidly.

Whatman paper powder, ball-milled in excess of 24 hours in aqueous suspension proved an adequate substitute for acid hydrolyzed cotton activity of M-32 plate colonies.

Avicel, a commercial hydrocellulose (American Viscose

Corporation) is described as being a microcrystalline cellulose prepared by treating a very pure α-cellulose with 2.5 N HCl for 15 minutes at 105 C (2). The hydrocellulose assay utilized a 2 mg / ml suspension of the above substrates. Varying concentrations were used in growth experiments.

Other Substrates. Carboxymethylcellulose, CMC-70-Premium High and CMC-70-Premium Low (Hercules Powder Company) were used as carbohydrate sources for M-32 cultures indicated the formation of glucose from culture cellulolytic activity.

P-Nitrophenyl-β-D-glucopyranoside (A grade, California Corporation for Biochemical Research) was used for the aryl-β-glucosidase (PNP-Gase) assay. Also, PNP-G was used to indicate PNP-Gase activity in culture of M-32.

Esculin, an esculetin-β-D-gluco pyranoside, (monohydrate, C. P.; Mann Biochemicals) was used to indicate the presence of a β-D-glucosidase in plate cultures, much in the same way as the solubilization of hydrocellulose, or hydrolysis of PNP-G. The hydrolysis products of esculin no longer fluoresce in short wave U.V.

The cellobiose (Calbiochem, A grade) and dextrose (Merck, reagent grade) was used in culture, standard curves, and cellobiase determinations. Other carbohydrates, listed in Tables 6 and 7 were used in the growth studies of M-32.

Human serum albumin (fraction V powder, C grade, Calbiochem)

was used to prepare the standard curve for the Lowry protein determination (21).

Enzyme Assays

Hydrocellulose Assay. This assay, measuring the solubilization of a finely dispersed hydrocellulose, was developed in principle from the turbidimetric assay of Norkrans (23), who had used a regenerated cellulose sol as substrate. Flora (9) modified this assay to accomodate the use of the hydrocellulose, further modified by using buffer at pH 6.8 instead of 4.8 by this author. One unit of activity was defined to produce a Δ Klett of 10 in one hour under the described assay conditions. Meicellase (Meiji Seika Co. Ltd.) was used as a standard of activity in this assay at a level of 4 mg / ml enzyme solution.

PNP-Gase Assay. The assay of Eberhart (8) was followed without modification. (Discontinuous assay.)

Analytical Procedures

Glucose (range, 0 to 100 µg) was determined by an enzymatic (Notatin) method modified from Saifer and Gerstenfeld (29). The commercial preparation called "Glucostat" (Worthington Biochemicals) was used according to the suggested procedure. Unknowns were compared to a series of glucose standards run simultaneously.

Total carbohydrate material (range, 0 to 150 µg) was determined by the phenol-sulfuric acid method described by Timell (34).

Protein (range, 0 to 300 µg, human serum albumin standard) was determined according to the method of Lowry, et al. (21) and the biuret procedure (13).

Special Equipment

Cultures were incubated at 30 C with an added humidity source to prevent excessive moisture losses from plate cultures.

Larger liquid cultures (to 200 ml) were grown in a constant temperature bath-shaker (Blue M Electric Co.). Bulk cultures (to 5 L) were provided with aeration apparatus and stirred magnetically at 25 C.

Cultures and stains were examined by a Zeiss GFL microscope, equipped for phase observation.

A Klett-Summerson colorimeter, Baush and Lomb "Spectronic 20" colorimeter, and Beckman Spinco 151 colorimeter were available for analytical procedures involving spectrophotometry. A model DB Beckman spectrophotometer was made available by the Chemistry Department.

Direct counts of M-32 cultures were facilitated by the use of a Spencer "Bright Line" Hemocytometer. Accurate counts were maintained by dilution of cultures. Observation was facilitated by including a few drops of crystal violet and counting under phase optics (Zeiss Neofluar).

Cell Disruption

Several methods of disruption have been attempted in order to obtain a cell-free preparation to study cellulolytic activity of such a system. The methods attempted have proved most unsatisfactory. Lysozyme, sodium lauryl sulfate, desoxycholate, trituration with alumina (two grades: Norton's levigated and Alcoa N-40), homogenation with glass beads, and sonication have all met with disappointing results.

Lysozyme (Nutritional Biochemicals, 3X Crystalline) was used with the method of Shugar, D. (30).

Trituration, homogenation and sonication were attempted both with and without abrasives, glass beads, or surface active agents for periods up to an hour according to the methods suggested in Methods in Enzymology (6) with no apparent success when observed microscopically or increase in protein (Biuret Assay).

Homogenation was attempted with an Omni-Mixer (Ivan Sorvall, Inc.) at 16,000 rpm. A Branson Sonicator (Heat Systems, Inc.) was the sonic oscillator that was utilized in other disruption techniques. All attempts at disruption of M-32 cells were unsuccessful in obtaining cellulolytic extracts due to the resistant nature of the cells.

IV. RESULTS AND DISCUSSION

Morphology

Culture M-32 occurs as short and long rods arranged singly and in pairs depending on the age of the culture. They range from 0.4 to 0.8 μ in diameter and 0.9 to 3.0 μ in length, with rounded ends. M-32 stains gram-positive with the Hucker and Kopeloff gram stain (26) in cultures up to fifteen days old. After fifteen days gram-negative forms begin to appear, probably as dead organisms that are no longer capable of retaining the gentian violet. (See "Cultural Cycles")

Capsules are probably not present. Endospores have never been observed via phase microscopy of wet mounts from liquid and solid media for all ages. Involution forms are present in limited numbers in all media with increasing numbers on restrictive media. They occur mainly as long cells (3.0 μ) with branching. In deficient broth, growth occurs as suspended granular microcolonies.

Motility has never been observed. Hanging drop, wet mounts and semi-solid media (S I M, Difco) have been employed to detect motility.

Dry Cells

The weight of a single M-32 cell has been determined to be 6.32×10^{-14} g. These cells had been grown in T-SOY media for 100 hours. The cells in a 1 ml sample of T-SOY media weighed 0.0278 g. (27.8 g./liter). This culture was assayed for a bacterial count by a direct count and photometrically. The count was 4.4×10^{8} cells / mm^3. The cells were dried in analytical weighing bottles at 100 C for 24 hours and cooled in a dessicator before weighing.

Culture Characteristics

Growth after five days on T-SOY (Difco) agar at 30 C produces a transparent to opaque white colony which yellows with age. These colonies become visible in 36 hours. They are glistening with a beaded form, smooth, convex surface, and a round, entire edge. M-32 colonies are of moderate size, rarely exceeding 5 mm. The yellow pigment increases with age and is water insoluble. Inclusion of cellulose in any medium seems to enhance this yellow color.

Biochemical Data

The optimum temperature for growth is 30 C with a range from 22 to 40 C. Growth curves for 30 C and 37 C are represented in figures 2 - 7.

In T-SOY broth after four days at 30 C, M-32 displays no surface growth, but produces a moderate to heavily turbid medium that is uniform. There is a moderate to heavy amount of sediment that begins to appear after two days. The temperature requirements are the same for broth and for solid media. The optimum pH seems to be 7.0 with a range from 6.5 to 7.6. Terminal pH on glucose broth was 5.2 after a three-week incubation at 30 C.

No growth occurs on either nutrient broth or agar. Desoxycholate agar and E M B agar do not support growth. M-32 has never been observed to liquify gelatin in this laboratory, although conflicting reports exist (3). No H_2S has been observed by means of S I M and Kligler's Iron Agar (Difco).

Growth on Russell's Double Sugar Agar (Difco) demonstrates a preference for glucose over lactose with an acid slant that turns alkaline after ten days--no visible butt growth, yet turning acid after a week.

No growth was observed on litmus or skim milk, both remaining at neutral pH after ten days. Urea is not hydrolyzed.

Citrate is not utilized as a carbon source. No growth was observed when inoculated in Koser's Citrate broth. Methyl red and Voges-Proskauer tests are negative after seven days. Kovac's test for indole is negative after seven days. The test for coagulase is negative after two days.

M-32 is catalase positive. Small amounts of ammonia are produced in Sugar-free Basal and other minimal media after a week.

Ammonia was detected by the direct nesslerization of a culture and the supernatant following cell sedimentation by high-speed centrifugation.

Blood was not hemolyzed after five days at 30 C, but some hemolysis is produced after five days at 37 C.

A rat was inoculated orally with 1.0 ml from a three day T-SOY culture of M-32. No reaction was observed after a three week incubation period. No autopsy was performed.

Lactate was not converted. (Moore, W. E. C., personal communication)

Growth in a T-SOY agar deep at 30 C demonstrated the absence of anaerobic growth. While slow growth was possible in a microaerophillic environment, aerobic growth clearly was the best.

Carbohydrate growth data appear more extensively in a separate section, but the following data appear in Table 1 after determination on Difco Phenol Red Agar Base. Carbohydrate levels were 0.5%.

Table 1. Fermentation activity of M-32 in Phenol Red Carbohydrate Agar (Difco).

SUGAR	FIRST APPEARANCE OF ACID (day)	FIRST APPEARANCE OF ALKALI (day)	GAS
Glucose	1	--	0
Lactose	2	14	0
Sucrose	1	--	0
Mannitol	2	4	0
Dulcitol	2	--	0
Inulin	2	4	0
Maltose	2	--	0

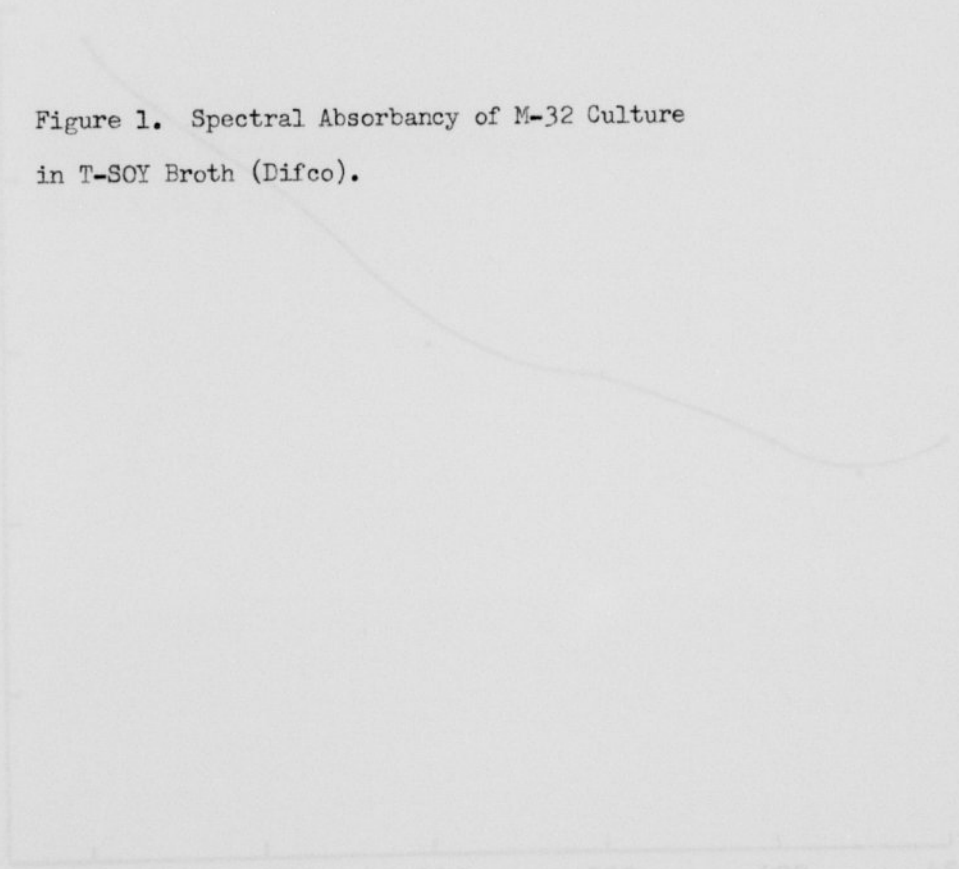
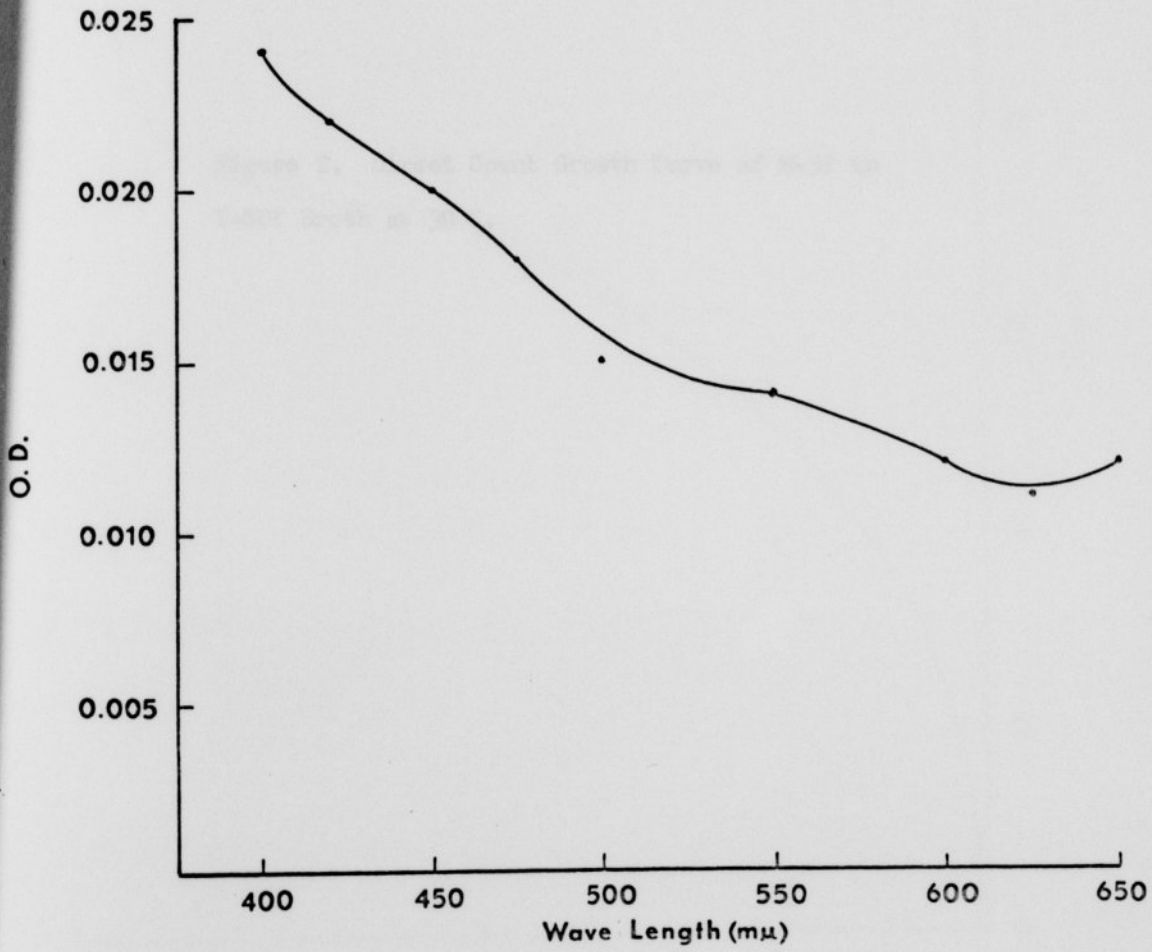


Figure 1. Spectral Absorbancy of M-32 Culture in T-SOY Broth (Difco).


