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METABOLISM, MORPHOGENESIS AND PIGMENTATION IN *NOCARDIA CORALLINA*

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METABOLISM, MORPHOGENESIS AND PIGMENTATION IN NOCARDIA CORALLINA

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CHAPTER I

INTRODUCTION

Nocardia corallina is a member of the order *Actinomycetales*, and its morphological development is typical of the genus *Nocardia*, Group II (McClung, 1949). The characteristic cyclic changes in the cellular morphology of this organism have been carefully studied (Webb, 1956). Initial growth occurs by a process described as germination and is followed by a period of hyphal development which is terminated by fragmentation to short forms. Under appropriate conditions, approximately 90% synchrony of cell type has been achieved (Webb, 1956); therefore several morphologically distinct cell types can be isolated for comparative studies. For example, hyphae which exhibit karyokinesis without cytokinesis are available for comparison with cells in which both processes occur. A system for studying the

factors associated with cross-wall formation is thus afforded.

Although the growth cycles of several other species of Nocardiae have been studied in detail, the causes for the morphological changes in this genus remain unknown. However, Webb (1956) published evidence to indicate that stale (used) medium contained factors which stimulated fragmentation. Clark and Aldridge (1960) reported that changes in fat metabolism appeared to be correlated with the growth cycle, and Hittle (1963) reported differences in the free amino acid pools when hyphae and coccoids were compared.

Morphogenesis surely is mediated through metabolic changes, and it appears that attempts to explain morphogenesis at this level should yield meaningful results. However, knowledge of the metabolism is essential as a basis for such correlation studies, and relatively little has been published concerning the intermediary metabolic pathways in this organism. Evidence for carbon dioxide fixation by a reversible, nucleotide dependent oxaloacetic carboxylase has been presented (Baugh et al., 1960). Midwinter and Batt (1960) reported a high level of oxidative assimilation and evidence that the endogenous metabolism continued during the oxidation of acetate by Nocardia corallina, strain S. Previous work (Brown, 1960) indicates that the carbohydrate metabolism of

Nocardia corallina occurs by an aerobic process and is accompanied by a high level of oxidative assimilation. Consistent with these results, evidence for the operation of the Kreb's cycle and the glyoxylate by-pass was found. In addition, fructose stimulated oxygen uptake and carbon dioxide evolution to a greater extent than did glucose. Evidence for formation of adaptive permeases for several compounds has also been reported (Brown, 1960; Midwinter and Batt, 1960).

To expand our knowledge of the basic metabolism of Nocardia corallina, the pentose cycle was chosen for study. Evidence has been reported for this cycle in the related actinomycete, Streptomyces coelicolor by Cochrane, Peck and Harrison (1953), and Cochrane and Hawley (1956) as well as for Corynebacterium cretinovorans (Ghiretti and Guzman Barron, 1954). The production of pentose cycle intermediates and their accumulation in the growth medium has been reported for Nocardia opaca and other saprophytic Nocardiae and Mycobacteria by Duff and Webley (1959).

During the course of the study, the author had occasion to observe morphogenesis under a variety of conditions, and certain differences from the published life cycle (Webb, 1956) were noted. Since the extent and duration of hyphal development have been used as criteria for delineation of the

genus as well as for speciation (McClung, 1949 and 1954), the variability in the growth cycles and some of the parameters affecting morphogenesis were studied.

This organism, like many species of Nocardia, produces pigments whose structures are unknown; hence their interrelationships as well as their relation to other bacterial pigments is undetermined. These pigments have been convenient taxonomic aids; in some cases, speciation is based on pigmentation differences alone (Breed, Murray and Smith, 1957). Nocardia corallina appeared to be well suited for an initial study of pigment in this genus for several reasons. Welton (1953) reported that the pigments of a related organism, Proactinomyces ruber (now called Nocardia rubra) appeared to have characteristics which were closely related to those of the carotenoid group. Preliminary work by the author indicated that the pigment of Nocardia corallina also had many of the characteristics of the carotenoids; hence it appeared that extraction and identification might be simplified because of the large amount of published information concerning this class of pigments. Also, a number of color mutants are available for future studies concerning the biosynthesis and function of the pigment.

For clarity, the dissertation has been divided into

three phases: (1) morphogenesis, (2) metabolism, and (3) pigmentation. The first two phases were studied to add to the basic knowledge of both morphogenesis and metabolism as a basis for future correlation studies. The pigment was investigated to determine procedures for isolation, and to attempt chemical identification. Applications of the pigment study await further development of knowledge.

CHAPTER II

MATERIALS AND METHODS

Culture. Nocardia corallina (ATCC 4273) was used throughout this study. Stock cultures were grown at 29 C on nutrient agar containing ½% fructose. Where correlations between metabolic activity and culture age were sought, the inoculum was taken from 48 hour old cultures which had been transferred for a minimum of two transfers at 48 hour intervals.

Morphogenesis

Observations of growing cultures. Agar block cultures were grown under coverslips on hanging drop slides using procedures similar to those described by McClung (1949). The agar blocks were cut from nutrient agar containing ½% glucose, and the slides were incubated at room temperature (25-26 C). The coverslips were sealed on only two sides and were positioned off-center to provide an opening for

diffusion of air into the chamber. The development of individual cells was microscopically observed and photographed at intervals. Although growth conditions were not optimum, microcolonies composed of fragmentation products were produced.

Photomicrography. Photomicrographs were taken on Kodak High Contrast, 35 mm film using an apochromatic lens system (limiting n.a., 1.3). For the photographs of living cells, the substage condenser diaphragm was almost closed to give maximum contrast and to expose the cells to a minimum of light. Phase microscopy was attempted but a higher level of light intensity was required and the cells failed to germinate; either because of the light, per se, or because of the heat produced.

Assay procedure for fragmentation factor. In order to measure the effects of stale growth medium upon morphogenesis, it was necessary to compare the morphological development of control cultures with that of cultures grown in aliquots taken from developing cultures at various stages of the growth cycle. It was necessary to reduce variability in the control cultures and desirable to have techniques whereby an increase in cross-wall formation could be easily evaluated. Preliminary assays were done using the method described by

Webb (1956), which employs broth cultures. However, cultures grown on the surface of solid media have simpler growth cycles with more synchronous fragmentation; therefore the effects of filtrates are more easily evaluated.

The following procedure was devised using assay cultures growing on the surface of Millipore filters. Forty-eight hour growth was harvested from fructose agar and washed three times with physiological saline solution by alternate suspension and centrifugation in a Servall refrigerated centrifuge at 4 C and 10,000 X g for 10 minutes. A standard curve relating optical density and number of viable cells was prepared by making plate counts from appropriate dilutions of known optical densities which were determined using a Beckman, Model DU spectrophotometer with a wavelength setting of 475 mμ. From the slope of the curve, the constant 8.5×10^5 cells per optical density unit was obtained. The function was a straight line over the optical density range, .070 to .600. Equal aliquots were filtered through Millipore filters (HAW, 0.45 μ), producing a series of filters inoculated with equal numbers of evenly distributed cells. The filters were transferred to sterile Millipore pads saturated with fresh fructose broth for the control cultures, or with aliquots of stale medium taken from various phases of the

growth cycle for determining fragmentation stimulation activity. The aliquots of stale medium had been centrifuged and filtered to remove the original population. After intervals of incubation, slides were prepared and stained by the Webb cell-wall procedure (Webb, 1954). The extent of cross-wall formation in cultures grown in stale medium was compared with that found in control cultures of the same age. An inoculum of 10^7 cells per filter with evaluation of slides after 6 hours was found to be adequate for the assay.

Detection of diffusable fragmentation inducing factor from growing cultures. During the study, evidence accumulated indicating that filtrates from fragmenting cultures had a maximum stimulatory effect upon the fragmentation of assay inocula; therefore, experiments were done to test the effects of one culture in the fragmenting stage upon the development of an assay culture of coccoids, grown in close proximity. This was accomplished using a double layer of Millipore filters. The bottom filter was inoculated with 9×10^8 cells from a culture in the fragmenting stage (about 20 hours old). The top filter was inoculated with about 9×10^6 cells which were coccoids from 48 hour old cultures. The two filters were incubated, one on top of the other, on Millipore pads saturated with fresh fructose broth. The

results were evaluated by comparison with controls, as described above.

Growth cycle studies. To determine morphological development in liquid media, cultures were grown in 2-liter flasks containing nutrient broth plus ½% fructose. Plate counts were performed using standard techniques. Optical densities were read using a Beckman, Model DU spectrophotometer at a wavelength of 475 mμ. The growth curve from solid medium was obtained using Millipore filters which were inoculated with equal numbers of organisms and incubated at 29 C on the surface of nutrient agar containing ½% fructose. The filters were removed after various intervals of incubation and placed in screw cap tubes containing physiological saline and large beads. The organisms were freed from the filter and evenly suspended by mixing with the Vortex Jr. mixer (Scientific Industries, Inc., Queens Village, N. Y.) and the Disontegrator (Ultrasonic Industries, Inc., Albertson, L. I., N. Y.). Standard plate counting procedures were used to determine the number of viable cells present on the filters. In experiments where the effects of inoculum size were sought, the filters were inoculated with suspensions containing various numbers of cells, as indicated in context. Slides were made at intervals and stained by

either the Chance nuclear stain (Chance, 1952) or the Webb cell-wall stain (Webb, 1954).

Metabolism

Cell-free extracts. Cells were harvested in saline and washed three times by alternate centrifugation and suspension in .01 M phosphate buffer, pH 7.3. Five ml of a heavy suspension of cells in buffer was transferred to Mickel cells containing 2.5 ml beads (Ballotini, No. 12). Disintegration was carried out for 20 minutes at 2 C in a Mickel disintegrator (Manufactured by Gomsholl, Surry, England). The resultant suspension was centrifuged twice at 7,000 X g for 15 minutes at 2 C and stored at -20 C.

Manometric techniques. Oxygen uptake was measured at 29 C using standard techniques (Umbreit et al., 1964). The flasks contained: .2 ml of 10% KOH; .1 ml of .1 M substrate and 2.7 ml of cell suspension with air as the gas phase. Endogenous metabolism was subtracted. Results were reported as microliters of oxygen uptake per mg dry weight per hour. Cells were prepared by washing as described above except that .01 M phosphate buffer, pH 6.8 was used.

Chemical procedures. Spectrophotometric measurements were made with a Beckman, Model DU spectrophotometer at room temperature (24-25 C). DPN, TPN, glucose 6-phosphate,

and fructose 6-phosphate were obtained from Nutritional Biochemicals Corporation. Ribose 5-phosphate and gluconic acid 6-phosphate (neutralized before use) were obtained from Calbiochem.