0.025
0.020
0.015
0.010
0.005
O.D.
400
450
500
550
600
650
Wave Length (mμ)

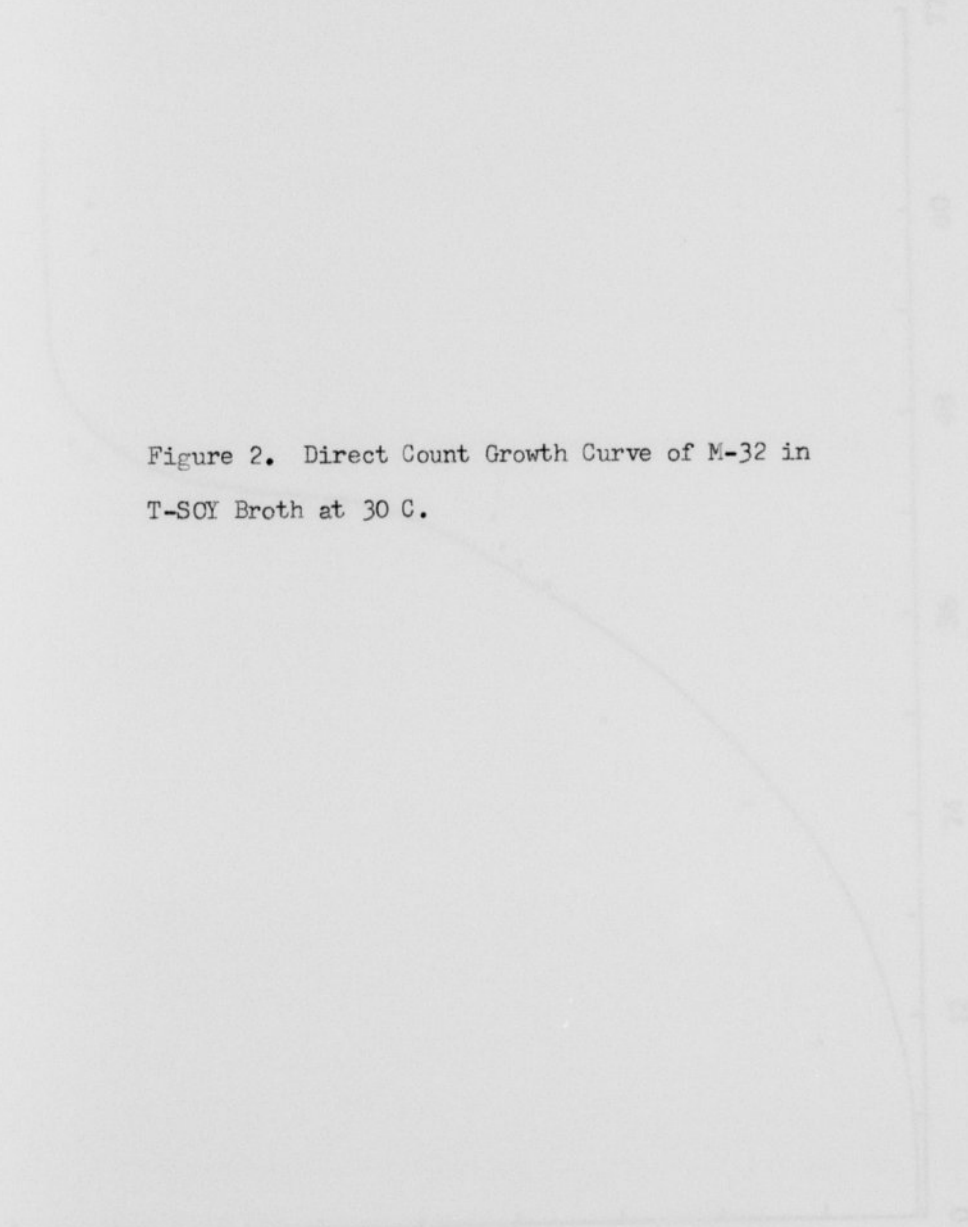
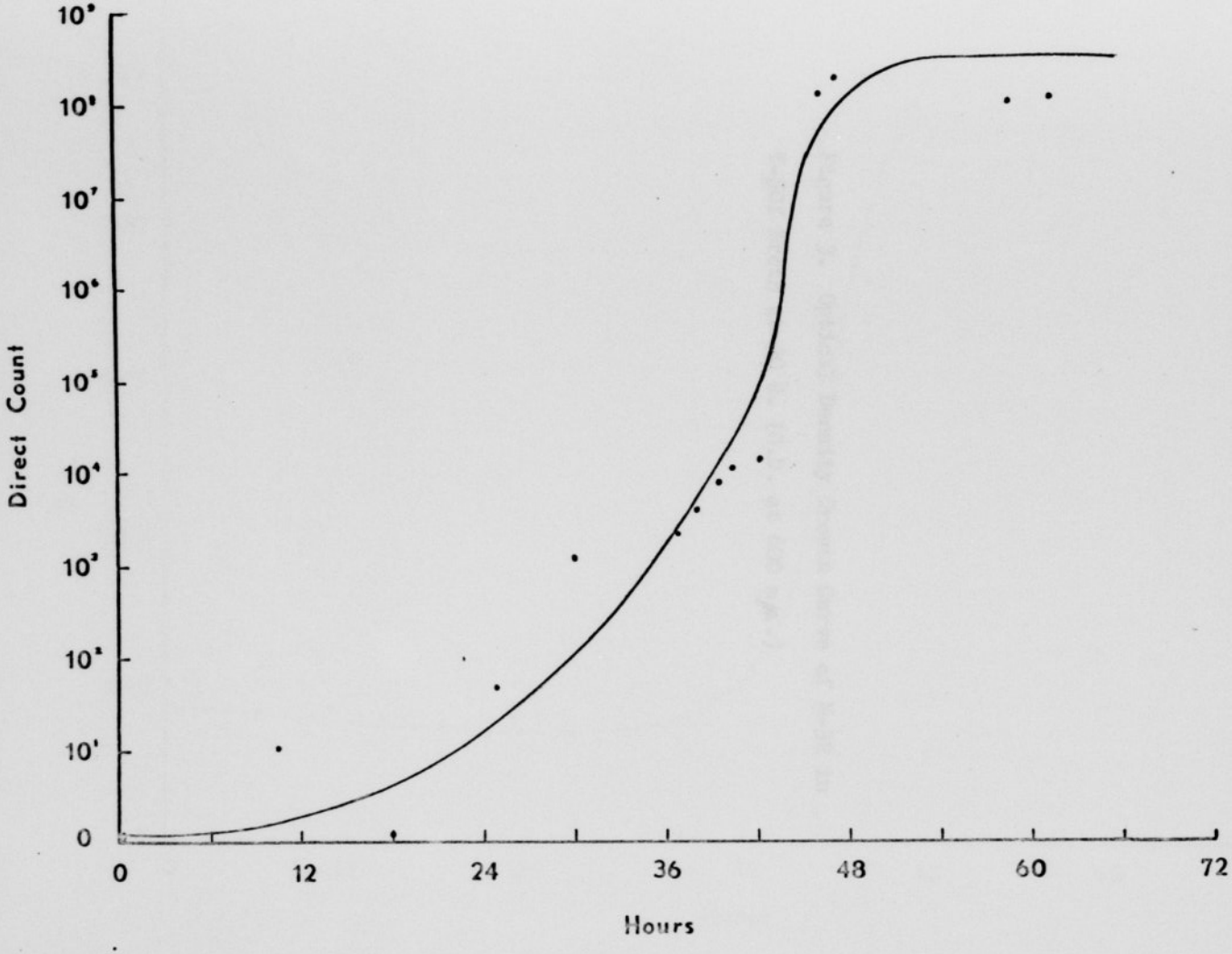


Figure 2. Direct Count Growth Curve of M-32 in T-SOY Broth at 30 C.


Direct Count
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
0
0
12
24
36
48
60
72
Hours

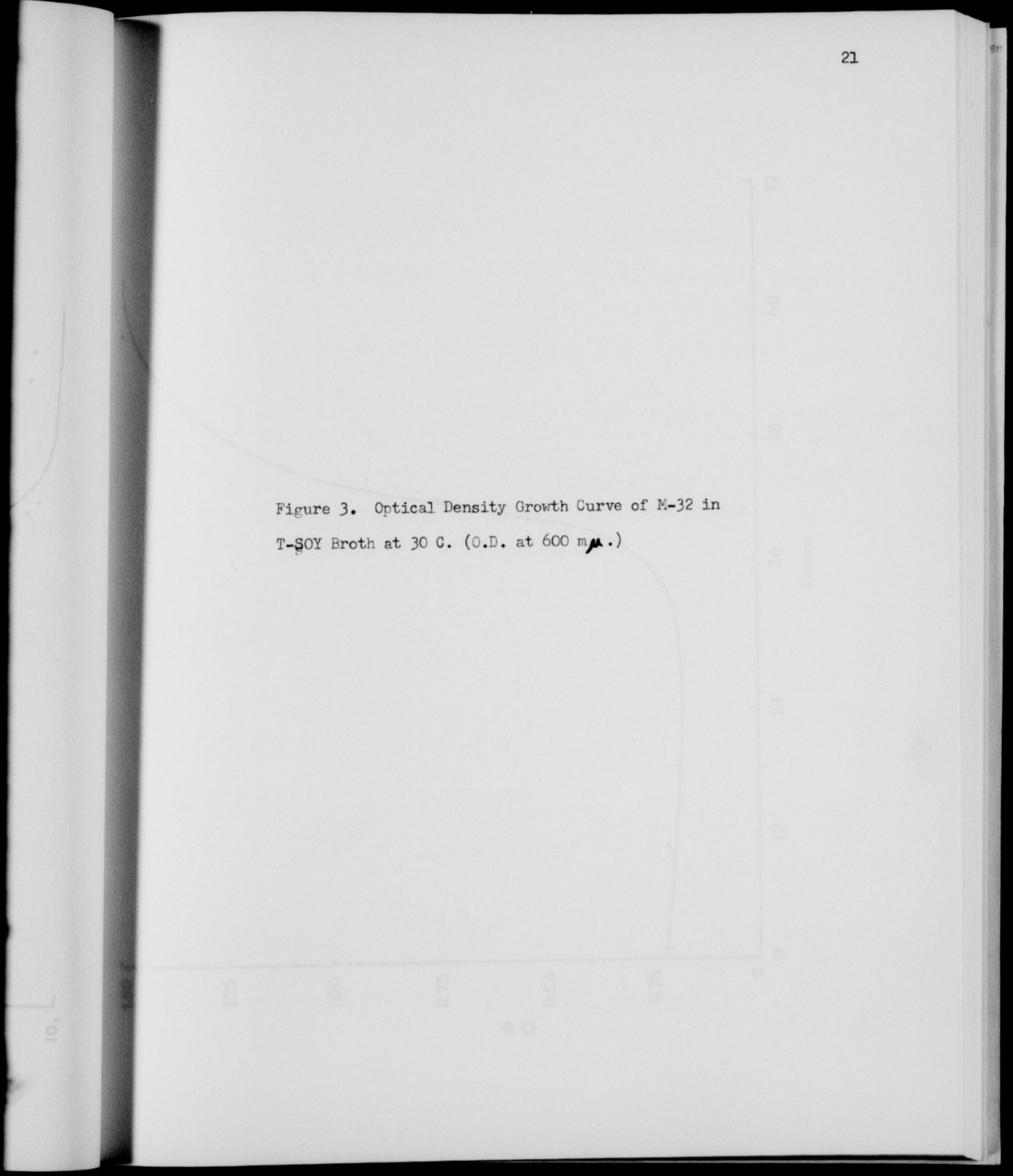
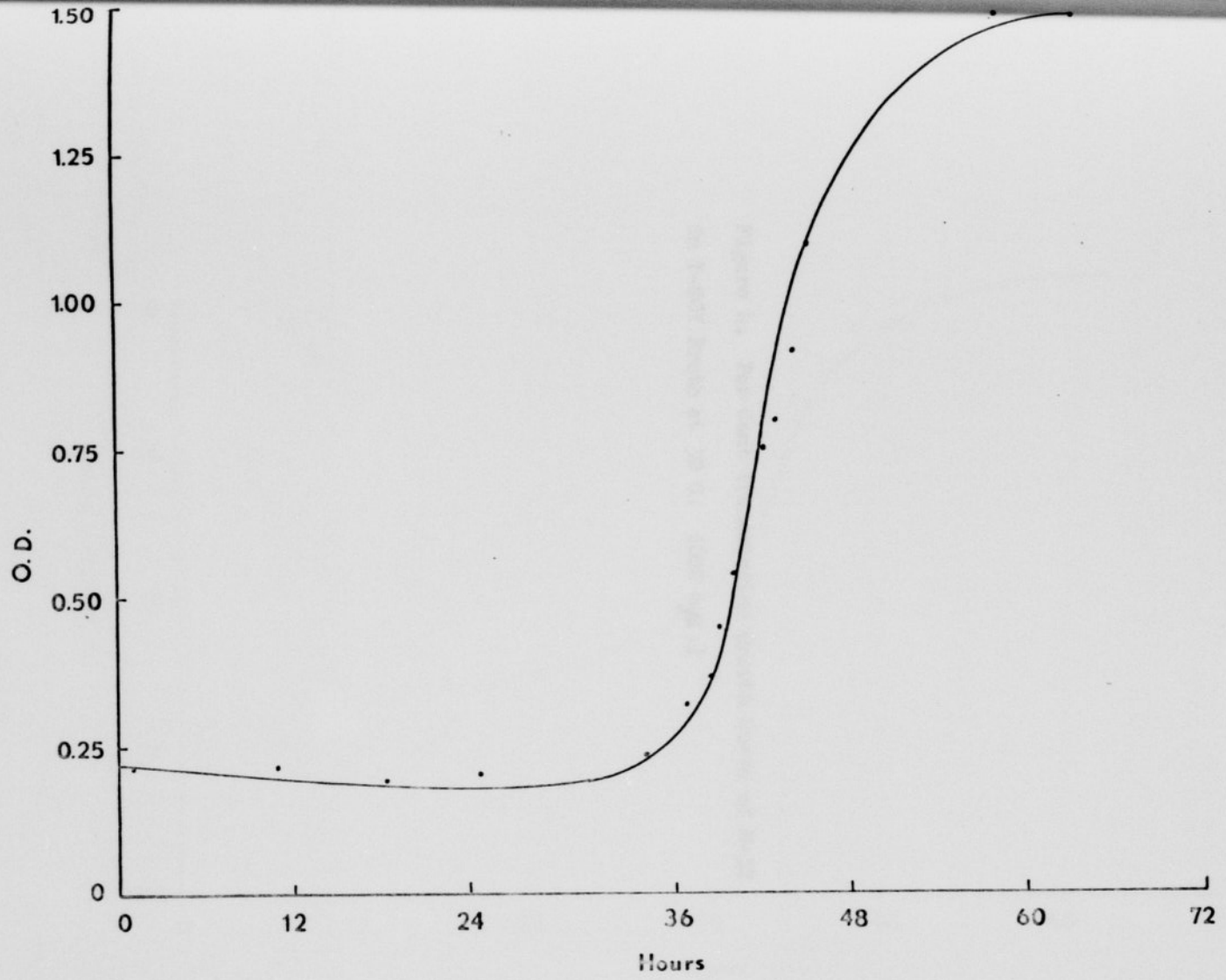


Figure 3. Optical Density Growth Curve of M-32 in T-SOY Broth at 30 C. (O.D. at 600 mμ.)


1.50
1.25
1.00
0.75
0.50
0.25
0
O. D.
0
12
24
36
48
60
72
Hours

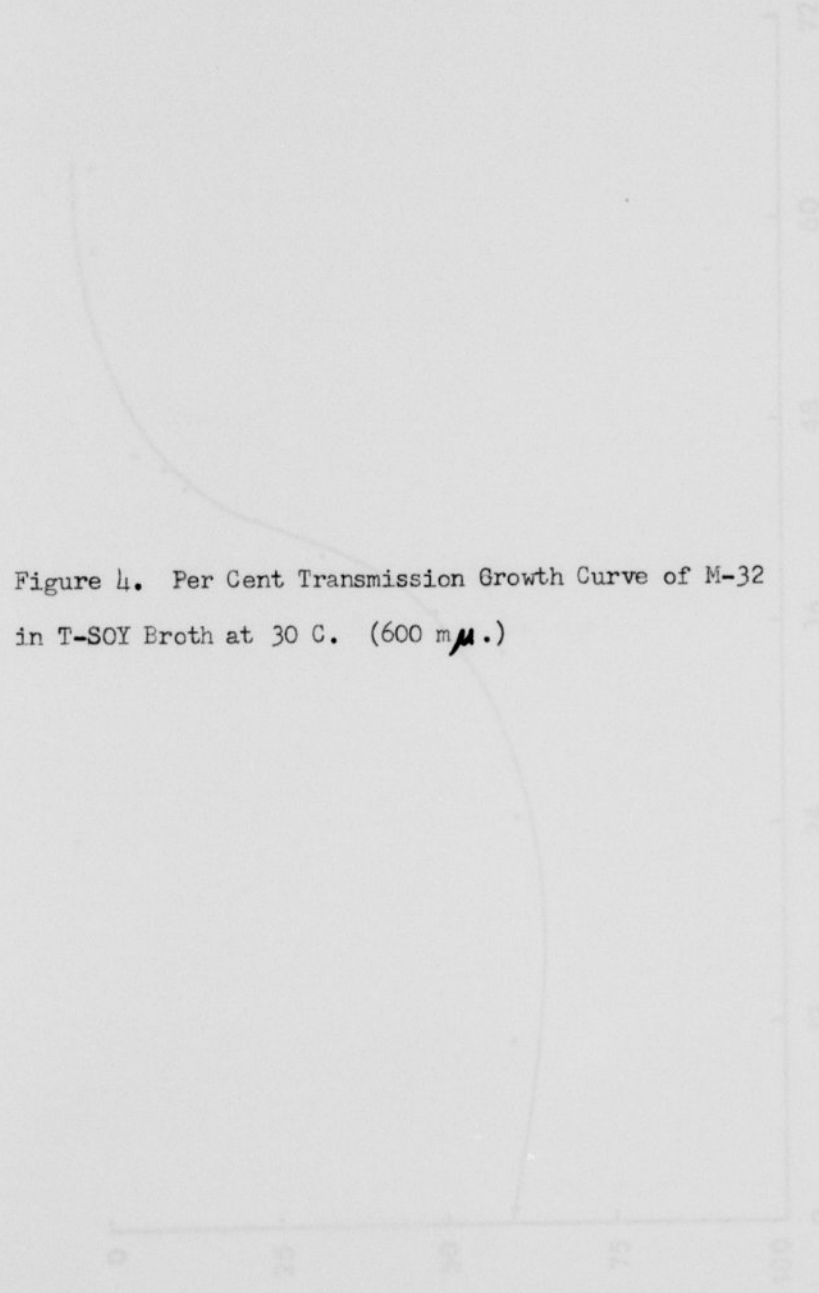


Figure 4. Per Cent Transmission Growth Curve of M-32 in T-SOY Broth at 30 C. (600 mμ.)

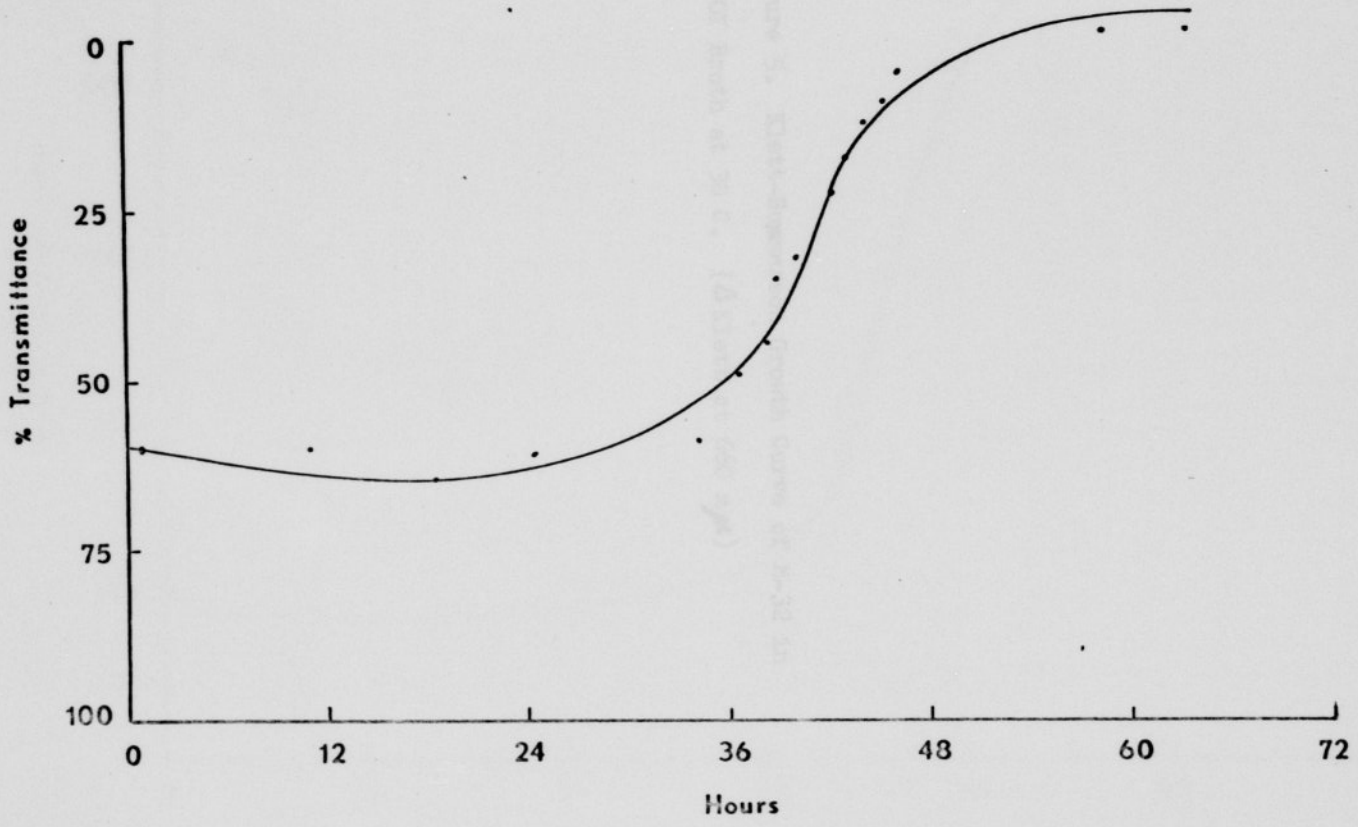
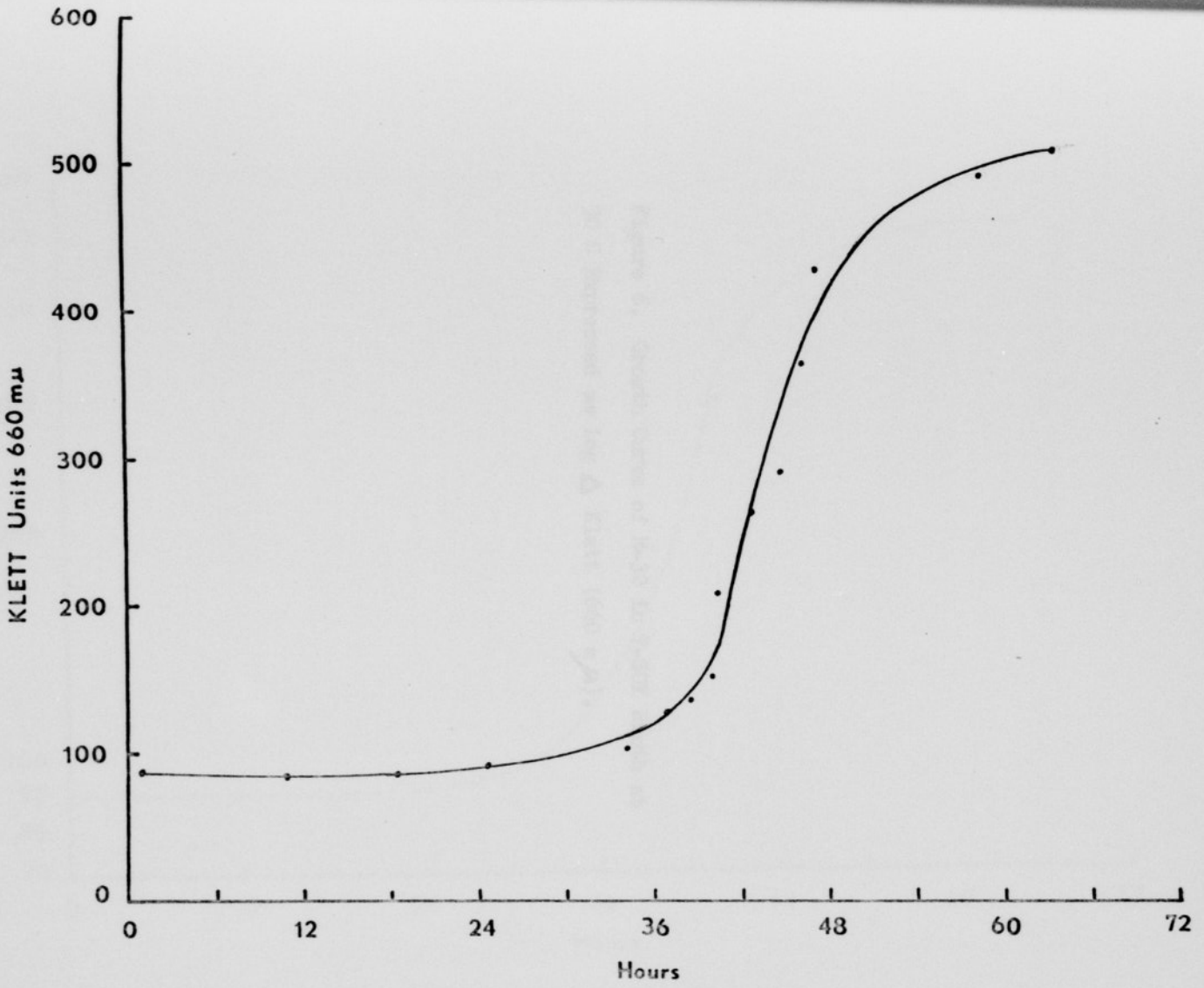

0
25
50
75
100
% Transmittance
0
12
24
36
48
60
72
Hours

Figure 5. Klett-Summerson Growth Curve of M-32 in T-SOY Broth at 30 C. (Δ Klett at 660 mμ)


600
500
400
300
200
100
0
KLETT Units 660 mμ
0
12
24
36
48
60
72
Hours

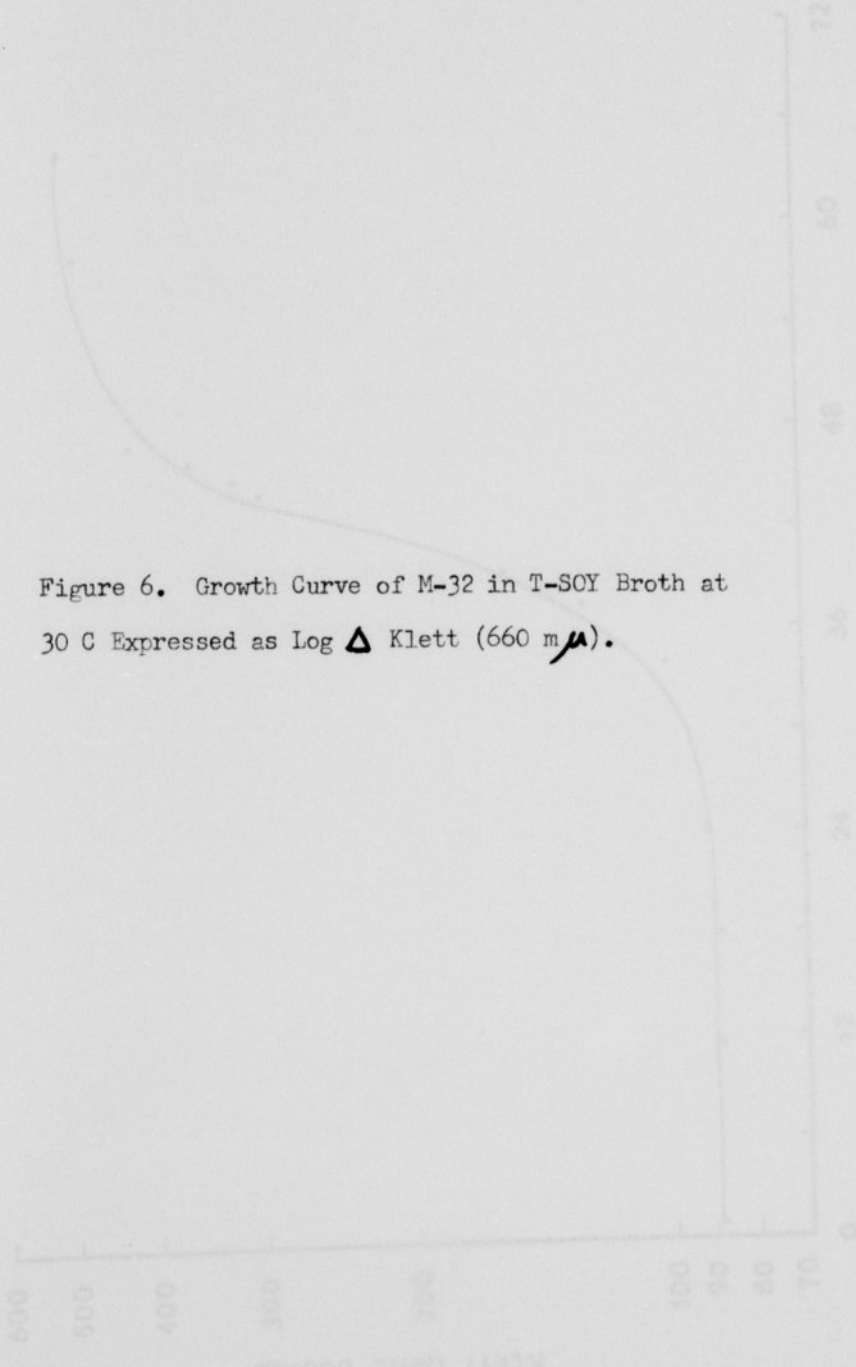
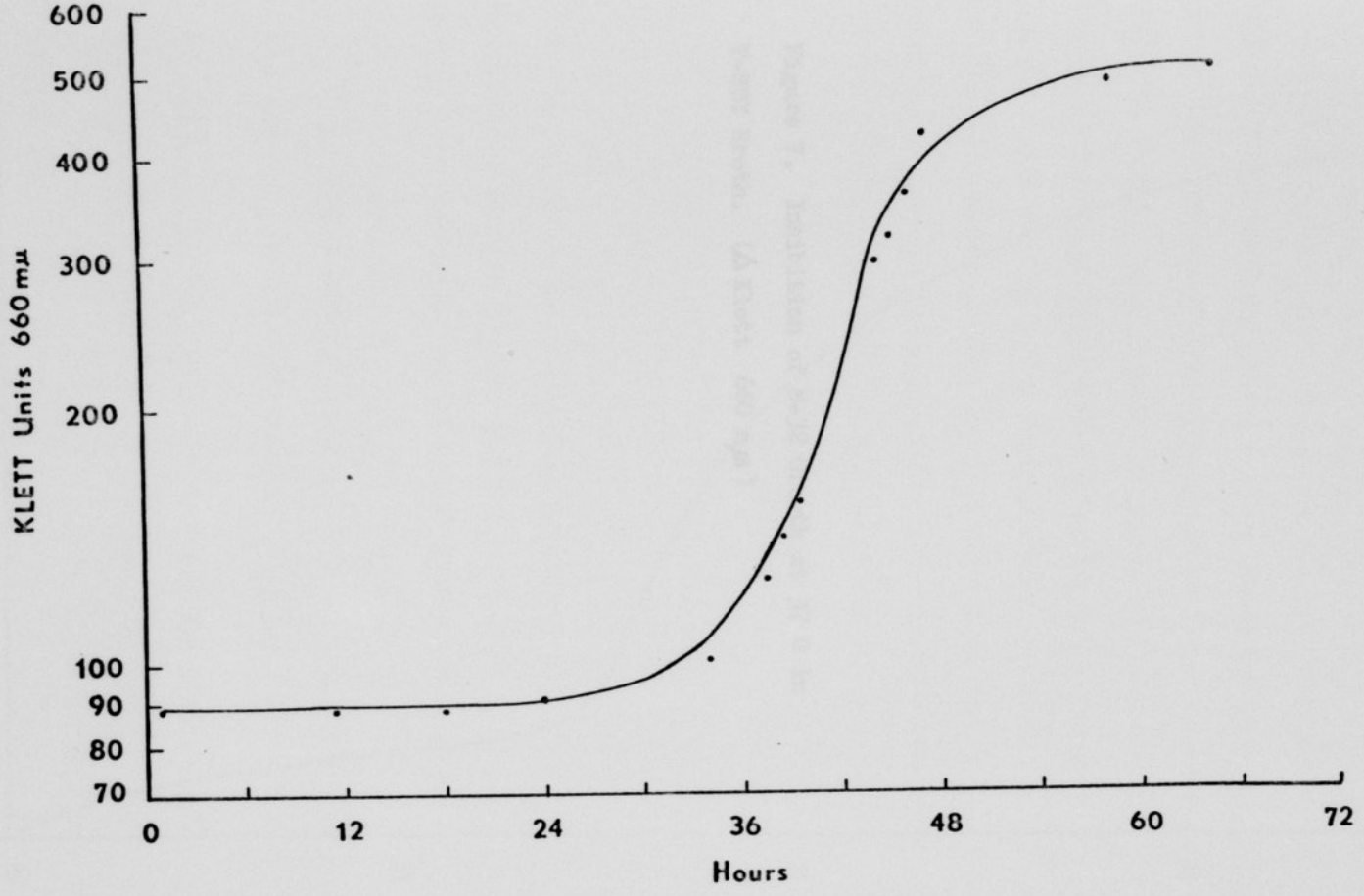