The method of DeMoss (1955) was used to estimate dehydrogenase activity. Results were expressed either as optical density at 340 mμ or as the change in optical density per minute per mg protein. Evidence for the formation of sedoheptulose, the product of transketolase activity, was obtained by the method of Dische, Shettles and Osnos (1949) as modified by Axelrod et al. (1953). Keto sugars were detected by the method of Dische and Bornfreund (1951) as modified by Axelrod and Jang (1953).

The products formed during the incubation of cell-free extracts with ribose 5-phosphate were determined using the orcinol reagent as modified by Brown (1946), and quantitated by a procedure similar to that used by Horecker (1953). The optical density was read at 520 mμ, 580 mμ, and 670 mμ and the concentrations of hexose, pentose and heptulose calculated by the simultaneous solution of equations derived using Beer's law and calibration constants obtained from standard curves for each sugar at the three wavelengths. Some decay occurred in the color reagent even when stored at

4 C; therefore calibration curves were prepared for each experiment. Reaction mixtures were prepared as described by Rao, Ramakrishnan and Sirsi (1960). Heptulose formation was confirmed by ascending paper chromatography using the orcinol spray (Klevestrand and Nordal, 1950) for detection.

Protein determinations were made using the Biuret reaction (Gornell, Bardawill, and David, 1949) with crystalline bovine albumin as the standard.

Pigmentation

Extraction procedure. Cells from cultures one to two months old were harvested in saline, collected by centrifugation, and dehydrated by suspension in methanol. After centrifugation, the colorless methanol was discarded and the cells transferred to a foil-wrapped extraction flask containing methanolic KOH, 10%, and benzene (1:3). The flask was evacuated and the extraction carried out on a shaker in a water bath at 50 C for 12-18 hours. During extraction, two layers were formed due to extraction of water from the cells. The deep red benzene layer was removed and clarified by centrifugation and filtration with a Millipore filter (solvent, resistant, OSP, 0.45 μ). The pale yellow alcohol-water layer was centrifuged to remove cellular debris.

Purification procedure, benzene phase. The deep red

benzene solution was washed by dropwise addition from a pipette, to avoid colloid formation, into the bottom of a volumetric flask. The benzene solution was collected, dried with anhydrous Na_2SO_3 and filtered through a Millipore filter. After concentration to a small volume by low heat in a partial vacuum, an acetone insoluble residue was removed by transferring the benzene solution into cold acetone in a chilled Morton filter. The filtered solution containing the pigment was concentrated to dryness by low heat in high vacuum and taken up again in a small volume of benzene. Further purification was carried out by column chromatography. Good separations were achieved on a mixture of Sea Sorb 43 and Hyflo Super Cel (1:1). Separations were also obtained using $CaCO_3$. On the first mentioned adsorbant, a deep red pigment was tightly adsorbed near the surface of the column from benzene solution, and a pale yellow component moved rapidly down the column and was eluted by the addition of benzene. The effluent was concentrated by low heat in high vacuum and a viscous, yellow oil was obtained. The yellow oil was rechromatographed in benzene on Sea Sorb 43 and Hyflo Super Cel (1:1) and a small amount of purple material with a high ultraviolet absorption remained on the column while the yellow component was washed off with benzene. The purple band

was eluted with methanol. The yellow oil was taken up in methanol; and a white, insoluble, waxy precipitate was removed by filtration.

The original column containing the red pigment was extruded, and the red portion was mechanically separated. The red pigment was extracted with methanol by pulverizing the column in a Millipore filter and washing repeatedly with small volumes of methanol. The pigment was crystallized from methanol solution. Because of the small quantity of pigment obtained, the crystals were not washed and some impurities may have been retained.

Purification procedure, methanol phase. The basic methanol layer from the extraction flask contained a yellow pigment in solution and a waxy, orange residue at the surface. The residue was washed repeatedly with methanol; the washings were added to the basic methanol layer; and the residue was discarded. The basic methanol layer was neutralized with 1 N HCl and extracted with ether. A greenish, crystalline precipitate formed which was insoluble in organic solvents, including acetone.

The ether solution was concentrated to dryness, taken up in benzene and chromatographed using procedures previously described. No pigments other than those already described

were obtained.

Chemical and physical methods. Visible and ultraviolet absorption spectra were recorded using spectral grade solvents and a Beckman, Model DU spectrophotometer. Infrared spectra were obtained with a Beckman, Model IR-8 infrared spectrophotometer. Potassium bromide pellets, oil smears on sodium chloride crystals, or solutions of the pigments in 1 mm solvent cells were used for individual spectra determinations, as indicated. Optical rotation was determined in benzene solution at room temperature (approximately 24 C) using a Kern full-circle polarimeter and a sodium lamp. The Carr-Price test was performed as described by Karrer and Jucker (1950). Molecular weight of the phenylhydrazone was determined by the Rast method as described by Shriner, Fuson and Curtin (1956). Melting points were determined by the capillary method and were uncorrected. The phenylhydrazine test and the $FeCl_3$ test were run as described by Shriner, Fuson and Curtin (1956). The chemical analysis and molecular weight determination of the red pigment were done by Microanalytical Laboratory (Kaiser Wilhelm Platz-1, Mulkeim, Ruhr, Germany).

CHAPTER III

RESULTS

Morphogenesis

Germination. The morphological appearance of initial growth is dependent upon the age of the inoculum used. Old cultures, after multiplication has essentially ceased, are composed primarily of uninucleate coccoids. These cells typically germinate by the production of a single tubule from one end of the cell. As the tubule grows in length, its diameter increases to approximately that of the coccoidal cell. If cultures are not transferred when the uninucleate coccoid stage is reached, some growth continues and nuclear division without cross-wall formation occurs resulting in the formation of binucleate cells. Upon transfer, these binucleate cells typically germinate with the production of tubules from both ends. In cultures several months old, germination products including short hyphae have been observed.

Fragmentation. During studies of the fragmentation

process it was observed that synchronous fragmentation, resulting in rapid transformation from hyphae to bacillary and then coccoid forms, did not occur in broth cultures. Instead, fragmentation began with the production of a single cross-wall per hypha and the resultant fragments continued to grow and fragment randomly. In broth this phase was very extensive, and much multiplication occurred by this means as can be seen by comparing the growth curve (Fig. 1) with the photographs showing typical cell types which predominated at various stages of the growth cycle (Plate I, figures 1-4). Observations made at regular intervals indicated that germination followed the normal pattern, and the long hyphae which developed were predominant until 26-28 hours. Cross-walls were observed, usually toward the center of the hyphae, and some short hyphae, probably fragmentation products, were present. The percentage of short forms had increased by the 26 and 28 hour observations, and after 28 hours they were predominant. From 28 hours until the termination of observations at 147 hours, there was a gradual transition to shorter elements. During this period, single cells which appeared to be in a state of division were observed (Plate I, fig. 4).

Growth in the chemostat. The development of liquid cultures was also studied by use of the chemostat (Novic and

Figure 1. Growth curve of Nocardia corallina in aerated nutrient broth containing ½% fructose.

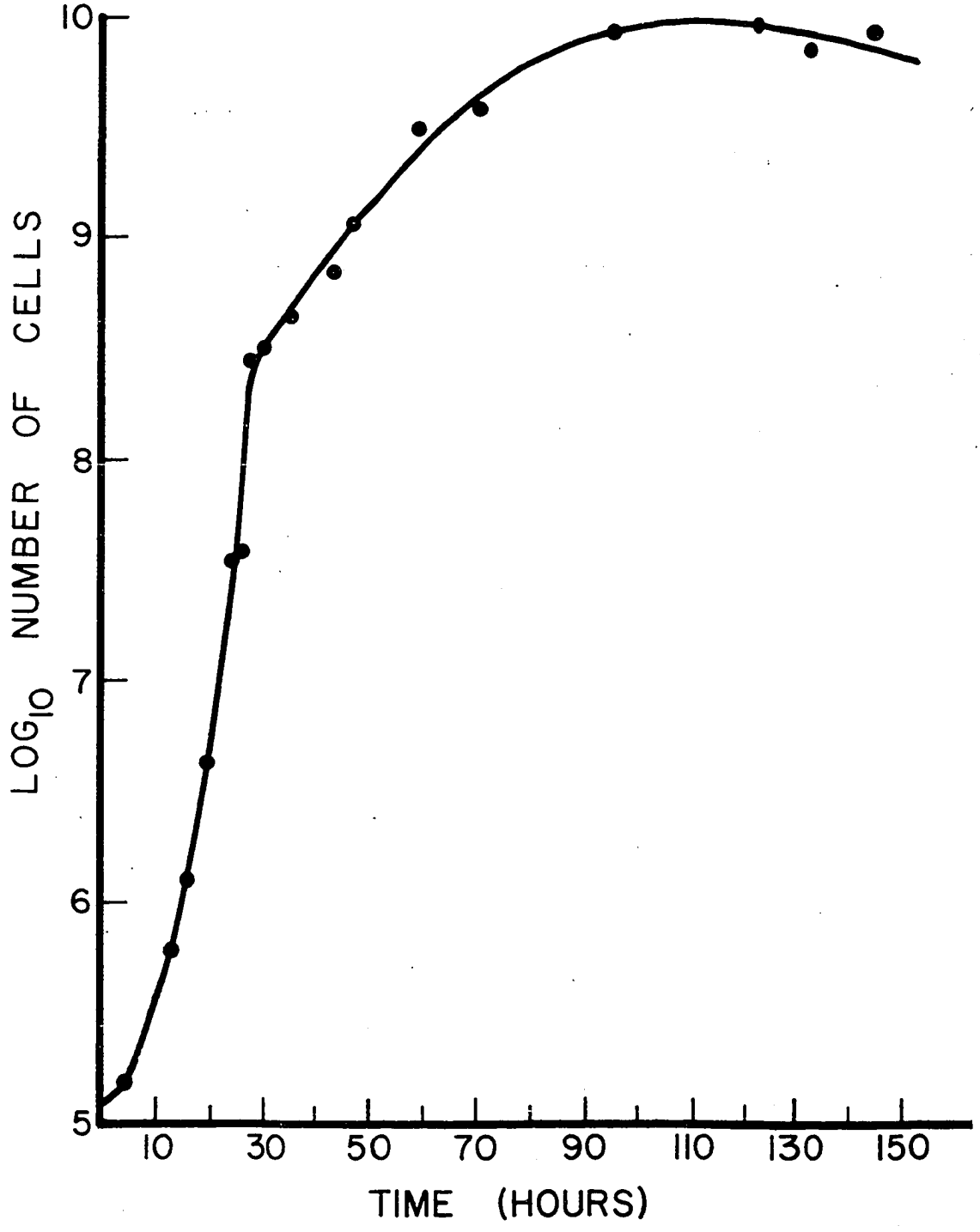
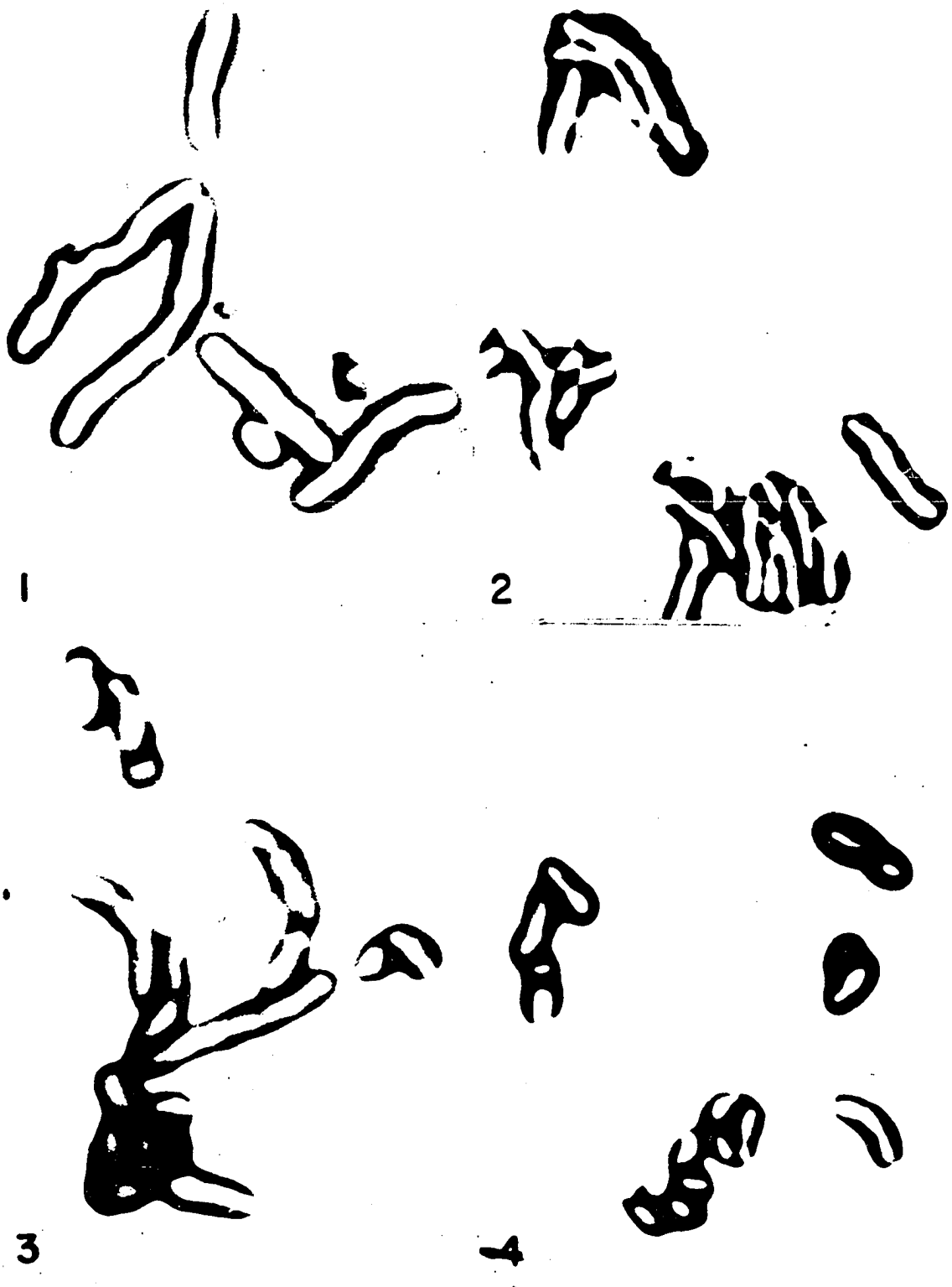

10
9
8
7
6
5
LOG10 NUMBER OF CELLS
10
30
50
70
90
110
130
150
TIME (HOURS)

PLATE I. Photomicrographs of Nocardia corallina, grown in nutrient broth containing ½% fructose and stained with the Webb cell-wall stain.

Figure 1. Eighteen hour hyphae
Figure 2. Thirty-six hour hyphae
Figure 3. Forty-eight hour cells
Figure 4. Ninety-six hour cells


1
2
3
4

Slizard, 1950). The reservoir was filled with nutrient broth containing ½% fructose, and various flow rates were investigated. With the flow rate adjusted to give maximum turbidity, growth in the chemostat resulted in the production of a culture which continued, as long as fresh medium was supplied, in a phase composed of short fragmentation products which were elongating and producing cross-walls at an equilibrium rate.

Observations of growing cultures. In order to study the fragmentation sequence in more detail, microscopic observations of developing slide cultures were made of individual cells, from germination through fragmentation to very short forms. Plates II, III, and IV show the development of a hypha from the formation of the first cross-wall through the production of very short fragmentation products. Growth occurred by elongation from the hyphae tips, including the new tips produced by fragmentation. Intercalary growth did not occur. This is more clearly shown in Figure 2 which shows typical results of measurements made with a filar micrometer on developing hyphae which had multiple branches that served as markers. As the culture developed, a stage was reached where multiple cross-wall formation resulted in the production of successively shorter fragmentation products.

PLATES II, III, and IV. Photomicrographs of living, unstained cells of Nocardia corallina.

The organisms were grown on slide cultures of nutrient agar plus ½% glucose at room temperature (24-25 C). The times listed below indicate the intervals after inoculation when the photographs were made.

PLATE II

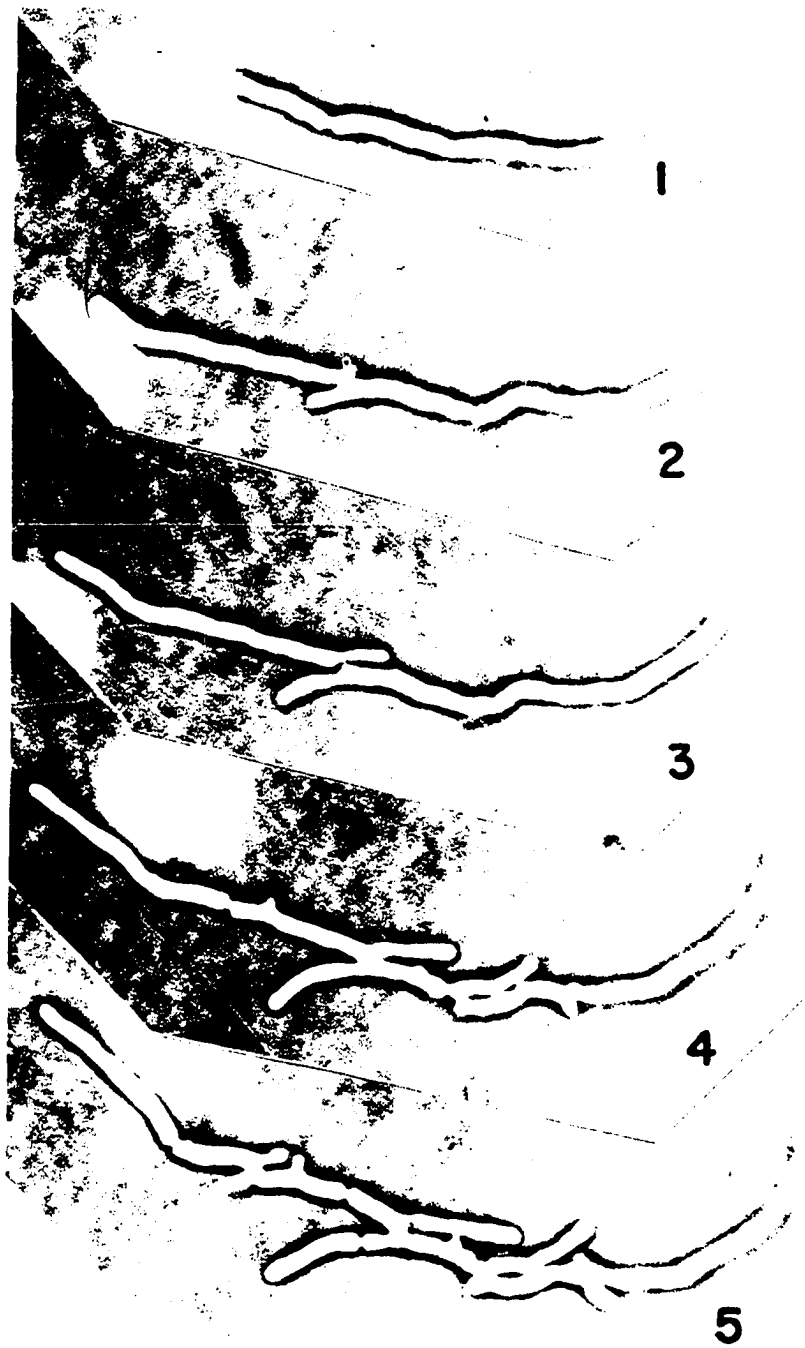
Figure 1. 12 hours
Figure 2. 13 hours, 50 minutes
Figure 3. 15 hours, 15 minutes
Figure 4. 16 hours, 55 minutes
Figure 5. 18 hours, 30 minutes

PLATE III

Figure 6. 20 hours, 45 minutes
Figure 7. 24 hours, 45 minutes
Figure 8. 27 hours, 10 minutes
Figure 9. 28 hours, 10 minutes

PLATE IV

Figure 10. 30 hours, 40 minutes
Figure 11. 32 hours, 40 minutes
Figure 12. 34 hours, 40 minutes
Figure 13. 39 hours, 40 minutes