Figure 6. Growth Curve of M-32 in T-SOY Broth at 30 C Expressed as Log Δ Klett (660 mμ).


600
500
400
300
200
100
90
80
70
KLETT Units 660mμ
0
12
24
36
48
60
72
Hours

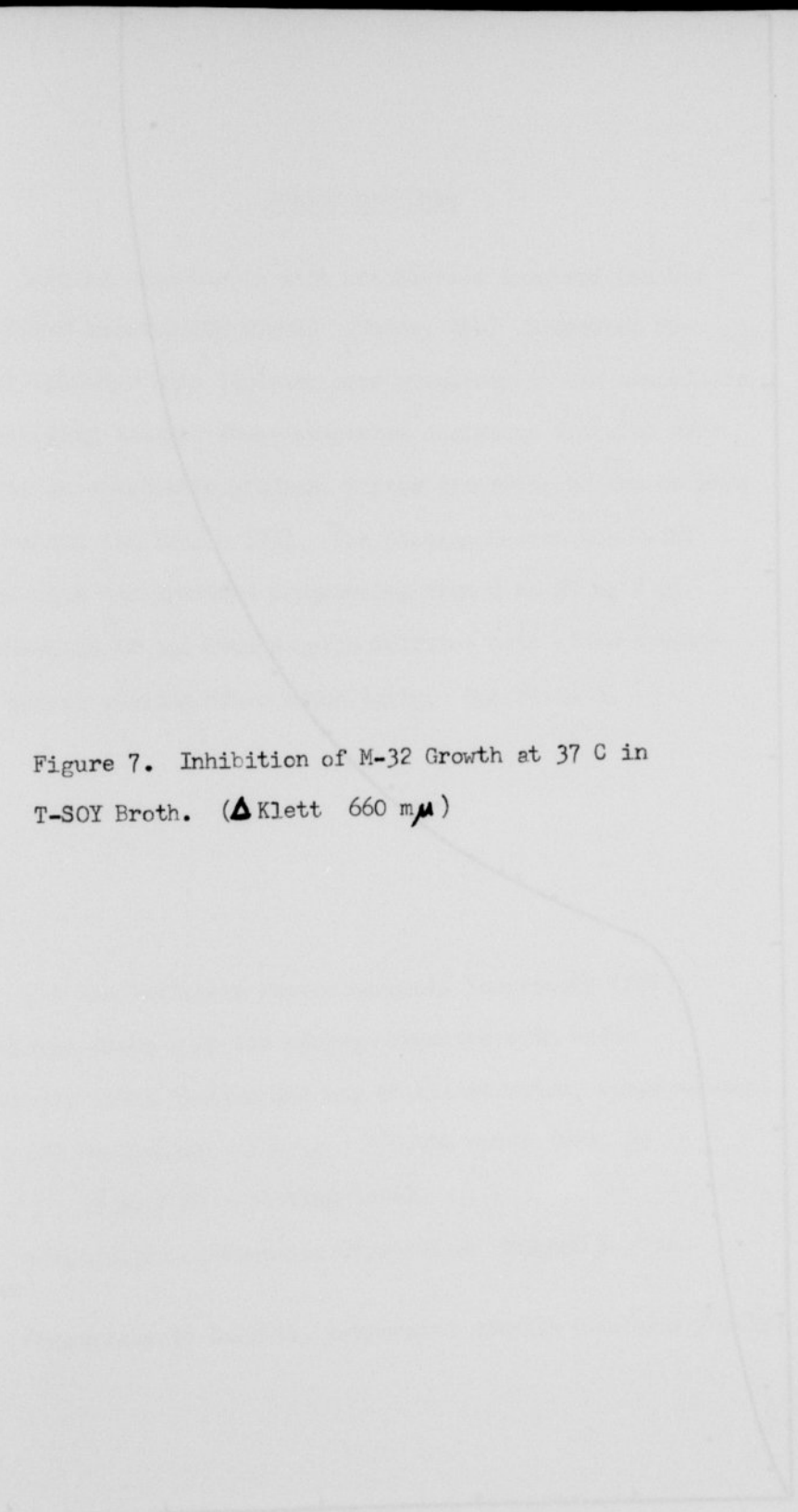
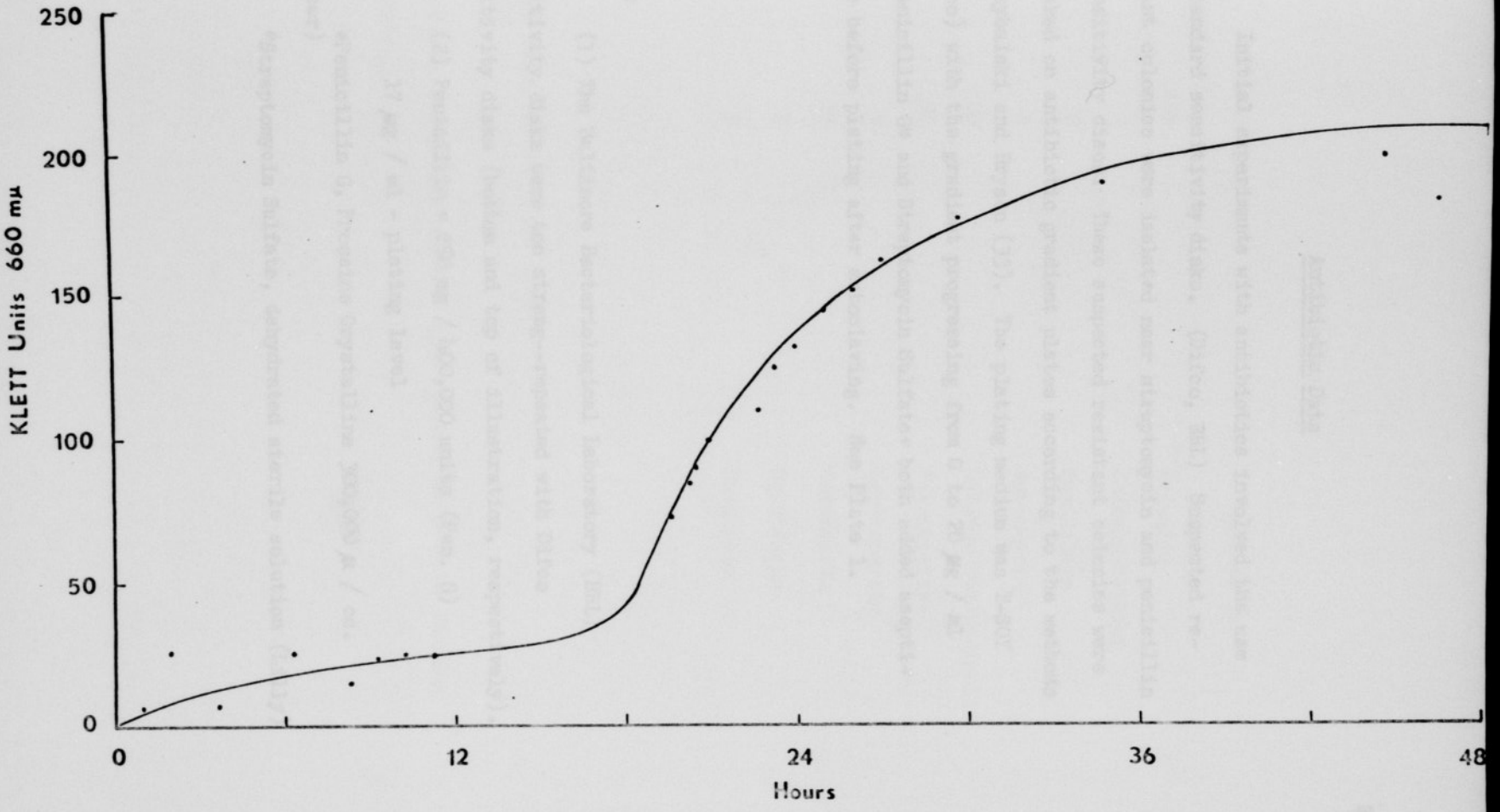


Figure 7. Inhibition of M-32 Growth at 37 C in T-SOY Broth. (Δ Klett 660 mμ)


250
200
150
100
50
0
KLETT Units 660 mμ
0
12
24
36
48
Hours

Antibiotic Data

Initial experiments with antibiotics involved the use of standard sensitivity disks. (Difco, BBL) Suspected resistant colonies were isolated near streptomycin and penicillin G sensitivity discs. These suspected resistant colonies were streaked on antibiotic gradient plates according to the methods of Szybalski and Bryson (33). The plating medium was T-SOY (Difco) with the gradient progressing from 0 to 20 µg / ml of Penicillin G* and Streptomycin Sulfate+ both added aseptically before plating after autoclaving. See Plate 1.

Note:

(1) The Baltimore Bacteriological Laboratory (BBL) sensitivity disks were too strong--repeated with Difco sensitivity disks (bottom and top of illustration, respectively).

(2) Penicillin = 250 mg / 400,000 units (Pen. G)

17 µg / ml - plating level

*Penicillin G, Procaine Crystalline 300,000 µ / cc. (Pfizer)

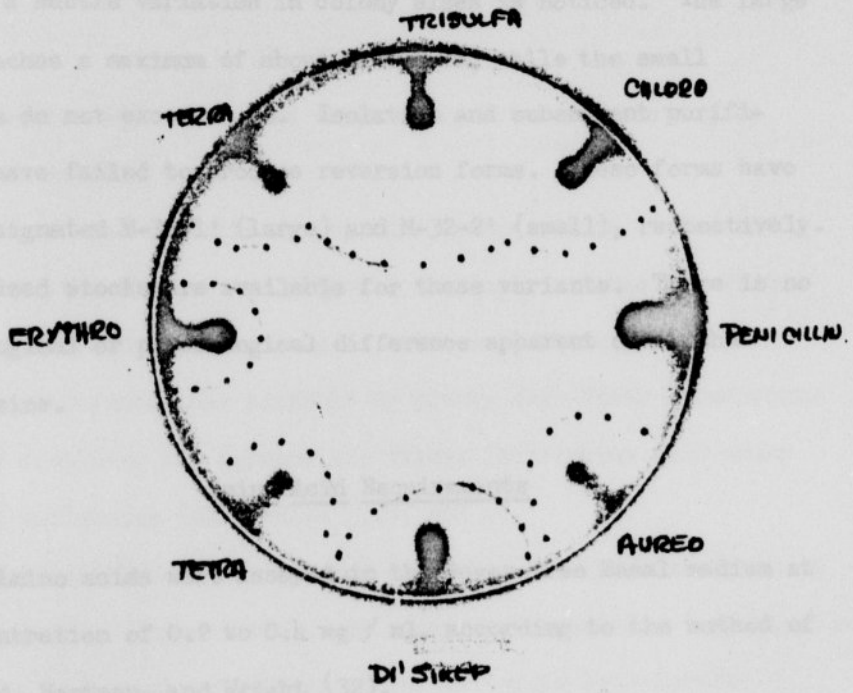
+Streptomycin Sulfate, dehydrated sterile solution (Lilly)

Plate 1

Abbreviation	Antibiotic
SSS	Trisulfa
C	Chloramphenicol
P	Penicillin
A	Aureomycin
DS	Dihydro Streptomycin
TC	Tetracycline
E	Erythromycin
TM	Terramycin
B	Bacitracin
K	Kanamycin

numbers refer to units per disc

M-32 growth inhibition on T-SOY agar (Difco) at 30 C by various antibiotics.



SSS
1.0

P2

K 30

B 10

DS 10

A
30

C 30

Mutants

When M-32 is plated (T-SOY or other suitable media for growth) a subtle variation in colony sizes is noticed. The large size reaches a maximum of about 5 to 6 mm, while the small colonies do not exceed 3 mm. Isolation and subsequent purification have failed to produce reversion forms. These forms have been designated M-32-1' (large) and M-32-2' (small), respectively. Lyophilized stocks are available for these variants. There is no morphological or physiological difference apparent other than colony size.

Amino Acid Requirements

Amino acids were assayed in the Sugar-free Basal medium at a concentration of 0.2 to 0.4 mg / ml. according to the method of Susskind, Hartman, and Wright (32).

Nine pools of seventeen amino acids and three nucleosides were arranged according to the following protocol:

Pool	
1	Adenosine, Histidine, Phenylalanine, Glutamic acid
2	Guanosine, Leucine, Tyrosine, Serine
3	Cysteine, Isoleucine, Tryptophan, Alanine
4	Methionine, Valine, Threonine, Aspartic acid
5	Thymidine, Lysine, Proline, Arginine
6	Adenosine, Guanosine, Cysteine, Methionine

7 Histidine, Leucine, Isoleucine, Valine, Lysine

8 Phenylalanine, Tyrosine, Tryptophan, Threonine, Proline

9 Glutamic acid, Serine, Alanine, Aspartic acid, Arginine

10 None

Sucrose (1%) was added aseptically after autoclaving as the carbon source.

Growth was measured as per cent transmittance at 600 and 420 mμ and the flasks were ranked accordingly. Since flasks 6 through 9 contained the same amino acids as 1 through 5, individual performance was possible by giving each flask a numerical rank and combining the appropriate values for ranking each amino acid and nucleoside (See Tables 2, 3, and 4).

Each amino acid was assayed separately at the same level in the same media. These tables were assayed by measuring transmission at 420 mμ, and compared with an enzymatic casein hydrolysate (Sheffield "N-Z Case").

These amino acids supported the growth of M-32 as well or better than the casein hydrolysate after 60 hours at 30 C.

1 Glutamic acid

2 Leucine

3 Isoleucine

4 Alanine

5 Methionine

6 Valine

7 Aspartic acid*

8 Lysine+

9 Proline*

10 Arginine+

*Effects greater than casein at 36 hours

+Effects greater than casein at 48 hours

Figures 8 - 12.

Certain brands of enzymatic casein hydrolysate do not support growth adequately, probably reflecting a required amino acid deficiency. "N-Z Case" (Sheffield) has proved most satisfactory for all purposes at a level of a gram per liter. Ammonium and nitrate salts, were not a sufficient nitrogen source.

Table 2. Protocol for Amino Acid Pool Assay (32).

Pool	1	2	3	4	5
6	Adenosine	Guanosine	Cysteine	Methionine	Thymidine
7	Histidine	Leucine	Isoleucine	Valine	Lysine
8	Phenylalanine	Tyrosine	Tryptophan	Threonine	Proline
9	Glutamic acid	Serine	Alanine	Aspartic acid	Arginine
10	--	--	--	--	--

Table 3. Growth of M-32 in Amino Acid Pool Assay after 12 hours at 30 C.

Results		+++	+	+++	+	+
	Pool	1	2	3	4	5
+	6	4	2	4	2	2
++	7	5	3	5	3	3
++	8	5	3	5	3	3
+++	9	6	4	6	4	4
-	10	-	-	-	-	-

Legend:

- No visually detectable growth

+ Visibly detectable growth

++ Light growth (transparent)

+++ Moderate turbidity (translucent)

++++ Heavy turbidity (barely translucent)

Table 4. Growth of M-32 in Amino Acid Pool Assay after 100 hours at 30 C.

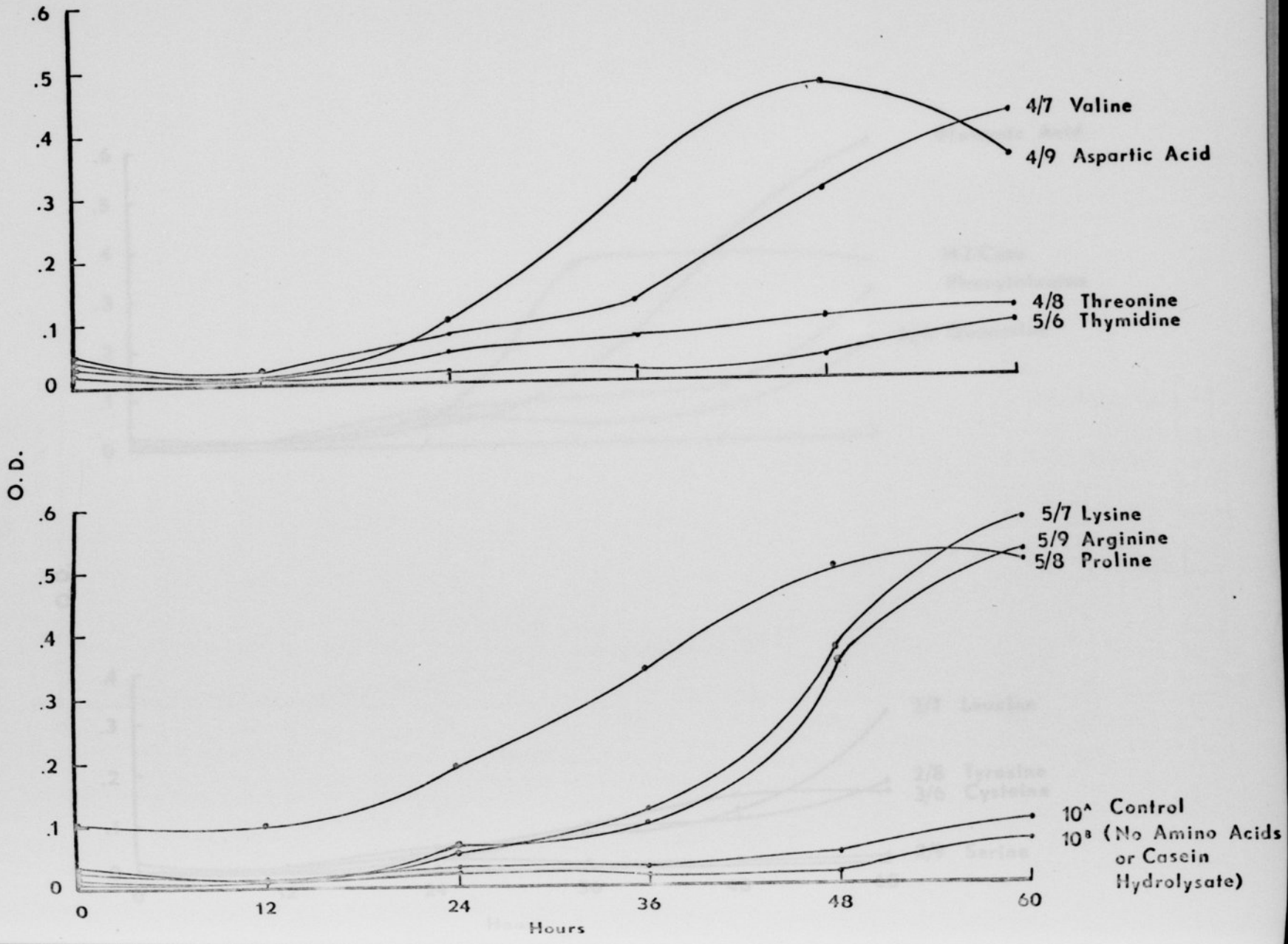
Pool		1	2	3	4	5
	Result	+++	-	+++	++	+++
6	++	5	2	5	4	5
7	++++	7	4	7	6	7
8	++++	7	4	7	6	7
9	+++	6	3	6	5	6
10	-	-	-	-	-	-

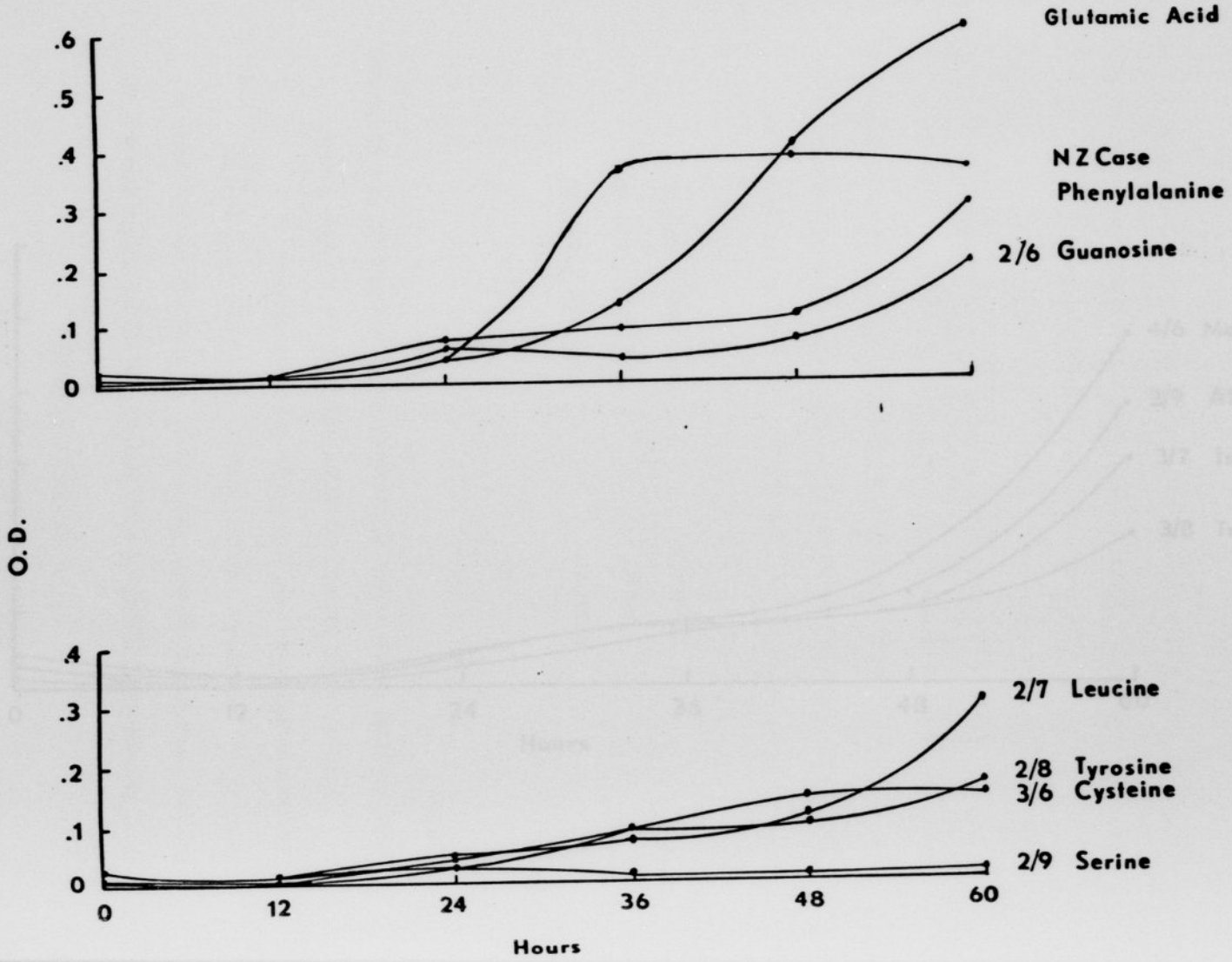
Assay:
From % transmittance at 420 and at 600 mμ

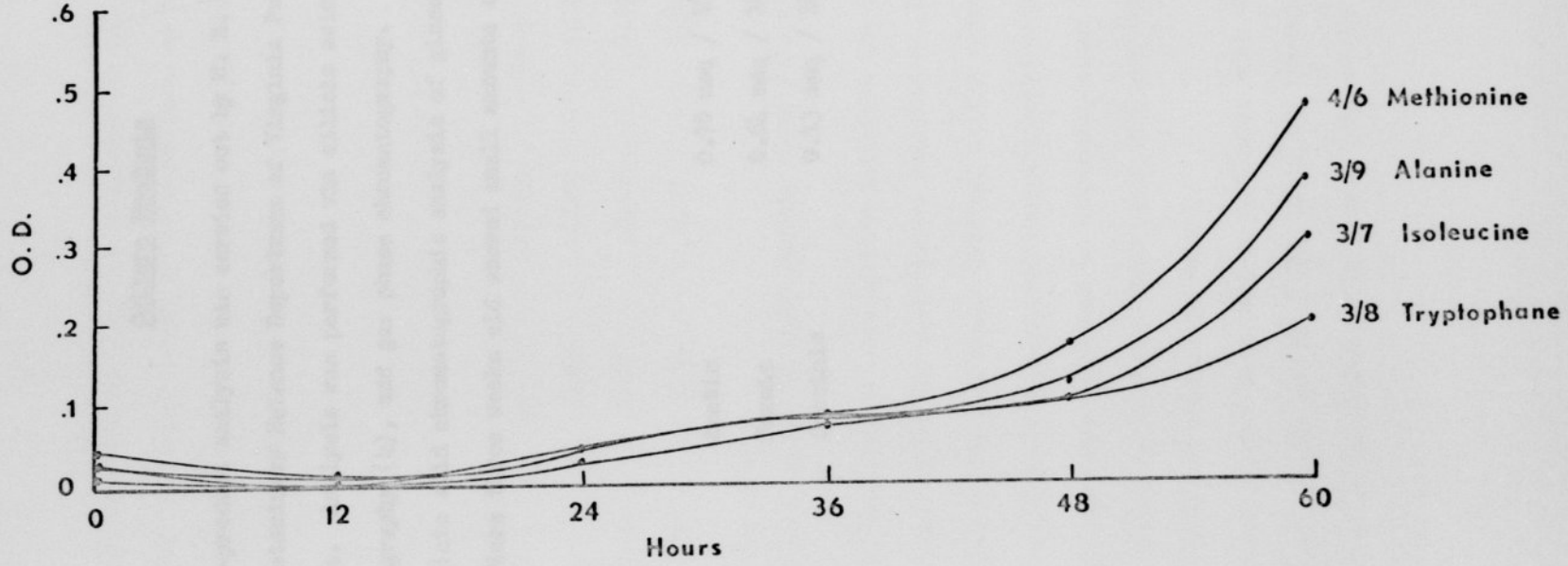
Legend:

- > 40% transmittance
+ 30 to 40% transmittance
++ 25 to 30% transmittance
+++ 20 to 25% transmittance
++++ < 20% transmittance

Figures 8, 9, 10. Growth response of M-32 in Amino Acid Assay Medium * expressed as optical density (600 mμ). Each curve represents a single culture.


.6
.5
.4
.3
.2
.1
0
O.D.
4/7 Valine
4/9 Aspartic Acid
4/8 Threonine
5/6 Thymidine
5/7 Lysine
5/9 Arginine
5/8 Proline
10^A Control
10^B (No Amino Acids or Casein Hydrolysate)
0
12
24
36
48
60
Hours


Glutamic Acid
NZ Case
Phenylalanine
2/6 Guanosine
2/7 Leucine
2/8 Tyrosine
3/6 Cysteine
2/9 Serine
O.D.
.6
.5
.4
.3
.2
.1
0
.4
.3
.2
.1
0
0
12
24
36
48
60
Hours


.6
.5
.4
.3
.2
.1
0
O. D.
0
12
24
36
48
60
Hours
4/6 Methionine
3/9 Alanine
3/7 Isoleucine
3/8 Tryptophane

Culture Products

End-product analysis was carried out by W. E. C. Moore of the Veterinary Science Department of Virginia Polytechnic Institute. Analysis was performed via silicic acid column chromatography (4), and gas phase chromatography.

Silicic acid chromatographic analysis of glucose broth M-32 culture three weeks old showed small amounts of the following acids:

Acetic	0.78 meq / 100 ml
Formic	0.64 meq / 100 ml
Succinic	0.13 meq / 100 ml

More interesting from the standpoint of identification are the changes in concentration of these products as the culture ages. Cultures of three days and 16 days were analyzed and compared to sterile T-SOY media on which M-32 was grown. This data appears below.

Uninoculated Broth	Acetic	Formic	Succinic	Lactic
	0.04	0.00	0.11	0.13
+ 3 days	0.27	0.22	0.16	0.00
+16 days	0.60	0.65	0.15	0.00

Values are meq / 100 ml above amounts found before inoculation.

For analysis of carbohydrate cellulose degradation products from culture fluids the quantitative analysis of oligosaccharide polymer series was followed. Charcoal celite columns coated with stearic acid adsorb oligosaccharides quantitatively from water and the sugars can then be eluted in increasing order of degree of polymerization by selective desorption with gradually increasing concentrations of ethanol in water.