1
2
3
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5


6
7
8
9

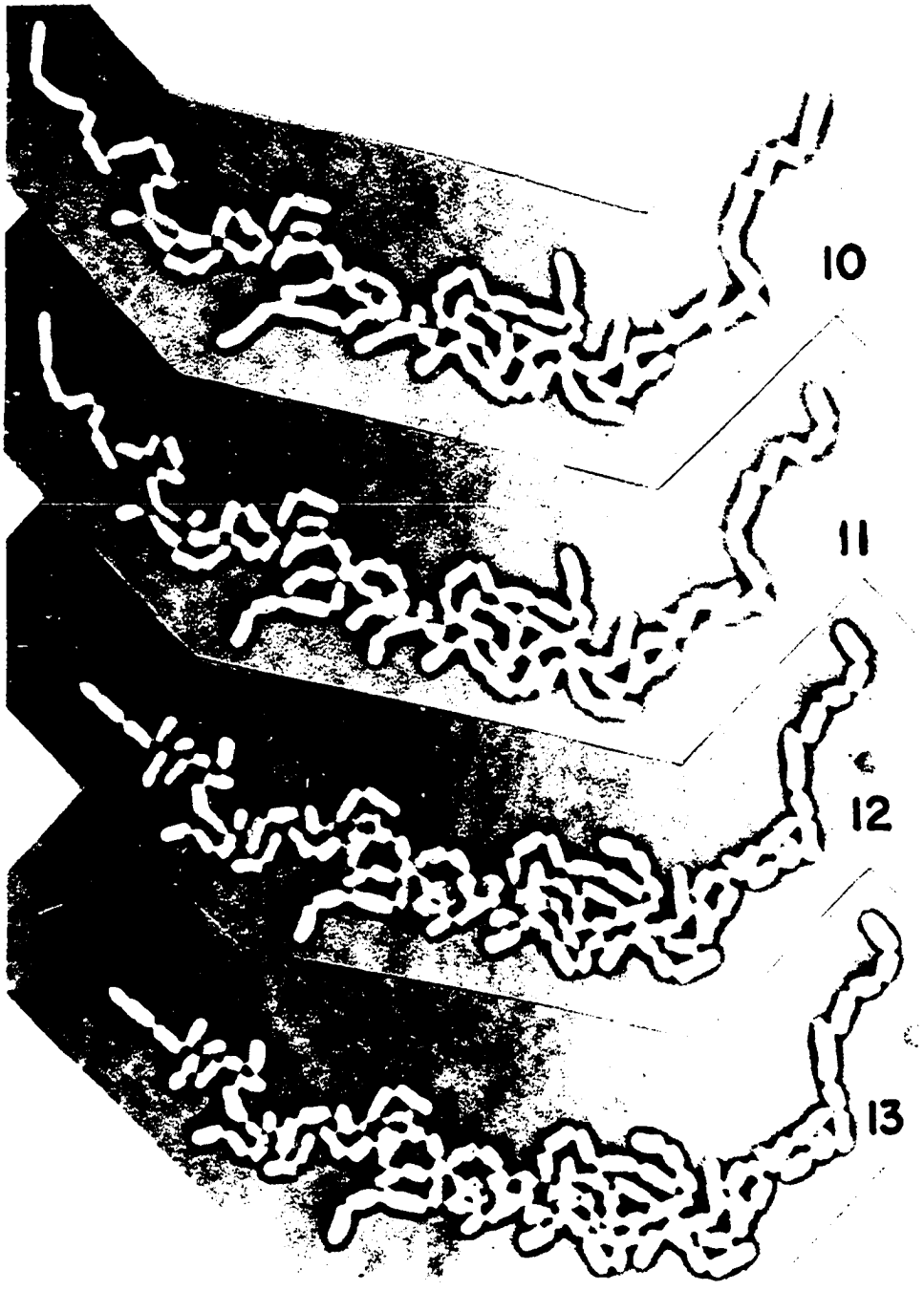
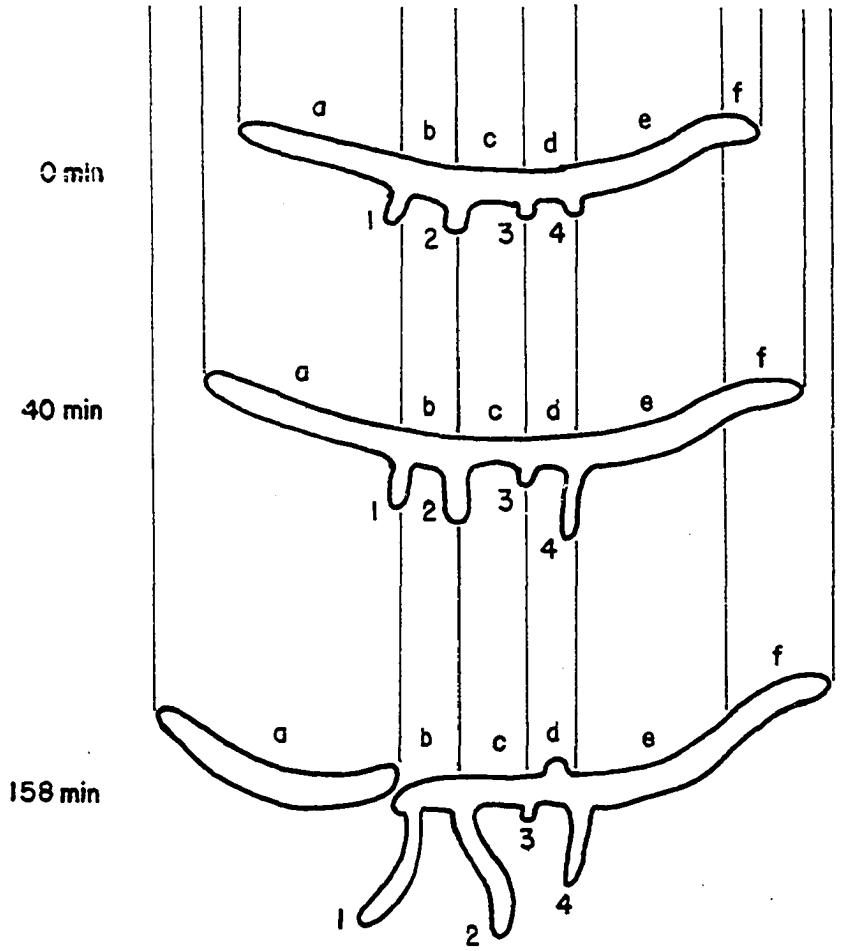

10
11
12
13

Figure 2. Growth of hyphae by elongation from the tips.

The values listed below give the lengths, and the changes in length (in microns) for the indicated portions of the hyphae shown in the accompanying diagram.

Reference no. or letter	Length of portion at:			Change in length (microns)
	0 min	40 min	158 min	
a.	5.34	6.10	8.45	+3.11
b.	2.12	2.07	2.09	-0.03
c.	1.91	2.38	2.02	+0.11
d.	1.69	1.45	1.48	-0.21
e.	3.39	3.49	3.37	-0.02
f.	1.57	2.03	4.67	+3.10
1.	1.67	2.16	4.30	+2.63
2.	1.73	2.50	4.52	+2.79
3.	0.57	0.71	0.57	0.00
4.	0.89	1.01	2.92	+2.03


0 min
40 min
158 min
a
b
c
d
e
f
1
2
3
4

Bacillary cells and some cells which appear to be coccoid can be seen in Plate IV, figure 13. The importance of the rate of cross-wall formation, relative to growth and nuclear division, upon morphogenesis is shown by these photographs (Plates II, III, and IV).

If hyphal fragments are transferred to begin a new culture, they grow by elongation from both tips in a manner analogous to that shown by the fragments in Plates III and IV. Since the diameters of the growing tips are approximately equal to that of the body of the hyphae, the process does not resemble germination. Cultures may be kept in the hyphal phase if transferred before extensive fragmentation occurs. Random cross-wall formation, usually toward the center of the hyphae, limits maximum hyphae length to approximately that found in the normal life cycle.

Assay of fragmentation stimulation factor. During growth in the chemostat, in aerated broth, and on agar, a stage is reached when the incidence of cross-wall formation increases to the extent that hyphae with multiple cross-walls result. Fragmentation produces short forms and multiplication continues by elongation and cross-wall formation. Previous work (Webb, 1956) provides evidence that fragmentation factors are produced and accumulate during growth. In order to

test this further, studies were made of the effects of stale broth upon the fragmentation processes of fresh inocula.

Table 1 shows that broth from cultures 2-4 days old has the maximum effect of increasing the rate of cross-wall formation in assay inocula. Heating the stale media to boiling lessened the effect but did not destroy it. Fragmentation was not stimulated by media from young cultures nor by media from week-old cultures. Some growth stimulation occurred in the 22-hour stale medium. Growth was poor in the week-old stale medium but germination to long hyphae did occur. The effects of addition of concentrated, fresh broth to the stale medium is shown in Figure 3. The inhibition of germination produced by week-old stale medium appeared to be due to depletion of nutrients; the inhibition by 3-day old medium appeared to be due to other causes.

Assay of fragmentation stimulation by Millipore technique. The fragmentation process in broth cultures was not synchronized, and the inception of cross-wall formation was not a sharply defined event. Agar surface cultures showed more synchrony in fragmentation and the effects of factors upon the process were more easily evaluated. Using a procedure envolving growth on Millipore filters, as described in Materials and Methods, it was possible to show that stale

TABLE 1

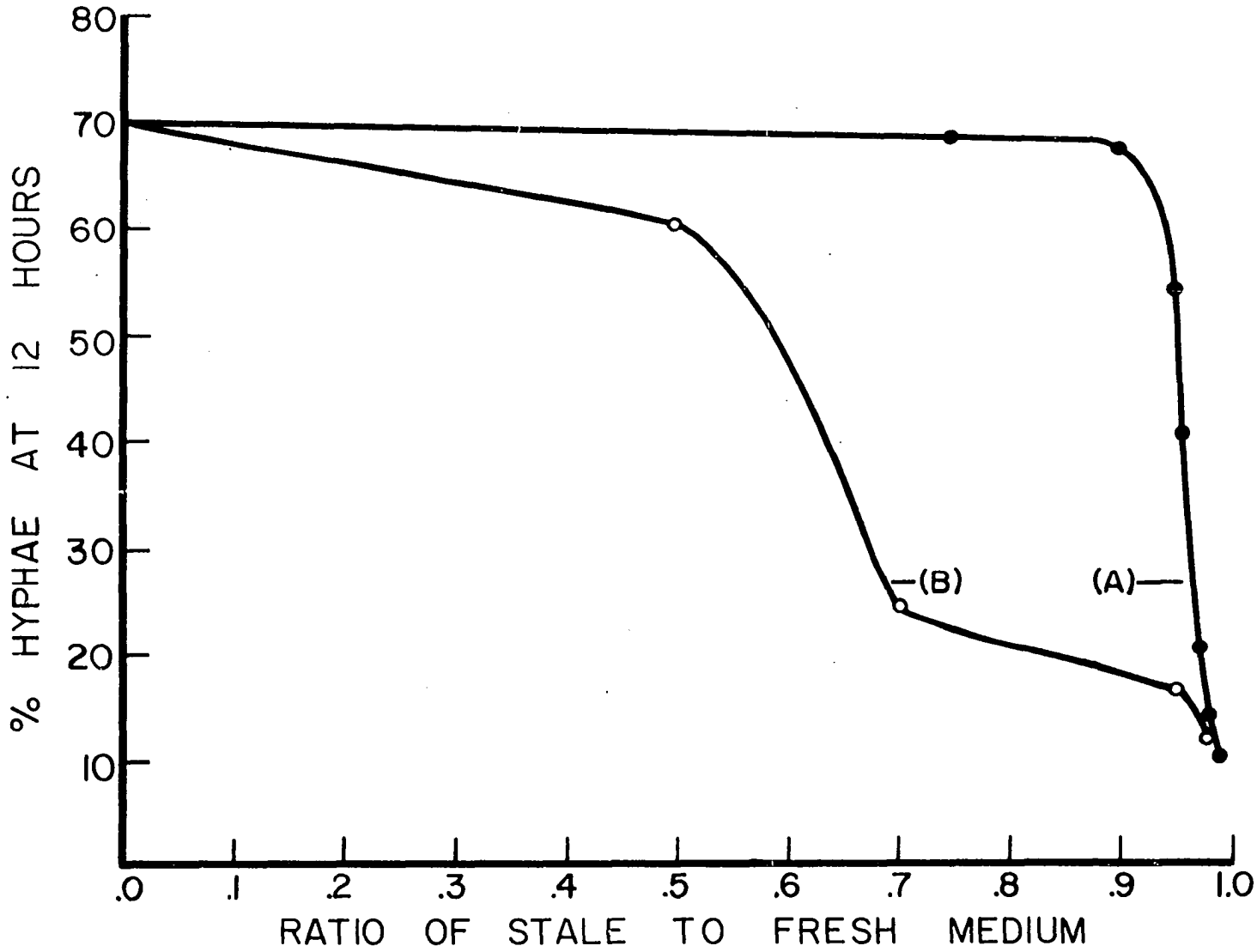
Morphological development in stale medium (heated* and unheated) taken from cultures of various ages