The procedure used here is basically that of Miller (22) as modified by Cole and King (5) using the phenol-sulfuric acid analysis for sugar described by Timell (24) as the detecting system.

A 50 ml culture of M-32 on Dubos media (16) with Avicel (American Viscose Corporation) as carbon source grown for six days was harvested by centrifugation and the supernatant filtered through a 0.45 μ millipore filter. The filtrate was boiled 15 minutes to denature proteins. One milliliter of this preparation contained 850γ of soluble carbohydrate. (Phenol-sulfuric assay) A sample of 0.4 ml was applied to the Miller column and elution begun. Two hundred 0.5 ml samples were collected and dried. A single peak was evident in tubes one through six, where glucose elutes from the Miller column, with the fourth as the peak tube. These aliquots were negative for glucose when assayed by the enzymatic determination (Notatin) of glucose (29).

Even a sample of supernatant was negative for glucose when assayed by the Notatin procedure. The nature of the carbohydrate present in the culture supernatant as a result of the solubilization of Avicel remains a question.

Carbohydrate Growth Data

Several attempts were made to determine the growth of M-32 on various carbohydrates. In the initial studies, 10% stock solutions were prepared and frozen. The media was Sugar-free Basal, which is carbohydrate-free. Each sugar was added via filter sterilization to sterile assay tubes in order to prevent the usual interactions that occur during autoclaving.

Transmittance measurements were taken daily at 600 mμ. The control was inoculated broth containing water added instead of carbohydrates. The sugar level in the assay was 1% (see Table 5).

Final readings were obtained on 72 hour cultures incubated at 30 C.

A more diverse study was carried out auxanographically on Sugar-free Basal plus agar with a few crystals of four carbohydrates added on each plate of seeded agar (see Table 6).

An attempt was made to determine whether a disaccharide preference described by Hulcher (16) for Cellvibrio gilvus existed in M-32. Sterile 10% glucose and cellobiose were added to sterile Sugar-free Basal Medium to a final 1% carbohydrate in 500 ml culture flasks. These flasks had specially designed sidearms permitting Klett-Summerson colorimeter assay of turbidity during growth. Two flasks contained cellobiose only, while glucose was included in two more. Finally, two flasks were included that contained T-SOY medium. The inoculum was obtained from cultures that had been maintained on cellobiose and glucose (1%) and Sugar-free Basal. A series of 10 sequential transfers had been made over a period of a month without changing the carbon source. Each 200 ml culture was inoculated with 0.5 ml of 48 hour M-32 culture maintained in this manner.

Two groups of cultures were inoculated as follows:

Cellobiose Culture Inoculum--
Cellobiose A, Glucose A, T-SOY A

Glucose Culture Inoculum--
Cellobiose B, Glucose B, T-SOY B

A comparison of the growth on cellobiose and glucose appears in Figure 11. The T-SOY cultures were included to demonstrate the equivalence of the culture inoculum.

Apparently there is no disaccharide preference as demonstrated by Hulcher (17) with Cellvibrio gilvus under these conditions. Continual transfer on glucose or cellobiose makes little if any difference when glucose is substituted for cellobiose and vice versa. This may reflect the reported constitutive nature of cellulolytic enzymes in Cellulomonas flavigena as reported by Hammerstrom, et al. (14). There seems to be a slight indication of higher turbidity at stationary phase of those cultures inoculated from cultures maintained on glucose. To verify the constitutive nature of the cellulolytic enzymes of M-32 would clearly require a more comprehensive study.

Table 5. Carbohydrate growth stimulation of M-32 at 30 C of 1% sugar levels.

	Carbohydrate Assay				
1	Arabinose	-	16	Melezitose	-
2	Cellobiose	++++	17	Melibiose	+
3	Dextrin	-	18	Raffinose	-
4	Dulcitol	-	19	Rhamnose	-
5	Esculin	++++	20	Ribose	-
6	Fructose	+	21	Salicin	+++
7	Galactose	++++	22	Sorbitol	++
8	Glucose	+++	23	Sorbose	-
9	Glycerol	+	24	Starch	+
10	Inositol	-	25	Sucrose	++++
11	Inulin	+	26	Trehalose	++++
12	Lactose	++	27	Xylose	+++
13	Maltose	++++	28	C.M.C.-7-LP	+
14	Mannitol	-	29	Avicel	+
15	Mannose	++	30	H_2O (control)	-

Assay: 4.5 ml Sugar-free Basal broth
0.5 ml sterile 10% carbohydrate
(triplicate tubes, readings above 72 hours)

Legend: % Transmittance (600 m)

- < 5%
+ < 25%
++ < 50%
+++ < 75%
++++ > 75%

Table 6. Auxanographic growth stimulation of M-32 on Sugar-free Basal* Agar at 30 C after seven days.

		Auxanography Results	
	SUGAR	DENSITY	SIZE
1	Salicin	-	-
2	Laminaribiose	tr	-
3	D-Ribose	-	-
4	Glucocapparin	-	-
5	Methyl-β-L-Arabinose	-	-
6	Gentiobiose	-	-
7	Amygdalin	-	-
8	L-arabinose	tr	-
9	D-Galactose	++	+++
10	β-Gentiobiose Octaacetate	+++	+
11	Melibiose	-	-
12	Cellobiose	++	+++
13	D(+)-Trehalose	+	++
14	Arbutin	-	-
15	Cellotriose	++	++
16	L-Glucose	tr	-
17	C.M.C.-7-LP	++	++
18	D-Mannose	++	+++ (++INH)
19	D-Maltose	tr	-
20	Esculin	-	-
21	Glucose	tr	-
22	Raffinose	-	-

(con'd)

Auxanography Results (con'd)

	SUGAR	DENSITY	SIZE
23	L-Sorbose	-	-
24	Lactose	+	++++
25	D-Gluconate	tr	-
26	D-Xylose	+	++++
27	D-Fructose	tr	-
28	D(-) Mannitol	-	-
29	Sucrose	++	++++
30	Saccharose	-	-
31	Glycogen	++++	+
32	Dextrin	++++	++++
33	Starch	-	-
34	Dulcitol	-	-
35	Inulin	-	-
36	i-Inositol	-	-
37	Creatinine	-	-
38	D-Lyxose	tr	-
39	L-(+)Rhamnose	-	-
40	Turanose	tr	-
41	D(+) Melezitose	-	-
42	D-Arabinose	-	-
43	T-SOY	++	++

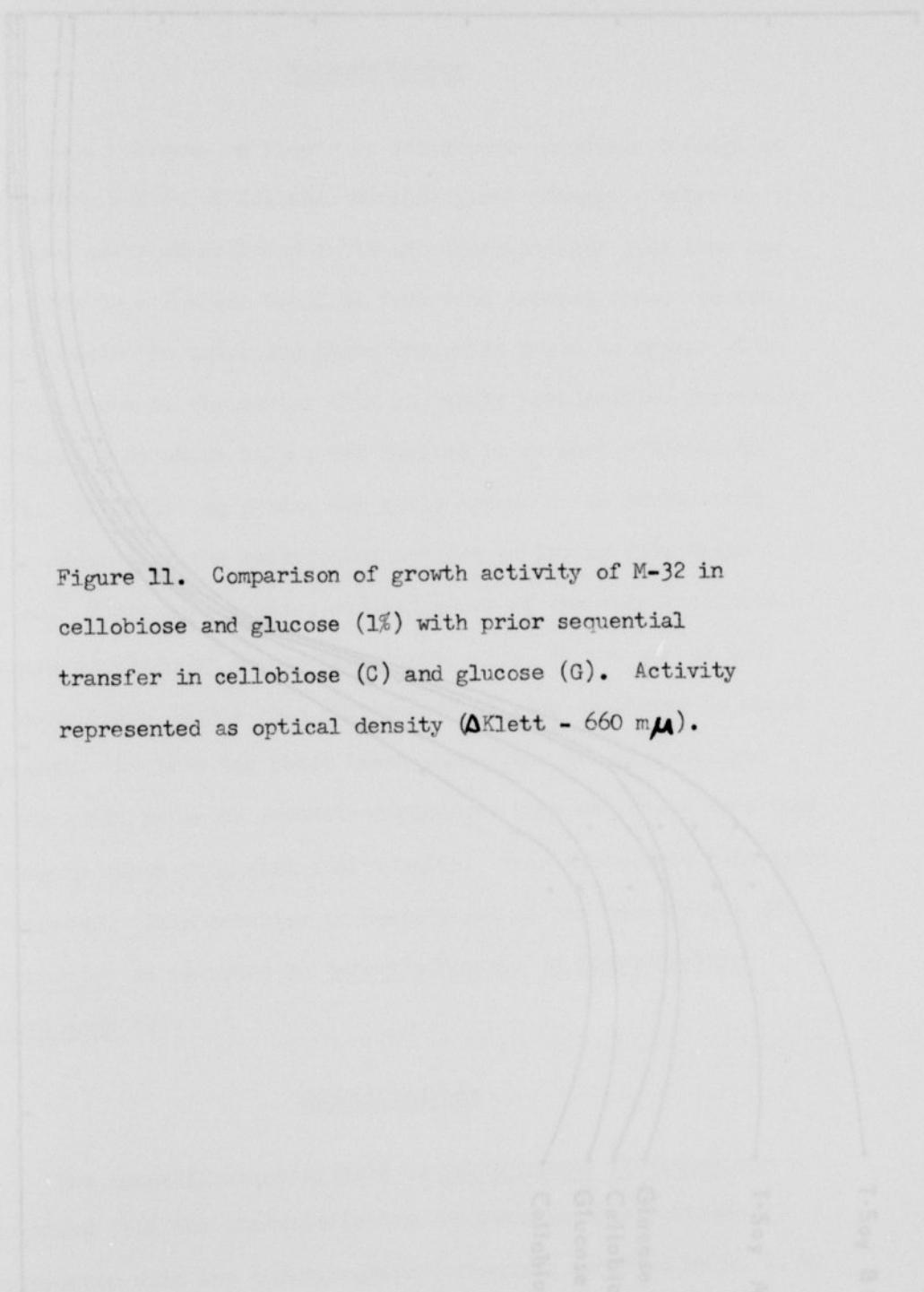
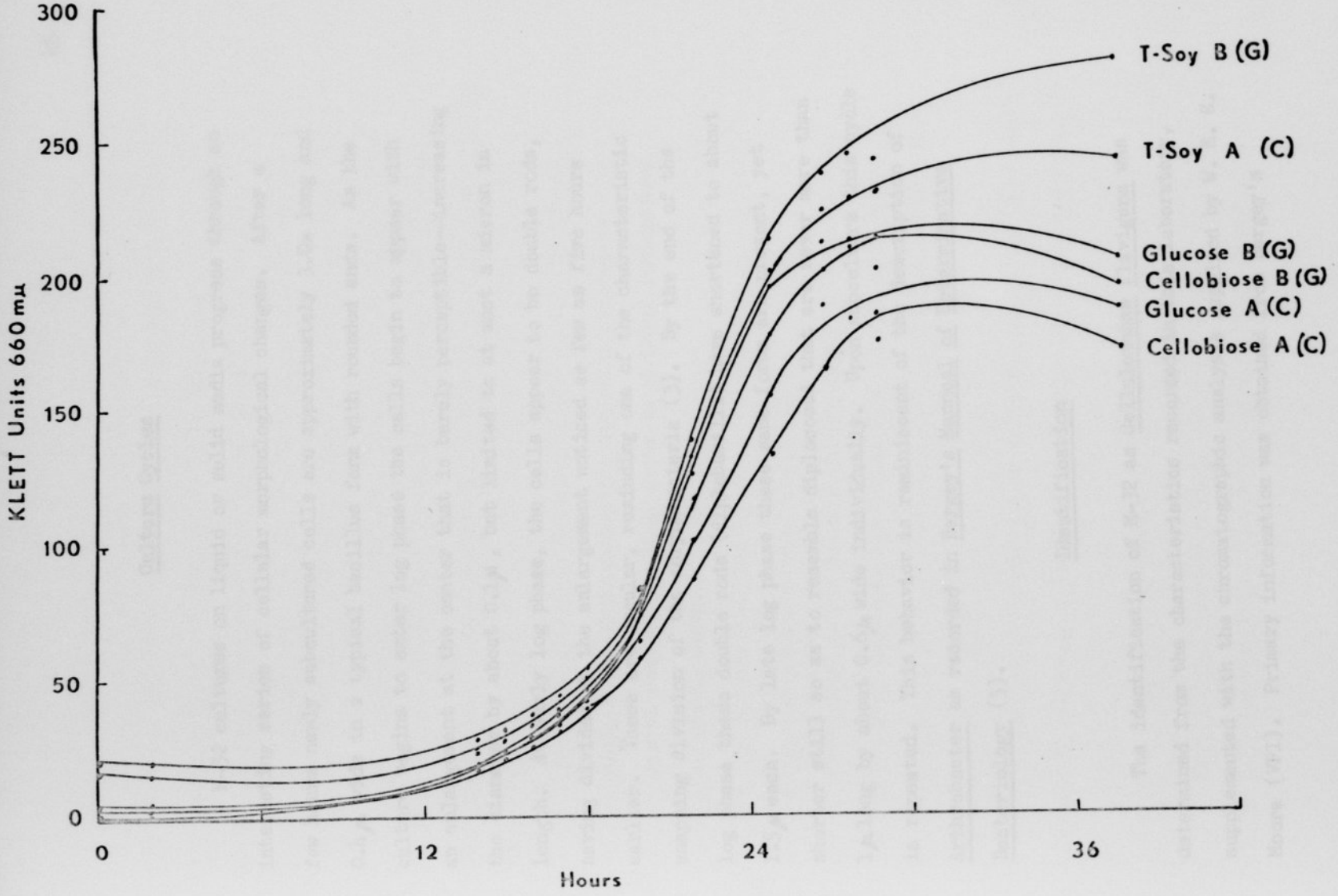


Figure 11. Comparison of growth activity of M-32 in cellobiose and glucose (1%) with prior sequential transfer in cellobiose (C) and glucose (G). Activity represented as optical density (ΔKlett - 660 mμ).


300
250
200
150
100
50
0
KLETT Units 660 mμ
0
12
24
36
Hours
T-Soy B (G)
T-Soy A (C)
Glucose B (G)
Cellobiose B (G)
Glucose A (C)
Cellobiose A (C)

Culture Cycles

M-32 cultures on liquid or solid media progress through an interesting series of cellular morphological changes. After a few hours newly subcultured cells are approximately 3.0μ long and 0.4μ wide in a typical bacillus form with rounded ends. As the culture begins to enter log phase the cells begin to appear with an enlargement at the center that is barely perceptible--increasing the diameter by about 0.1μ, but limited to at most a micron in length. At early log phase, the cells appear to be double rods, having divided at the enlargement noticed as few as five hours earlier. These are angular, reminding one of the characteristic snapping division of the corynebacteria (3). By the end of the log phase these double rods (diplobacilli) have shortened to about 1.5μ each. By late log phase these same forms are present, yet shorter still so as to resemble diplococci that are never more than 1μ long by about 0.6μ wide individually. Upon subculture this cycle is repeated. This behavior is reminiscent of the description of Arthrobacter as recorded in Bergey's Mannual of Determinative Bacteriology (3).

Identification

The identification of M-32 as Cellulomonas flavigena was determined from the characteristics recorded in this laboratory, supplemented with the chromatographic analysis supplied by W. E. C. Moore (VPI). Primary information was obtained from Bergey's

Plate 2 - M-32 morphological variation related to growth stages. Drawings refer to numbered indications on growth curve.

Manual (3). Fermentation products place this organism in the Corynebacteriaceae according to W. E. C. Moore (Personal Communication). Its ability to decompose cellulose exempts M-32 from Arthrobacter, yet its characteristic cultural changes are identical to those of Arthrobacter.

Microbacterium is eliminated since M-32 was isolated from soil and lacked lactic acid formation. Apparent lack of animal and plant pathogenicity eliminates the rest of the Corynebacteriaceae except Cellulomonas.

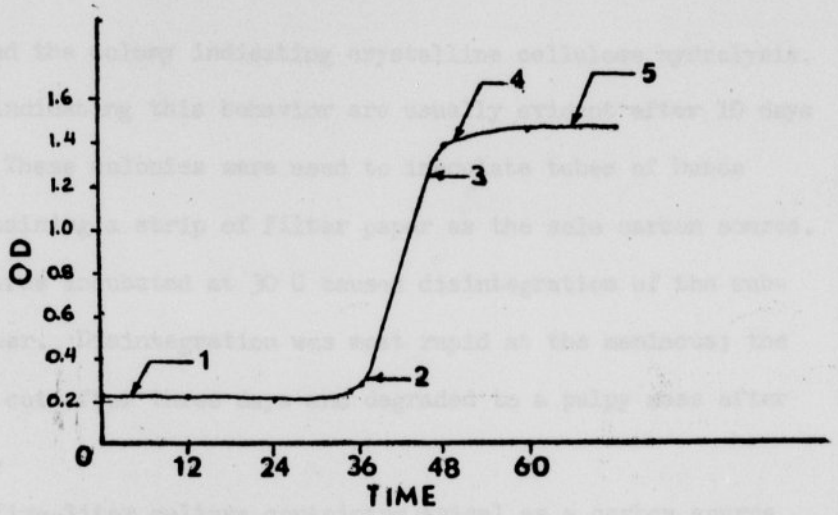
Since M-32 is not motile and ferments xylose, C. biazotea, C. cellasea, C. aurogena, and C. pusilla are eliminated. Production of nitrites from nitrates eliminates C. galba, C. gelida, and C. acidula. Motility eliminates C. fimi. C. uda is eliminated since it does not produce a yellow pigment. Cellulomonas flavigena fits all the necessary criteria for identification.

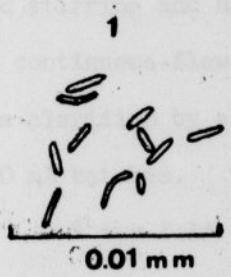
Differences in the description according to Bergey include the formation of small amounts of ammonia, gelatin hydrolysis (M-32 is negative for this characteristic), and a consistent gram-positive staining reaction.

Examination of literature of Kellerman and McBeth (19), the original description, failed to uncover signficant discrepancies.

Cellulolytic Activity

Cellulolytic activity of M-32 was first evident on plates of the modified Fuller and Norman medium (10) by producing a clear

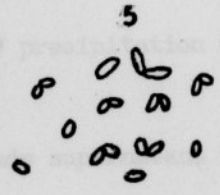

OD
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
12
24
36
48
60
TIME
1
2
3
4
5


1
0.01 mm


2


3


4


5

zone around the colony indicating crystalline cellulose hydrolysis. Colonies indicating this behavior are usually evident after 10 days at 30 C. These colonies were used to inoculate tubes of Dubos broth containing a strip of filter paper as the sole carbon source. M-32 cultures incubated at 30 C caused disintegration of the submerged paper. Disintegration was most rapid at the meniscus; the paper was cut after three days and degraded to a pulpy mass after five days.

A five-liter culture containing Avicel as a carbon source in Sugar-free Basal was grown at room temperature with aeration and stirring and harvested after five days. Harvest was effected by continuous-flow high-speech centrifugation. The supernatant was clarified by a second continuous flow ran and then frozen in 100 ml batches.

A two-fold concentration was carried out by slowly freezing the crude supernatant. Hydrocellulase (C_1) (9) activity of this preparation was double that of the crude supernatant when assayed by the method of Flora (9) exhibiting an activity of 1 unit / ml of the two fold concentrate.

Other concentration methods were attempted. A 50% acetone fractionation of the supernatant showed a small increase in activity while lyophilization seemed to destroy all activity. Ammonium sulfate (50%) precipitation and a 50 to 75% cut produced no activity.

Dialysis of crude supernatant in Carbowax 20M (Union Carbide)

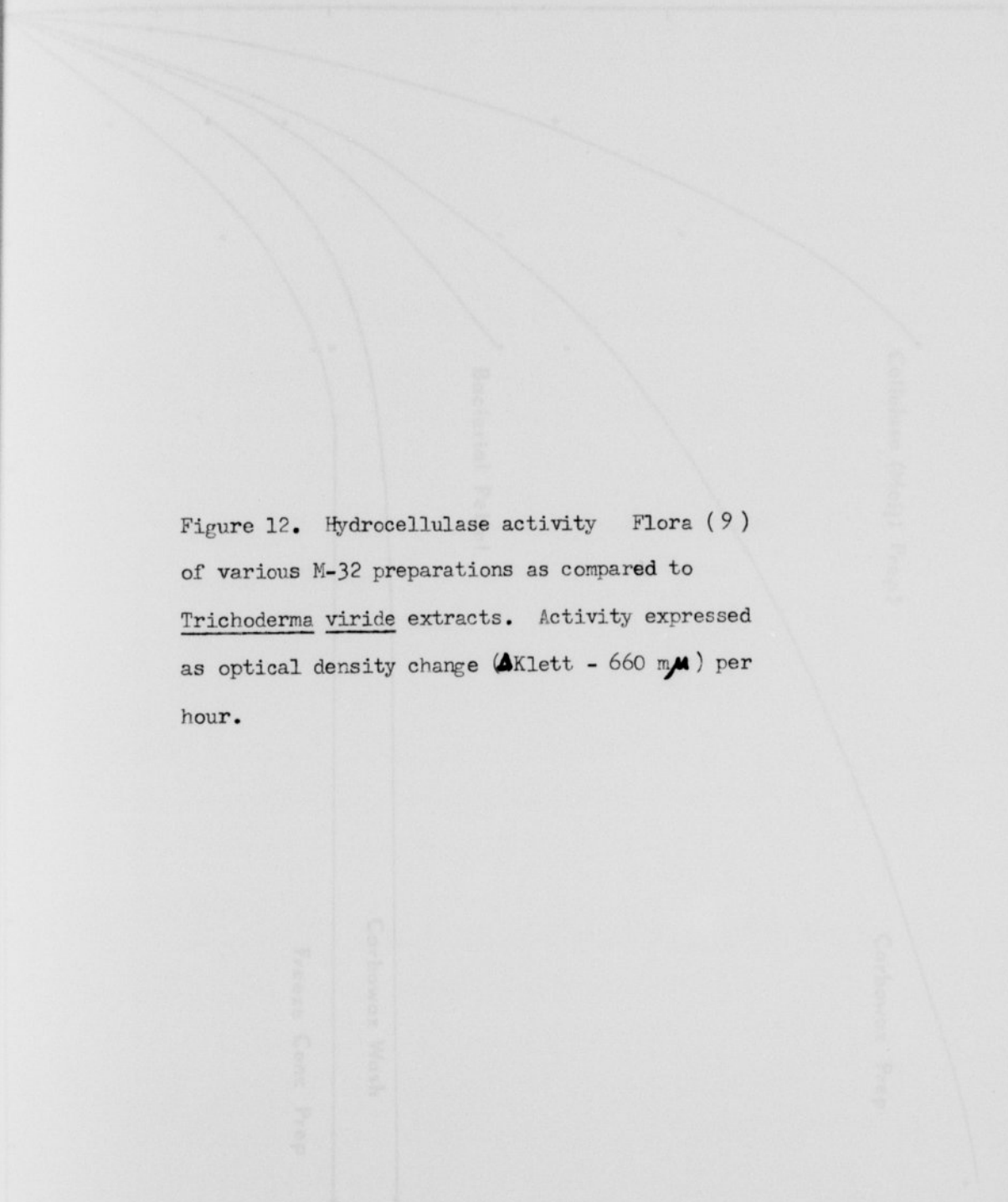
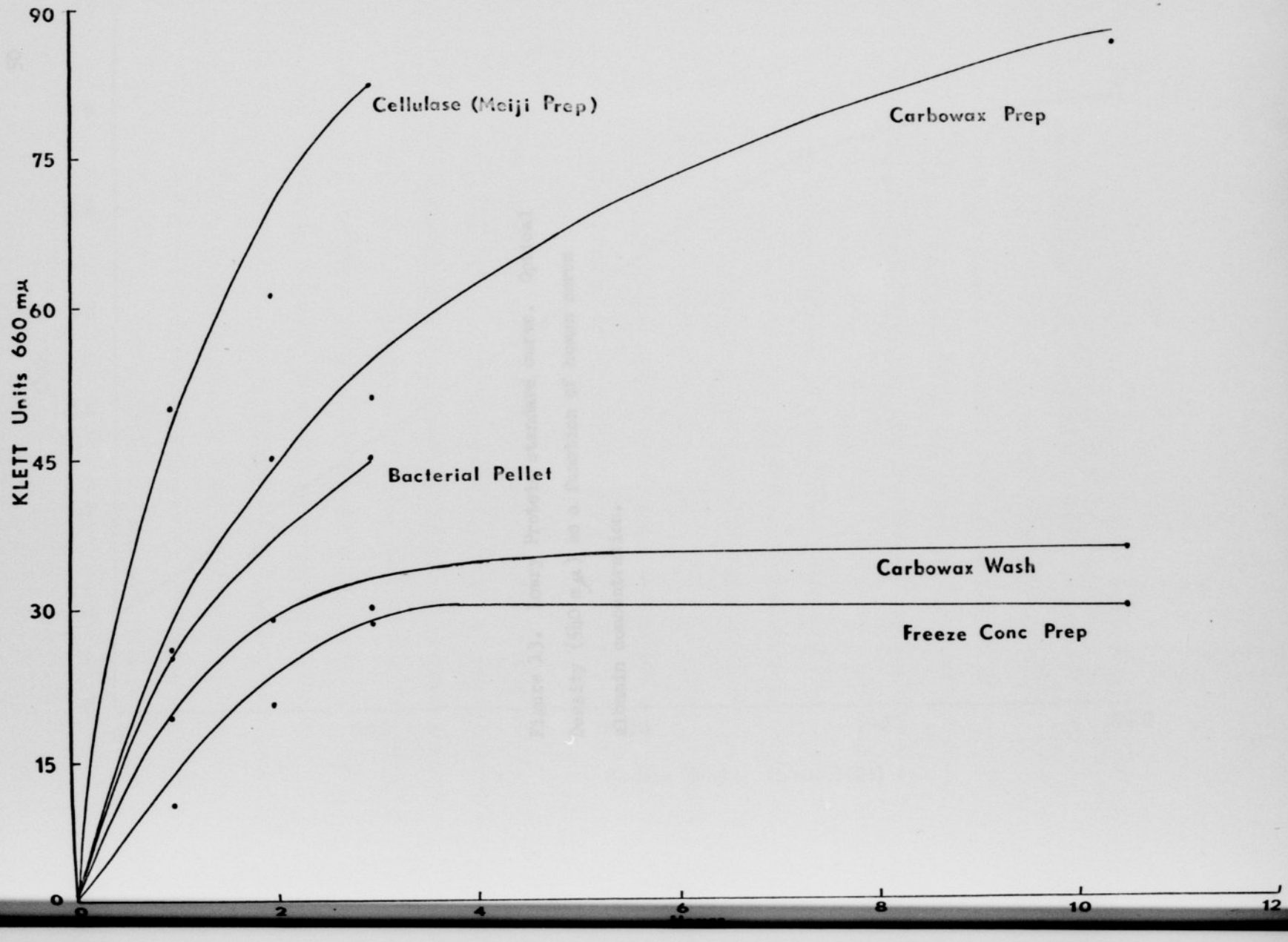


Figure 12. Hydrocellulase activity Flora (9) of various M-32 preparations as compared to Trichoderma viride extracts. Activity expressed as optical density change (ΔKlett - 660 mμ) per hour.


90
75
60
45
30
15
0
KLETT Units 660 mμ
Cellulase (Meiji Prep)
Carbowax Prep
Bacterial Pellet
Carbowax Wash
Freeze Conc Prep
0
2
4
6
8
10
12