Age of Culture	Morphological Development
22 Hr. (heated)	Development similar to that found in fresh broth.
22 Hr. (not heated)	Development similar to that found in fresh broth.
47 Hr. (heated)	Cross-wall formation stimulated; mostly coccoid by 69 hrs.
47 Hr. (not heated)	Cross-wall formation stimulated; mostly coccoid by 47 hrs.
70 Hr. (heated)	Similar to 47 hour, heated.
70 Hr. (not heated)	Few long hyphae formed; multiplication appears to occur by rapid cross-wall formation.
96 Hr. (heated)	Similar to 47 hour, heated.
96 Hr. (not heated)	Essentially no hyphae produced, multiplication occurs by rapid formation of cross-walls.
165 Hr. (heated)	Germination to long, coenocytic hyphae.
165 Hr. (not heated)	Germination to long, coenocytic hyphae.

*Heated to boiling for 5 minutes.

Figure 3. The effects of various concentrations of stale medium upon germination to hyphae.

Curve A shows the effects of stale medium from a 1 week old culture, and curve B gives the results obtained with medium from a 3-day culture. The assay inoculum was a 1 week old culture of coccoids.


80
70
60
50
40
30
20
10
% HYPHAE AT 12 HOURS
(B)
(A)
.0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
RATIO OF STALE TO FRESH MEDIUM

medium from actively fragmenting cultures stimulated fragmentation of a fresh inoculum (Plate V). Cross-wall formation was extensive at 6 hours in the culture containing the stale medium, while relatively few cross-walls were present in the control cultures.

Similar results were observed when assay cultures of coccoids, inoculated onto Millipore filters, were placed directly on top of a second filter which contained a culture in the fragmenting stage as described in Materials and Methods. The fragmenting culture apparently produced factors which diffused into the adjacent culture and stimulated earlier fragmentation.

Effects of inoculum size. Table 2 shows a summary of changes in morphological development which were produced by varying the size of the inoculum in aerated broth cultures. The results suggest that the rate of cross-wall formation relative to cell elongation and nuclear division increases as population density increases. Table 3-A shows results obtained when cells were grown on the surface of Millipore filters. When the number of cells inoculated per unit area of surface was increased, the culture went through the developmental cycle at a more rapid rate. When the inoculum was large enough to produce more than a monolayer of cells,

PLATE V. Stimulation of cross-wall formation by a cell-free filtrate from a fragmenting culture.

Cells were stained by the Webb cell-wall procedure.

Figure 1. A 6 hour control culture grown on a Millipore filter placed on a pad saturated with fresh fructose broth.

Figure 2. A 6 hour culture grown as indicated above except that the pad was saturated with stale broth from a fragmenting culture.


1
2

TABLE 2

The effects of inoculum size upon morphological development

Inoculum Size (percent)	Morphological Development
0.5	Cells germinate, elongate into hyphae and after about 15 hours, a few cross-walls are produced resulting in the formation of hyphae of irregular length. Most of the growth and multiplication occurs by hyphae elongation and random cross-wall formation.
5.0	Growth similar to above except that the ratio of cytokinesis to karyokinesis approaches one at about 48 hours, resulting in very short forms. Long hyphae may re-appear with further incubation.
50	Similar to the 5% except that the short fragmentation products predominate at about 24 hours. These short forms persist through 48 hours and with further incubation more long hyphae appear.
50	Very similar to 50% described above.
100	Germination occurs to produce hyphae, but within 12 hours cross-wall formation and fragmentation has begun. Growth and multiplication is produced, almost from the start by hyphae elongation and random cross-wall formation.
150	Initial development similar to 100%. Culture becomes a mixture of some long hyphae, fragmenting randomly, and many short chains of two and three cells.
300	At 12 hours, very few hyphae, many short chains of two, three or four cells. The ratio of cytokinesis to karyokinesis appears to be approximately one from the start. A few hyphae are observed, most have several cross-walls.

TABLE 3A

Effect of inoculum size upon morphological development of cultures grown on the surface of Millipore filters

Total No. of Cells per Filter	No. Cells per Sq. Micron Surface Area	Age of Culture and Morphological Description
4×10^5	.0042	12 hr- long hyphae 14 hr- long hyphae 17 hr- long hyphae 22 hr- fragmentation beginning
4×10^6	.042	12 hr- long hyphae 14 hr- fragmentation beginning 17 hr- many short fragmentation products 22 hr- short forms (coccoid like)
4×10^7	.42	12 hr- short hyphae 14 hr- hyphae fragments 17 hr- short hyphae fragments 22 hr- short forms (coccoid like)
4×10^8	4.2	no hyphae observed, cells appear to be dividing

TABLE 3B

Growth curve from Millipore filter

Time (hrs) after Inoculation	O.D.	Total Cells per Filter Times 10^{-8}
0*	.035	4.3
0	---	4.1
5.5	.128	6.5
8.25	.205	13.8
8.8	.207	20.9
20.5	.581	54.0
32.5	.964	91.6

*Optical density and plate counts made from suspension used to inoculate filters.

typical germination was not observed. Table 3-B indicates that multiplication did occur when this size inoculum was used.

Metabolism

Dehydrogenase activity. Figure 4 shows typical results obtained when crude, cell-free extracts of Nocardia corallina were incubated with either glucose 6-phosphate or gluconate 6-phosphate in the presence of either TPN or DPN. The increase in optical density at 340 mμ which occurred with TPN but not with DPN indicated that TPN specific dehydrogenases for both substrates were present. Extracts from cultures of various ages were tested and all were found to be active (Table 4) although the specific activity decreased during germination and hyphal growth. Figure 5 shows the reduction of TPN which occurred with fructose 6-phosphate as substrate. The change in the slope of the curve during the first two minutes of reaction suggests that fructose 6-phosphate was not dehydrogenated directly, but was first converted to some intermediate such as glucose 6-phosphate. The presence of hexose isomerase activity in the enzyme extract would have given results similar to these.

Products of ribose 5-phosphate metabolism. Figures

Figure 4. Glucose 6-phosphate and gluconate 6-phosphate dehydrogenase activities.

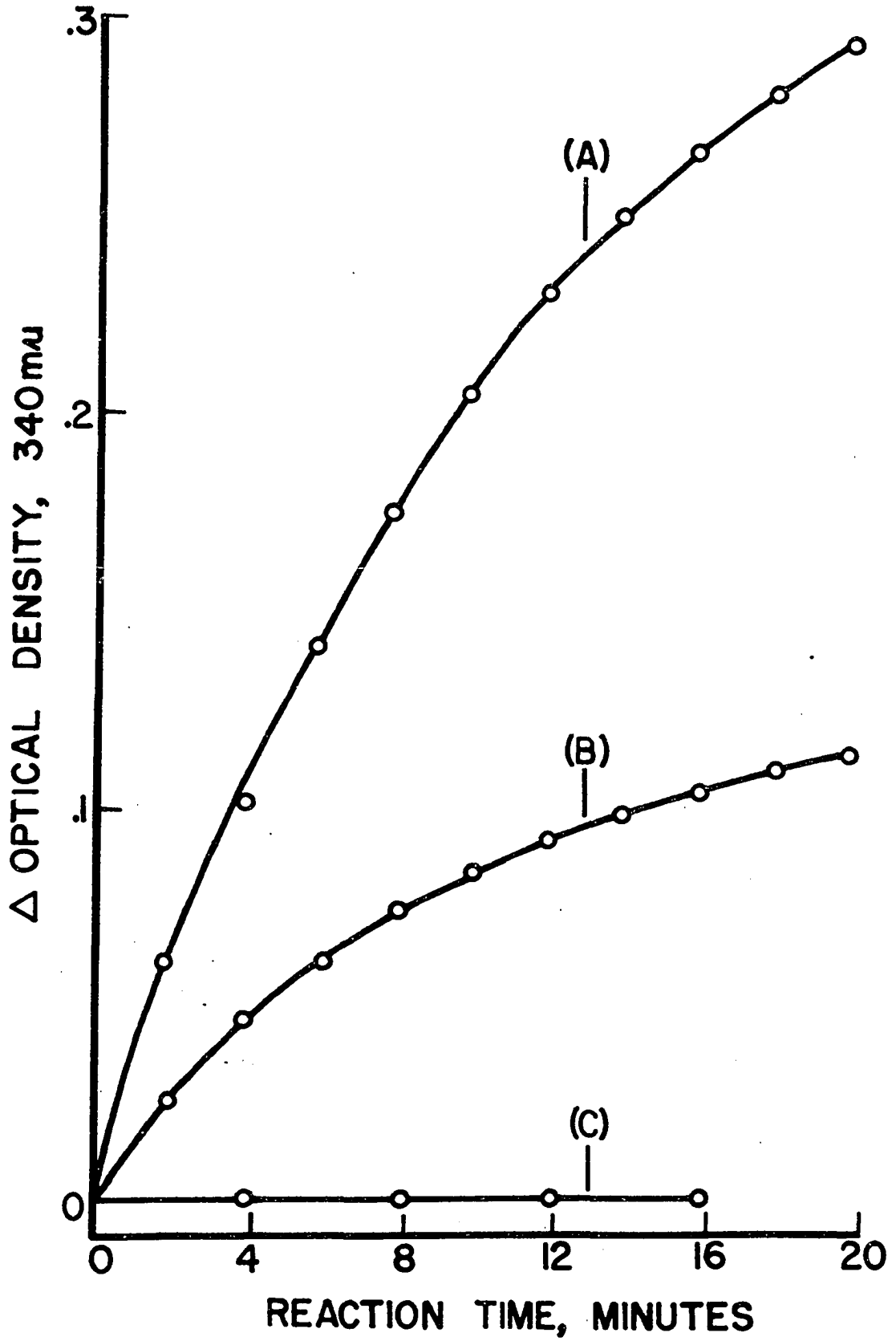
Additions to A: glucose 6-phosphate (0.025 M), 0.4 ml; $MgSO_4 \cdot 7H_2O$ (0.1 M), 0.2 ml; tris buffer (0.05 M) pH 7.9, 0.9 ml; cell-free extract from 22 hr. old culture (1.7 mg protein), 0.5 ml; TPN (1.0 mg per ml), 0.2 ml; volume adjusted to 3 ml with deionized water.