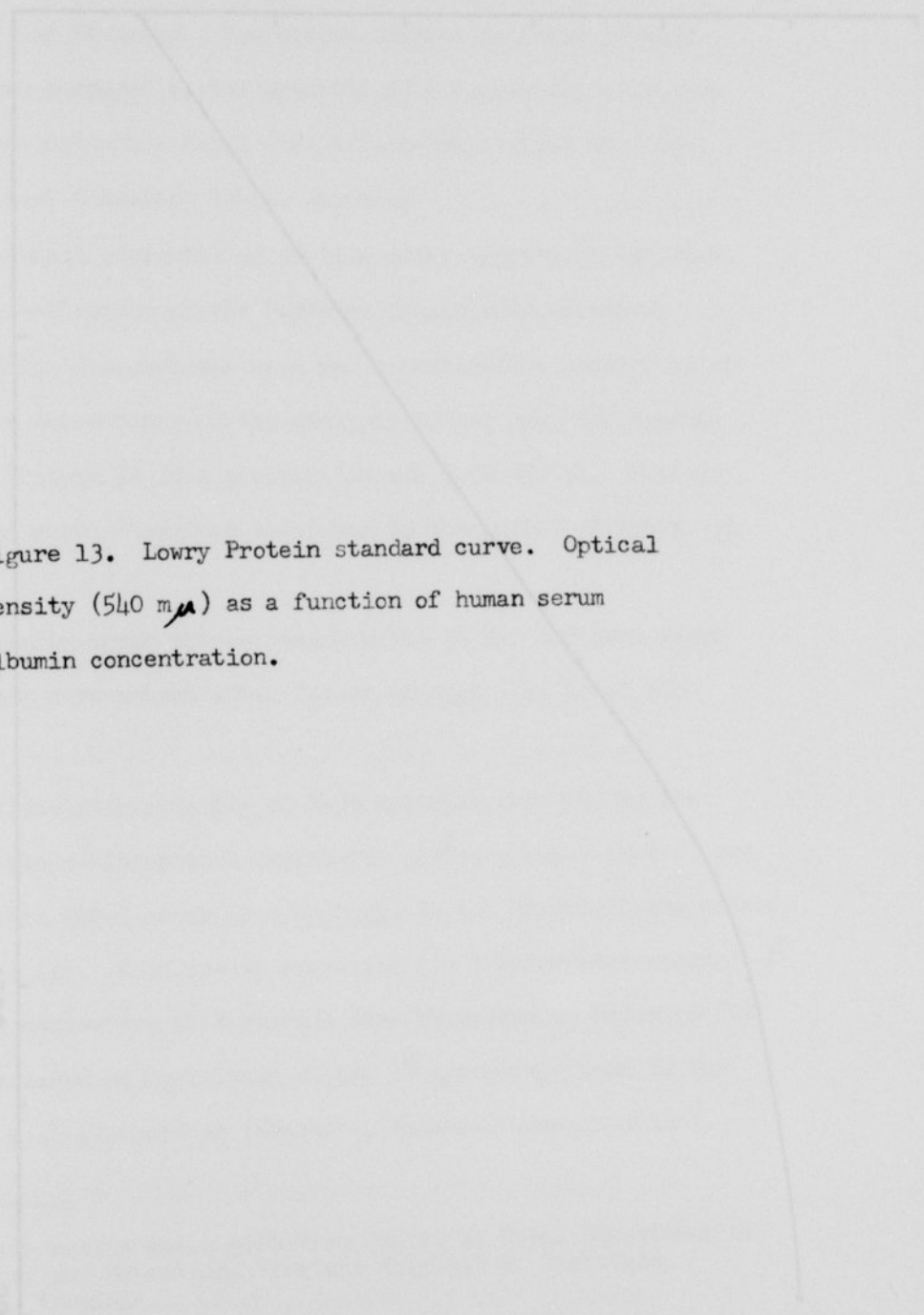
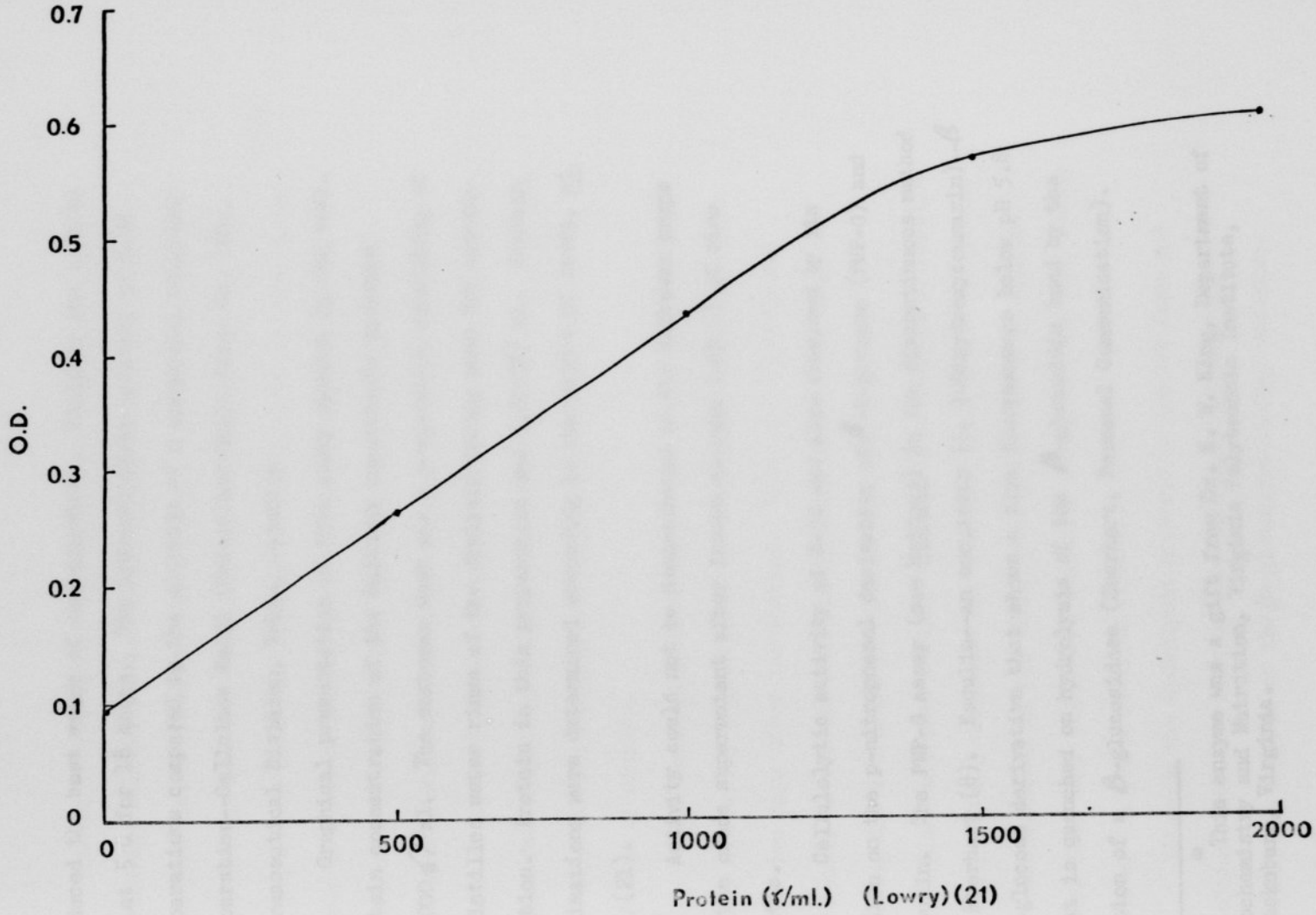


Figure 13. Lowry Protein standard curve. Optical Density (540 mμ) as a function of human serum albumin concentration.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D.
0
500
1000
1500
2000
Protein (γ/ml.) (Lowry) (21)

produced the best means of concentration. Dialysis was carried out at 5 C for 18 hours. The hydrocellulase activity of M-32 preparations compared to the activity of a commercial cellulase preparation--Cellulase Meiji (Meicellase-Meiji Seika Co. Ltd., Pharmaceutical Division, Tokyo, Japan).*

Graphical presentation of this assay appears in the text. Protein concentration of the Carbowax concentrate exceeded 10,000 γ/ ml. The carbowax wash was a preparation consisting of a distilled water rinse of the dialysis tubing used for concentration. Protein in this preparation was 5,000 γ/ ml. Protein estimations were determined according to the method of Lowry, et al. (21).

Activity could not be demonstrated in the carbowax preps or the crude supernatant after frozen storage (-20 C) of nine months.

Cellulolytic activity of M-32 was also observed by its action on the p-nitrophenol derivative of β-D-glucose (PNP-G) and esculin. The PNP-G assay (see Methods) is the discontinuous method of Eberhart (8). Esculin--an esculetin (6, 7-Dihydroxycoumarin)--β-D-glucose derivative that shows a blue fluorescence below pH 5.8 that is quenched on hydrolysis of the β-glucosidic bond by the action of a β-glucosidase (Eberhart, Personal Communication).

---

* This enzyme was a gift from Dr. K. W. King, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia.

Plates of Sugar-free Basal Agar with suspended microcrystalline cellulose were inoculated with M-32. After clear hydrolysis zones appeared around isolated colonies the plate was overlaid with a dilute solution of PNP-G (1 mg / ml) which was poured off after two minutes. Colonies and the clear zone around them became colored yellow after fifteen minutes due to the release of p-nitrophenol (1). Addition of "Tris" (tris-hydroxymethylaminomethane) 1.0 M brought the p-nitrophenol to the pH (about pH 8) where the yellow color was more intense and stopped the enzymatic action. Plate cultures that are more than four days old are completely yellow upon treatment due to the diffusion of enzymes.

Plates containing PNP-G in the media at a level of 0.5 mg / 5 ml display a development of a similar yellow color that begins two days after inoculation. This color spreads and eventually covers the whole plate after four to five days reflecting the diffusion of PNP-Gase activity.

Plate cultures sprayed with a dilute solution of esculin (0.5 g / 100 ml) displayed the characteristic blue fluorescence that was quenched in the vicinity of growth after 15 minutes. Plates four days old showed quenching in all but the most remote areas of the plate after 15 minutes.

Although the hydrolysis products of the PNP-Gase and esculinase activity were not isolated and identified, they are probably the results of the β-glucosidic linkage scission.

In other experiments PNP-G assay was carried out on cultures grown up on McBee minimal media (Methods) containing Avicel, glucose, or cellulose. Biuret (13) determination of protein was made simultaneously. Culture and supernatant activities were determined. Cultures were sedimented by centrifugation at 50,000 x G for 15 minutes.

Notatin assay (29) of the preceeding supernatants (except glucose) showed glucose to be present in detectable amounts. Although the outcome of the experiment was measured only visually, it demonstrates that glucose was produced in the utilization of these substrates, probably as a hydrolytic product.

V. SUMMARY

By following the routine tests for the identification of bacteria it was possible to classify the M-32 culture obtained from V. P. I. as Cellulomonas flavigena. This identification was facilitated by the data collected by Dr. W. E. C. Moore of V. P. I. The characteristics of M-32 were not without discrepancy as several minor differences became apparent. This variability is normal in well established cultures and this characterization lies within reasonable limits.

The establishment of a defined medium is probably the most signficant finding of this research. While the requirements of M-32 are now chemically defined assurance of the reliability of such a medium would require thorough and exhaustive testing by repeated transfer.

The formula for this chemically defined media would be that of Sugar-free Basal supplied with any of the adequate carbohydrates found in the carbohydrate section, Substitution of those stimulatory amino acids comparable to the casein hydrolysate should be an adequate chemically defined medium. Probably a combination of these amino acids would develop the best results.

While Garrison and Harris (11) have extensively described the proteolytic activity of Cellulomonas their work also presents comprehensive information regarding adequate carbon sources for Cellulomonas flavigena.

Also unique to this thesis is the description of the existence of a cyclic cultural plemorphism. A cyclic structural variation that according to Breed, et al. (3) takes place in members of the genus Arthrobacter is not a true pleomorphism. The presence of this phenomenon among members of Cellulomonas is not particularly surprising in that the main differentiating characteristic between the two genera is that Cellulomonas possesses the ability to digest cellulose. There are lesser distinguishing characteristics, but the overall classification is probably in need of review as these findings suggest.

A general description of the cellulolytic activity of M-32 was carried out in this study. Other than the discovery of decomposition of several diverse cellulosic substrates, little specific information was gathered. Degradation of hydrocellulose, CMC, PNP-G, esculin, cellobiose, and glucose seems to indicate the presence of a complete cellulolytic system as described by any of the theoretical pathways for the decomposition of native cellulose. Due to the nature of the assays for cellulose decomposition the presence of an extracellular enzyme is suggested.

Although exhaustive studies were not made, the evidence presented in this thesis supports the constitutive nature of the cellulases of this species as described by Hammerstrom and co-workers (14).

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VIII. APPENDICES

APPENDIX I

MEDIA

Sugar-free Basal

KNO_3	2.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$Fe_2(SO_4)_3 \cdot 7H_2O$	tr
K_2HPO_4	1.2 g
KH_2PO_4	0.14 g
N-Z Case	1 g
Vitamin stock*	10 ml
Water	1 liter

*See page 63.

Vitamin Stock (10 ml / liter)

(Eberhart, Personal Communication)

B_1 Thiamin (HCl or HNO_3)	100 mg
B_2 Riboflavin	30 mg
B_6 Pyridoxine	75 mg
Ca Pantothenate	200 mg
Para Amino Benzoic Acid	5 mg
Nicotinamide	75 mg
Choline, HCl	200 mg
Folic Acid	1 mg
Biotin	50
Inositol	1 g

Made up in 1 liter 50% Ethanol

Trace Elements Solution

(Eberhart, Personal Communication)

Citric Acid·$1H_2O$	5.0 g
$ZnSO_4 \cdot 7H_2O$	5.0 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	1.0 g
$CuSO_4 \cdot 5H_2O$	0.25g
$MnSO_4 \cdot 1H_2O$	0.05g
H_3BO_3(Anhydrous)	0.05g
$Na_2MoO_4 \cdot 2H_2O$	0.05g

Made in 95 ml distilled H_2O with 0.05 ml $CHCl_3$ added as preservative

Tryptic Soy Broth

(Difco T-SOY)

Bacto Tryptone (Pancreatic Digest of Casein USP)	17.0 g
Bacto Soytone (Soy Bean Peptone)	3.0 g
Bacto Dextrose	2.5 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Bacto agar (for solid media only)	20.0 g
H_2O (Distilled)	1 liter

Final pH 7.3 at 25 C.

Cellvibrio Medium

(Manual of Microbiological Methods, 1962) (26)

KNO_3	2.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$Fe_2(SO_4)_3 \cdot 7H_2O$	(trace)
KH_2PO_4	0.14g
K_2HOP_4	1.2 g
$CaCO_3$	(trace)
Yeast extract	(trace)
Cellobiose	1.0 g
H_2O (Distilled)	1 liter

Amino Acid Assay # 1 (24)

NH_4Cl	1.5 g
Glucose	2.5 g
Salts A	1.5 ml
Salts B	1.5 ml
H_2O (Distilled)	to make 225 ml

Salts A

$MgSO_4 \cdot 7H_2O$	1.00 g
NaCl	0.05 g
$FeSO_4 \cdot 7H_2O$	0.05 g
$MnSO_4 \cdot H_2O$	0.05 g
HCl (conc)	0.10 ml
H_2O (Distilled)	to make 25.0 ml

Salts B

K_2HPO_4	2.5 g
KH_2PO_4	2.5 g
H_2O (Distilled)	to make 25.0 ml

Basal Medium #2

Bacto Tryptose	1%
NaCl	0.5%
K_2HPO_4	0.1%
Add Filter-Sterilized Carbohydrate	

Cellvibrio Minimal Medium

Trace elements	1 ml / liter
Vitamin Mix*	10 ml / liter
Tryptose	1 g / liter
Casein Hydrolysate	1 g / liter
Salts, etc.	as usual for Cellvibrio medium

Carbohydrate Nutritional Experiment

Vitamin Mix*	1 ml
5% Casein Hydrolysate	0.02 ml (for 0.0010 g) or 1 ml stock solution (5 g / 250 ml)
Salts mixture	5 ml
Carbohydrate	0.1 g (1 ml--10% sol'n)
Water	2 ml

*See page 63.

Cellulose Medium

(Flora, thesis, 1964)(9)

KNO_3	0.60 g
KCl	0.16 g
$MgSO_4.7H_2O$	0.16 g
$CaCO_3$	trace
$Fe_2(SO_4)_3.7H_2O$	trace
Yeast extract	0.50 g
Casein Hydrol.	1.00 g
Cellobiose	0.05 g
Hydrocellulose (0.2%) or Whatman paper	1.00 g
Phosphate Buffer 1M H7	5.02 ml
Agar	7.50 g
(Distilled)	to make 500 ml

Mod. McBee *Cellulomonas sp.* Medium

(Hammerstrom, *et al.*, 1955) (14)

$Na_2HPO_4.12\ H_2O$	0.5 %	5.0 g
NaCl	0.4%	4.0 g
KH_2PO_4	0.1%	1.0 g
$(NH_4)_2SO_4$	0.03%	0.3 g
$MgSO_4.7H_2O$	0.01%	0.1 g
$CaCl_2$	0.01%	0.1 g
Substrate	0.2-1%	2-10 g
Vitamin Solution	0.01-0.1%	5-10 ml
Tap water	to make 1,000 ml	

Amino Acid Assay Media

KNO_3	2.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$Fe_2(SO_4)_3 \cdot 7H_2O$	trace
KH_2PO_4	0.14 g
K_2HPO_4	1.2 g
Vitamin stock*	10 ml
Water (tap)	500 ml
Each amino acid	20 g / ml
2% sucrose	500 ml

Assay: 10 ml cultures for each amino acid

*See page 63.

Appendix II

The gas chromatograph was a Beckman GC-2A using a helium carrier and thermal conductivity detection. The column was six feet long and 0.25 inches in diameter packed with Resoflex LAC-1-R-296 standard concentration (P). (Burrell Corporation) Analysis was carried out at 108 C with a 14 µl sample. The run requires 26 minutes with a 32 pound column pressure.

Culture material is prepared for chromatography by acidifying 4 ml of a stationary phase glucose broth with about 0.5 ml 50% H_2SO_4 to pH 2.0. A milliliter of ethyl ether is added and shaken vigorously. If an emulsion forms it is broken by centrifugation. The ether layer is then placed in a small tube with a pinch of $MgSO_4$ (anhydrous) and allowed to stand for five to ten minutes. A 14 µl sample is then placed on the column.

For alcohol analysis the above procedure is modified in that the culture aliquot is made to pH 8 to 9 with NaOH instead of acidification. The column temperature is changed to 80 C instead of 108 C.