Additions to B: the same as A, except that gluconate 6-phosphate was substituted for glucose 6-phosphate and enzyme extract contained 4.3 mg protein.

Additions to C: the same as either A or B except that DPN was substituted for TPN.

Figure 5. Dehydrogenase activity with fructose 6-phosphate as substrate.

Additions: the same as Figure 4 except that fructose 6-phosphate was used as substrate and a cell-free extract from a 12 hour old culture was used (7.4 mg protein).


.3
.2
.1
0
Δ OPTICAL DENSITY, 340mμ
(A)
(B)
(C)
0
4
8
12
16
20
REACTION TIME, MINUTES

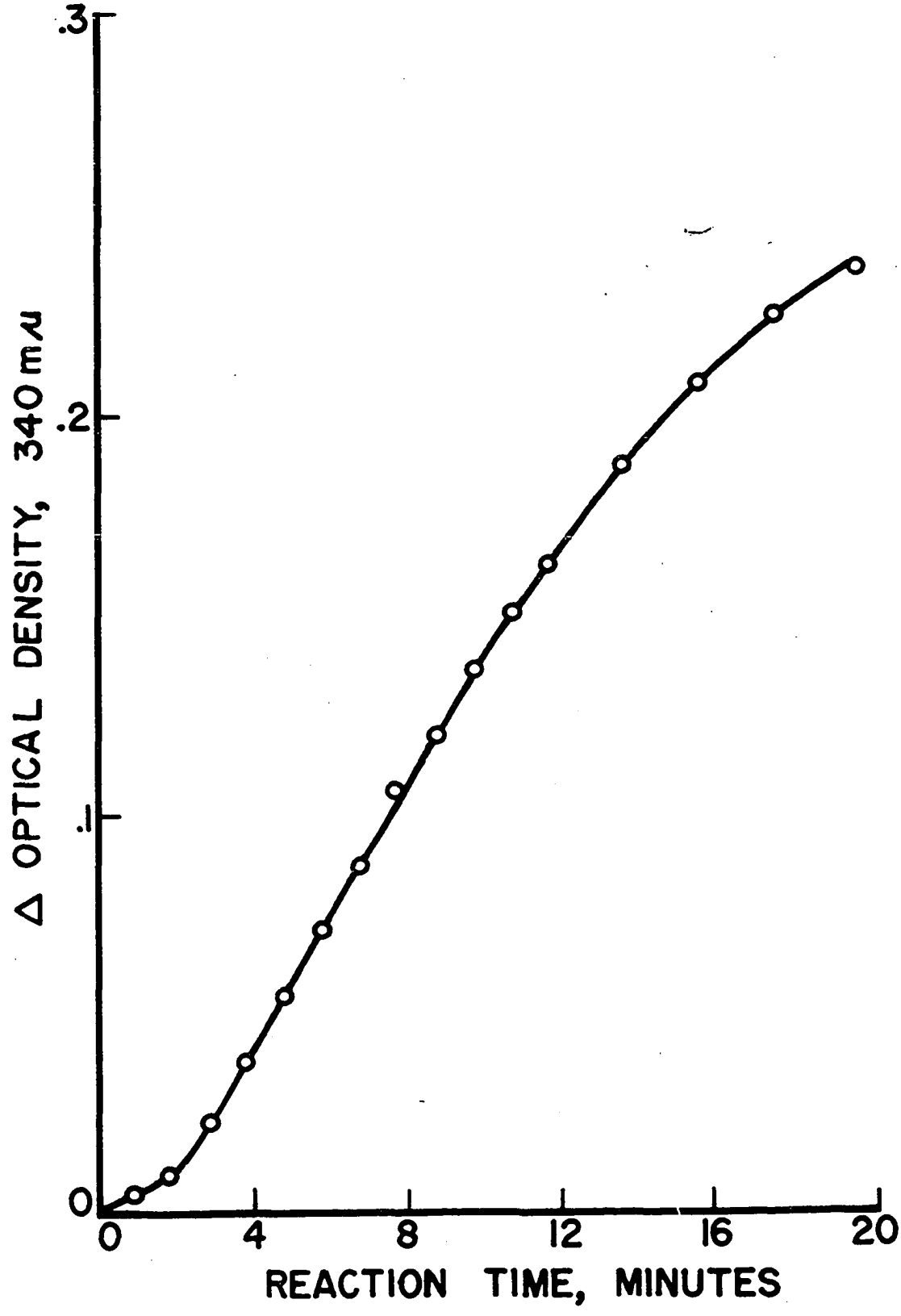

.3
.2
.1
0
Δ OPTICAL DENSITY, 340 mμ
0
4
8
12
16
20
REACTION TIME, MINUTES

TABLE 4

The effects of culture age upon QO_2 values

QO_2 values for Nocardia corallina grown on nutrient agar containing ½% glucose. Inoculum for all cultures was 48 hours old and had been transferred every 48 hours for a minimum of two growth cycles. Endogenous metabolism was subtracted.

Substrates	QO_2 Values									
	Age of cultures in hours									
	2.5	7	7.5	8.5	12	22	24	28	48	96
Endogenous	7.	12.9	11.2	20.4	8.8	4.5	5.6	2.9	3.1	5.2
Glucose	4.3	0	0	0	5.1	6.8	5.2	6.1	4.4	6.9
Fructose	10.4	7.8	11.2	19.9	17.4	18.9	16.4	11.9	6.6	7.2
Caproate	18.6	21.9	20.8	41.1	30.8	25.7	16.1	17.5	10.	---

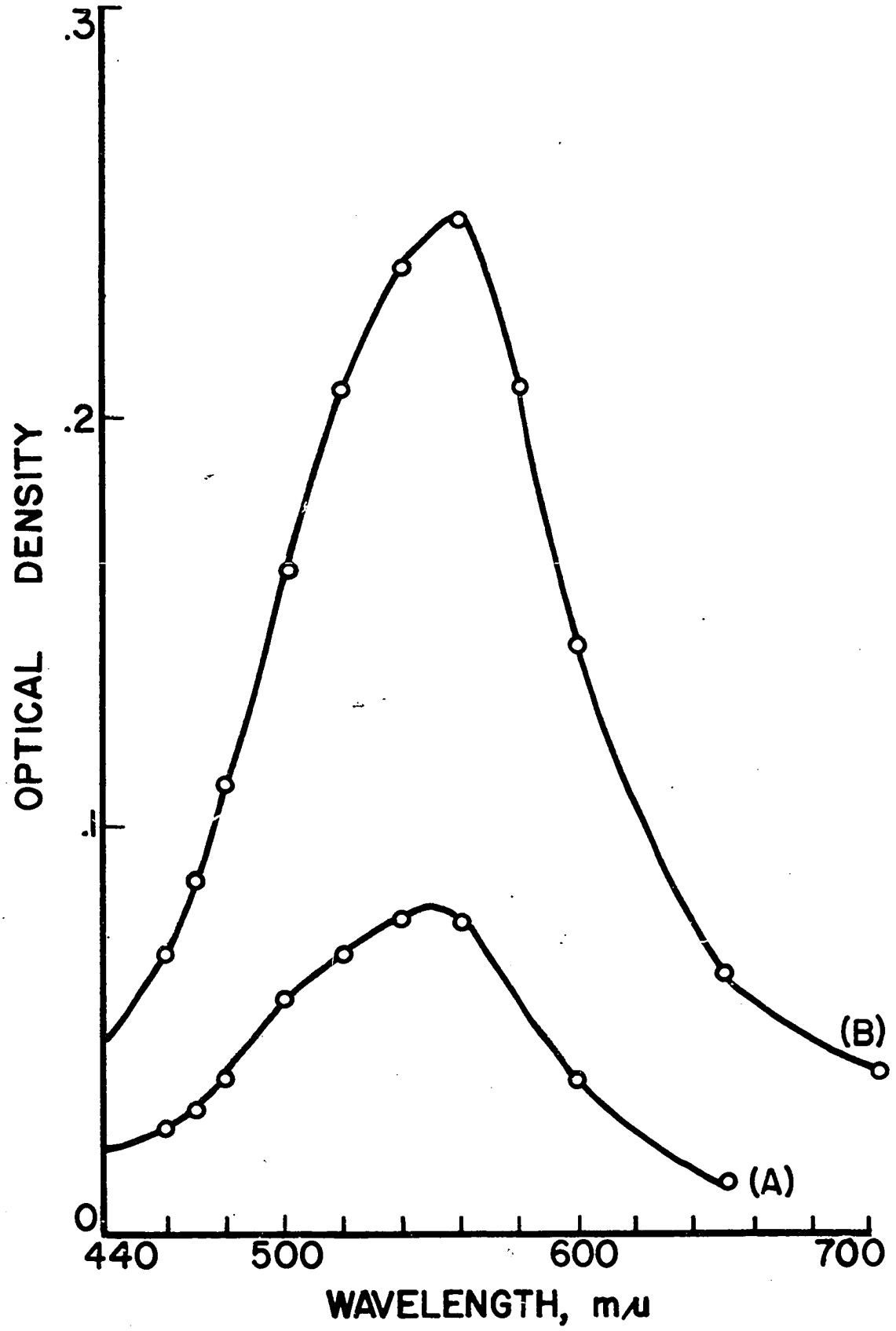
6, 7, and 8 show results obtained by analysis with the cysteine-carbazole reagent. Figure 6 shows the results given by the cell-free extract alone. The peak at 560 mμ is characteristic of ketohexoses, such as fructose (Cochrane and Hawley, 1956). Authentic samples of fructose were run in conjunction with the experiment and gave maximum absorption at 560 mμ. Ribose 5-phosphate also gave color with the cysteine-carbazole reagent, but the absorption maximum appeared slowly (Fig. 7). It should be noted that the concentrations of both enzyme extract and ribose 5-phosphate used in the control tests (Figures 6 and 7 were 3.3 times as high as were used in the reaction mixture (Fig. 8). Ketopentoses, such as ribulose 5-phosphate, produce color rapidly after the addition of the reagents and are about 60 times as reactive as are aldopentoses (Ashwell, 1957). The relatively high absorption at 540 mμ which appeared soon after the addition of the reagents (Fig. 8) is evidence for the formation of ketopentose.

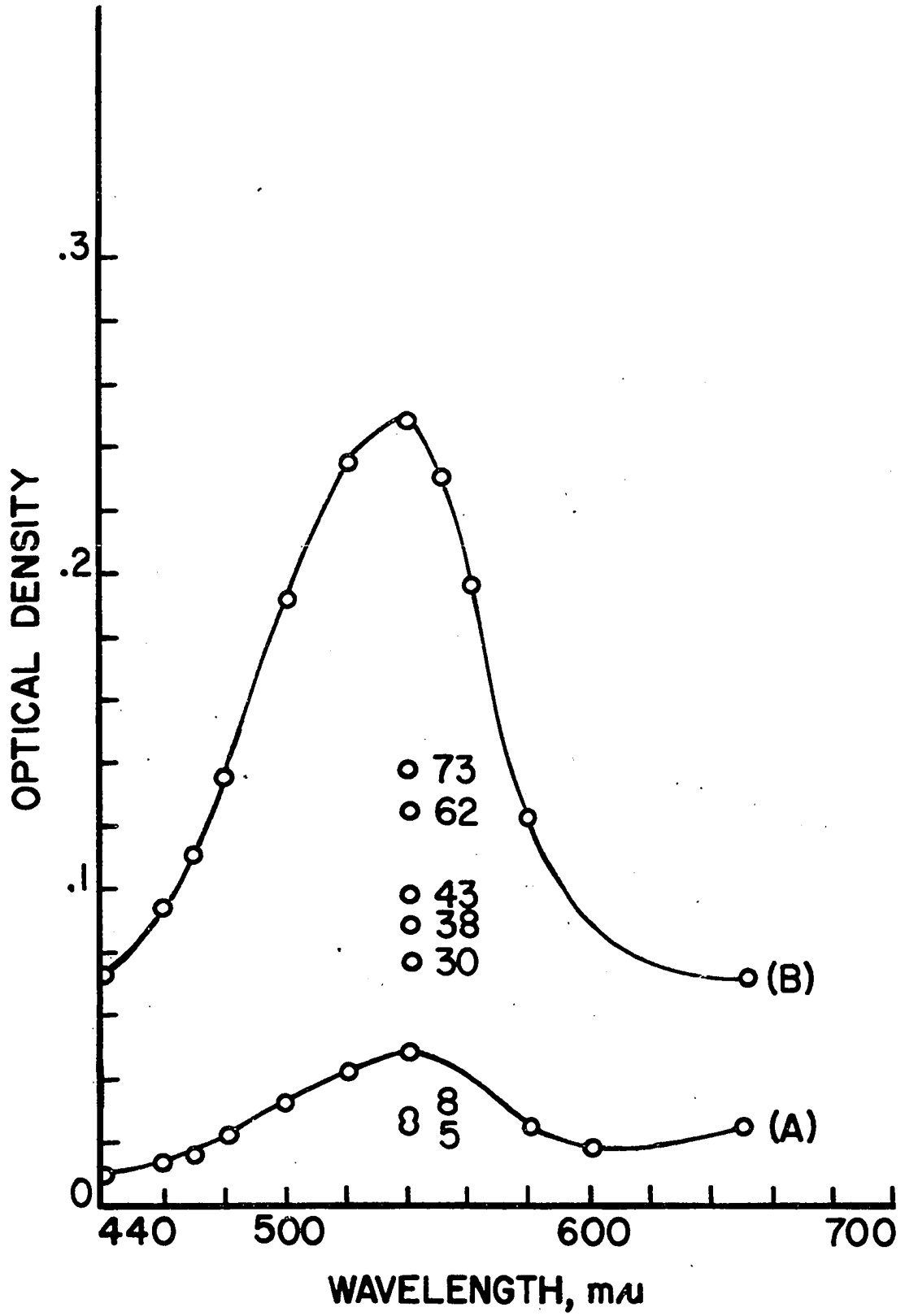
Figure 9 shows the absorption spectra obtained by the SCyRI Procedure (Dische, Shettles and Osnos, 1949). Curve A is the control and the absorption maxima at 600 mμ is further evidence for the presence of hexose in the cell-free extract. Curves B and C were obtained with the reaction

Figure 6. Results of analysis for keto sugar by the cysteine-carbazole procedure--the absorption spectrum given by ribose 5-phosphate. This is a substrate control for the data of Figure 8, and experimental conditions were identical except that cell-free extract was omitted, and ribose 5-phosphate concentration was 3.3 times as high. Curve A was recorded 16 minutes, and curve B, 12 hours after addition of color reagents.

Figure 7. Results of analysis for keto sugar by the cysteine-carbazole procedure--the absorption spectrum given by cell-free extracts. The experimental procedure was identical to that given in Figure 8 except that ribose 5-phosphate was omitted, and the enzyme concentration was 3.3 times as high. The numbers on Figures 7 and 8 indicate the time in minutes after the addition of color reagent when the optical densities were read.

Figure 8. Results of analysis for keto sugar by the cysteine-carbazole procedure--the absorption spectrum given by reaction mixtures. The reaction mixtures contained: tris buffer (0.1 M, pH 7), 0.6 ml; thiamine hydrochloride (0.15 M), 0.2 ml; $MgSO_4 \cdot 7H_2O$ (0.1 M), 0.2 ml; cell-free extract from 48 hour old culture (14.4 mg protein), 0.9 ml; ribose 5-phosphate (0.025 M), 0.9 ml; volume adjusted to 3.0 ml with deionized water. The reaction was incubated at 30 C and 0.1 ml was used for analysis.


.3
.2
.1
0
OPTICAL DENSITY
440
500
600
700
WAVELENGTH, mμ
(B)
(A)


OPTICAL DENSITY
.3
.2
.1
0
73
62
43
38
30
(B)
8
8
5
(A)
440
500
600
700
WAVELENGTH, mμ

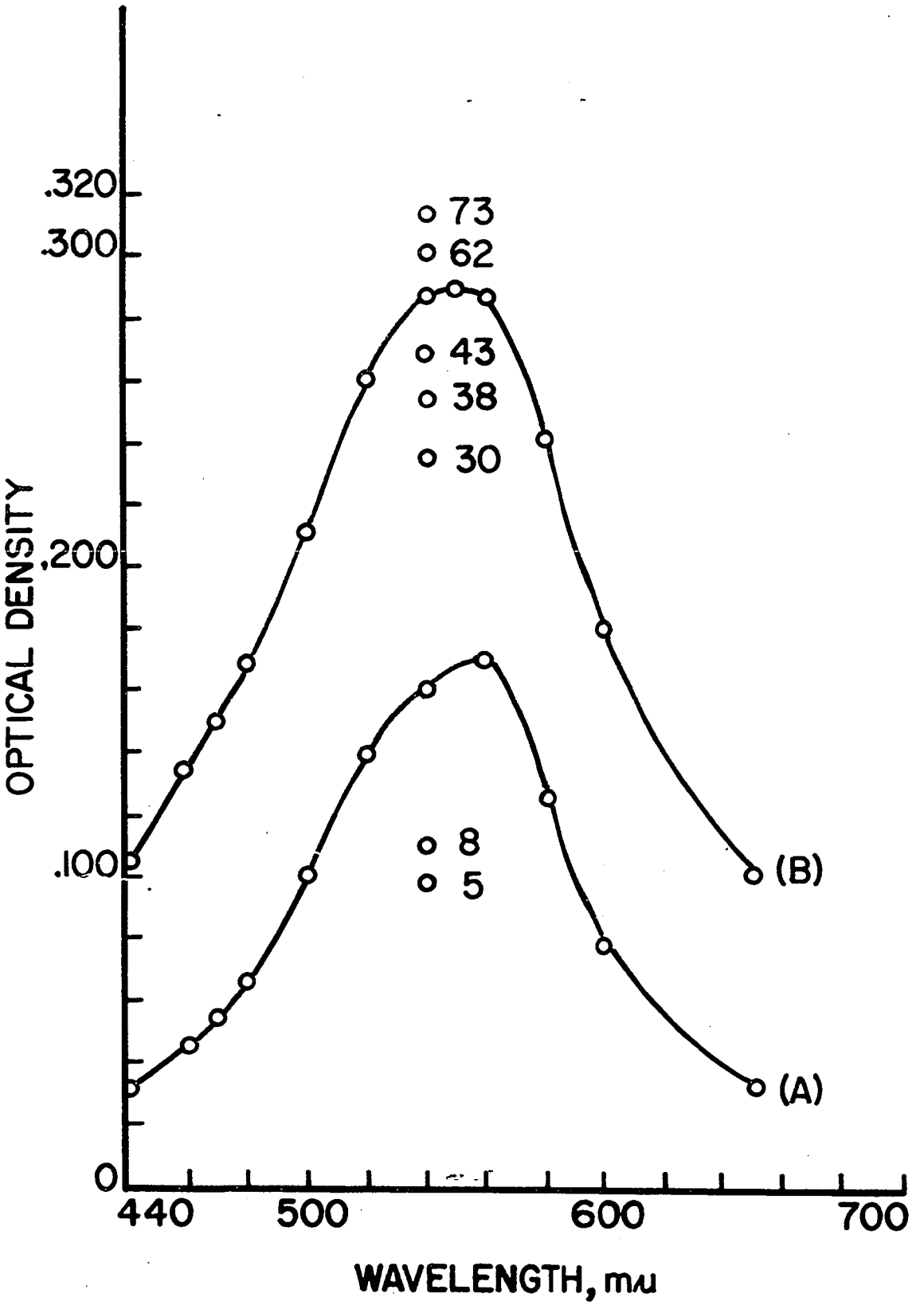
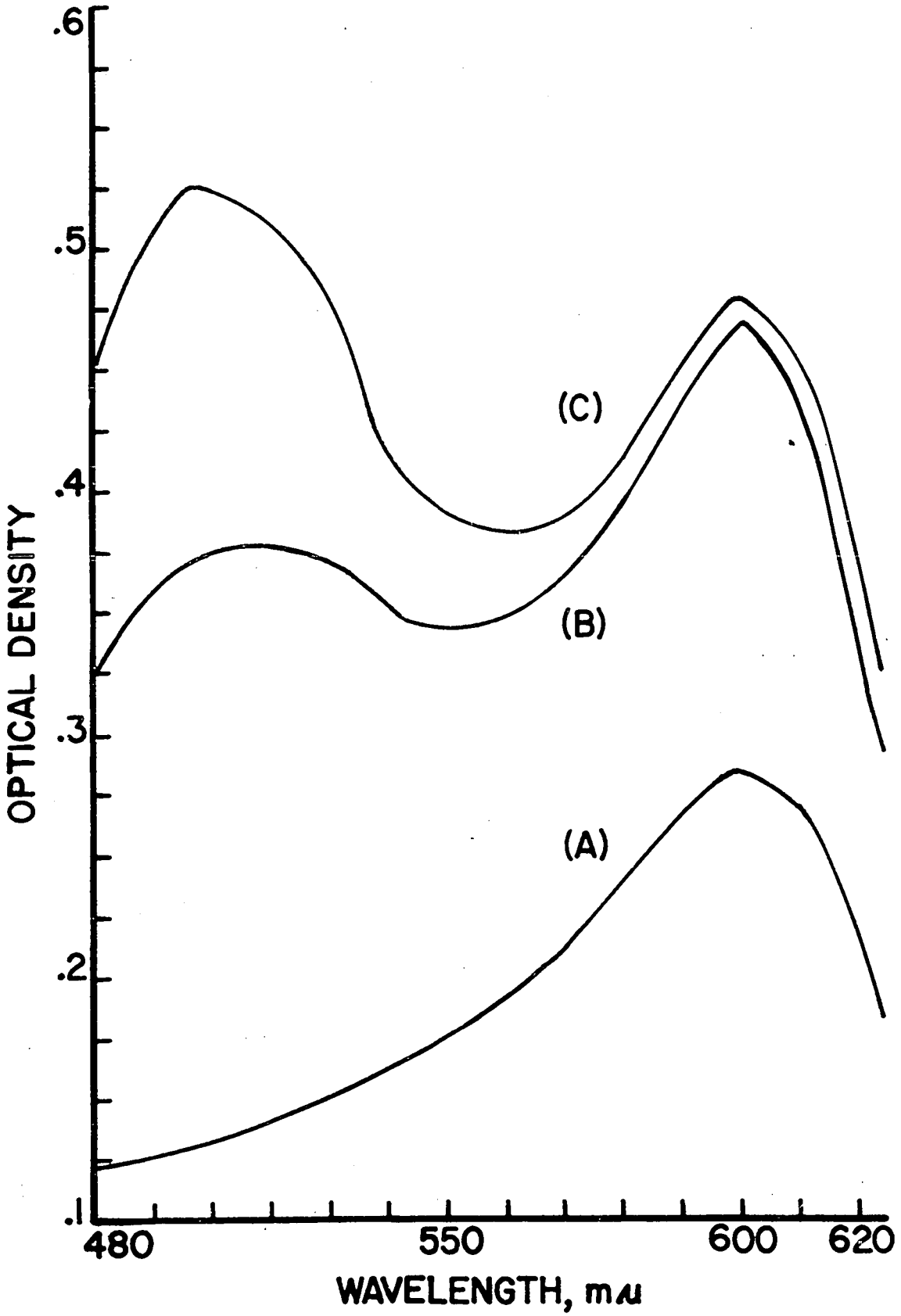

.320
.300
.200
.100
0
OPTICAL DENSITY
73
62
43
38
30
8
5
(B)
(A)
440
500
600
700
WAVELENGTH, mμ

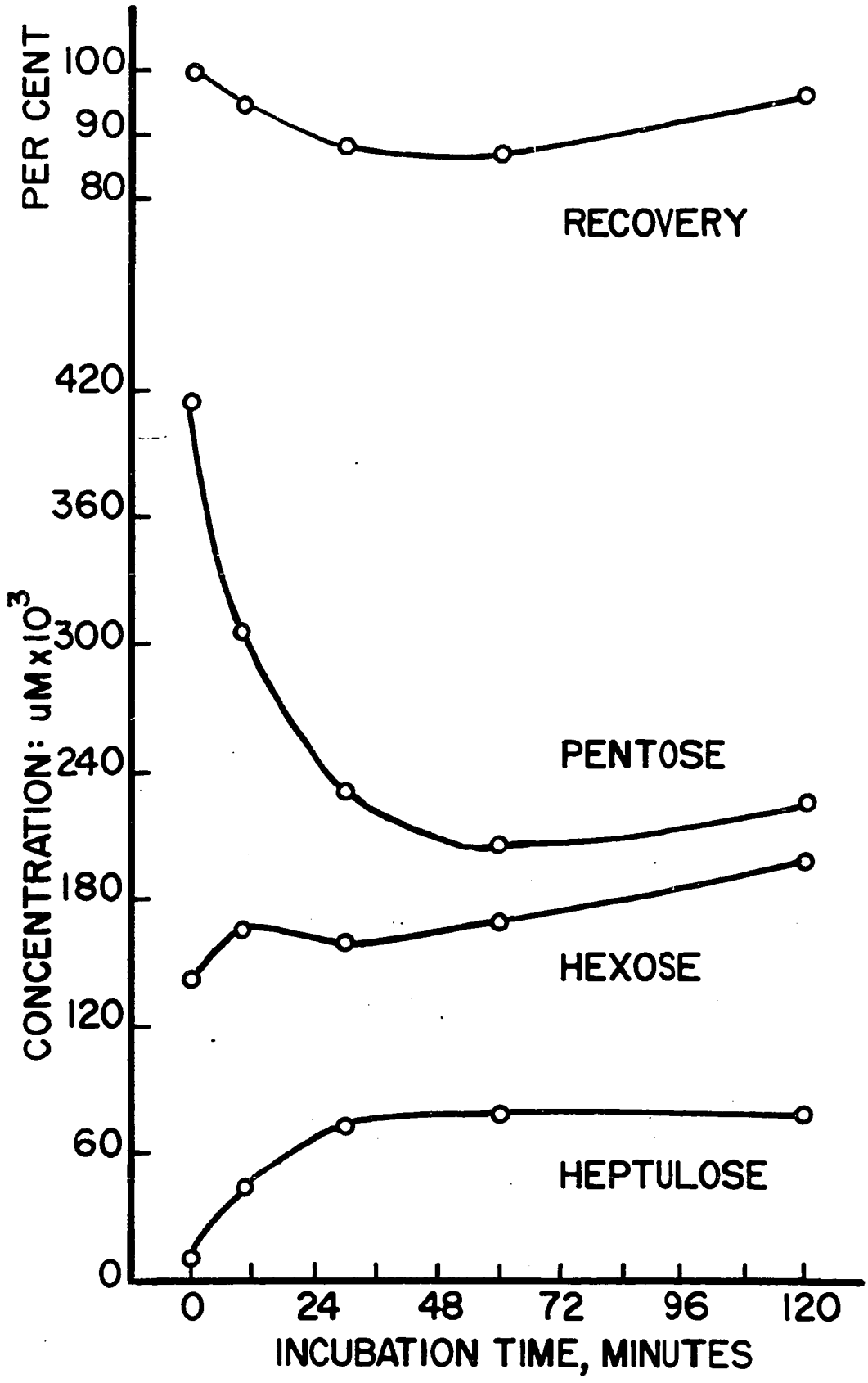
Figure 9. Typical absorption spectrum obtained during analysis of reaction mixtures by the SCyRI procedure. The reaction mixture was the same as described in Figure 8 except that 13.2 mg protein from a cell-free extract of an 18 hour old culture was used.


.6
.5
.4
.3
.2
.1
OPTICAL DENSITY
(C)
(B)
(A)
480
550
600
620
WAVELENGTH, mμ

products formed from incubation of cell-free extracts with ribose 5-phosphate and co-factors for 10 minutes and 120 minutes, respectively. The peak near 505 mμ is evidence for the formation of heptulose (Dische, Shettles, and Osnos, 1949). This procedure does not distinguish between gluco-, manno-, and sedoheptulose. The increase in optical density at 600 mμ indicates an increase in hexose.

Quantitative procedure. Figure 10 shows the results of analysis, using the orcinol reagent (Brown, 1946) on the products formed after various intervals of incubation of ribose 5-phosphate and cell-free extracts. There was a decrease in pentose, an increase in hexose and the appearance of heptulose. Hexose concentration was not zero initially because the enzyme extracts contained hexose. Percentage recovery, based on the sugar analysis at zero time, is included at the top of the graph. The formation of triose during the reaction could account for the decrease in percentage recovery during the first 30 minutes of incubation; however, no triose analysis was made. Heptulose reached an equilibrium concentration after about 40 minutes of incubation when about half of the pentose had been utilized. The synthesis of heptulose was confirmed by paper chromatography using the Klevstrand spray (1950) and comparison with the

Figure 10. Quantitation by the orcinol procedure, of the sugars present in a reaction mixture after various intervals of incubation. The reaction mixture was as described for Figure 8 except that a cell-free extract from a 20 hour old culture was used, and 1 ml of a 1/20 dilution was used for analysis.


PER CENT
100
90
80
RECOVERY
420
360
300
240
180
120
60
0
CONCENTRATION: uM x 10^3
PENTOSE
HEXOSE
HEPTULOSE
0
24
48
72
96
120
INCUBATION TIME, MINUTES

color obtained with sedoheptulose monohydrate.

Warburg studies. Table 5 shows comparative QO_2 values for endogenous and exogenous metabolism with glucose, fructose and caproate as substrates. Stimulation of oxygen uptake by glucose decreased during germination and reached a minimum in 7-9 hour old hyphae, when no stimulation was detected. During the same period, stimulation of oxygen uptake by fructose and caproate increased and reached a maximum in 7-9 hr hyphae. Old coccoids which have much lower endogenous metabolism were stimulated about equally by both glucose and fructose with caproate stimulation slightly higher. The ratio of QO_2-glucose to QO_2-fructose was not constant during the growth cycle (Fig. 11). The ratio approached one in 96 hour old coccoids and diminished to zero in hyphae.

Pigmentation

Extraction. Nocardia corallina has a high fat content and the pigment is difficult to extract. Continued extraction in a Soxhlet extractor with solvents such as alcohols, dimethyl ether and petroleum ether left much of the pigment still in the cells. Benzene and chloroform gave better results, but to be effective dried cells had to be used. Acetone worked effectively, especially if the cells were

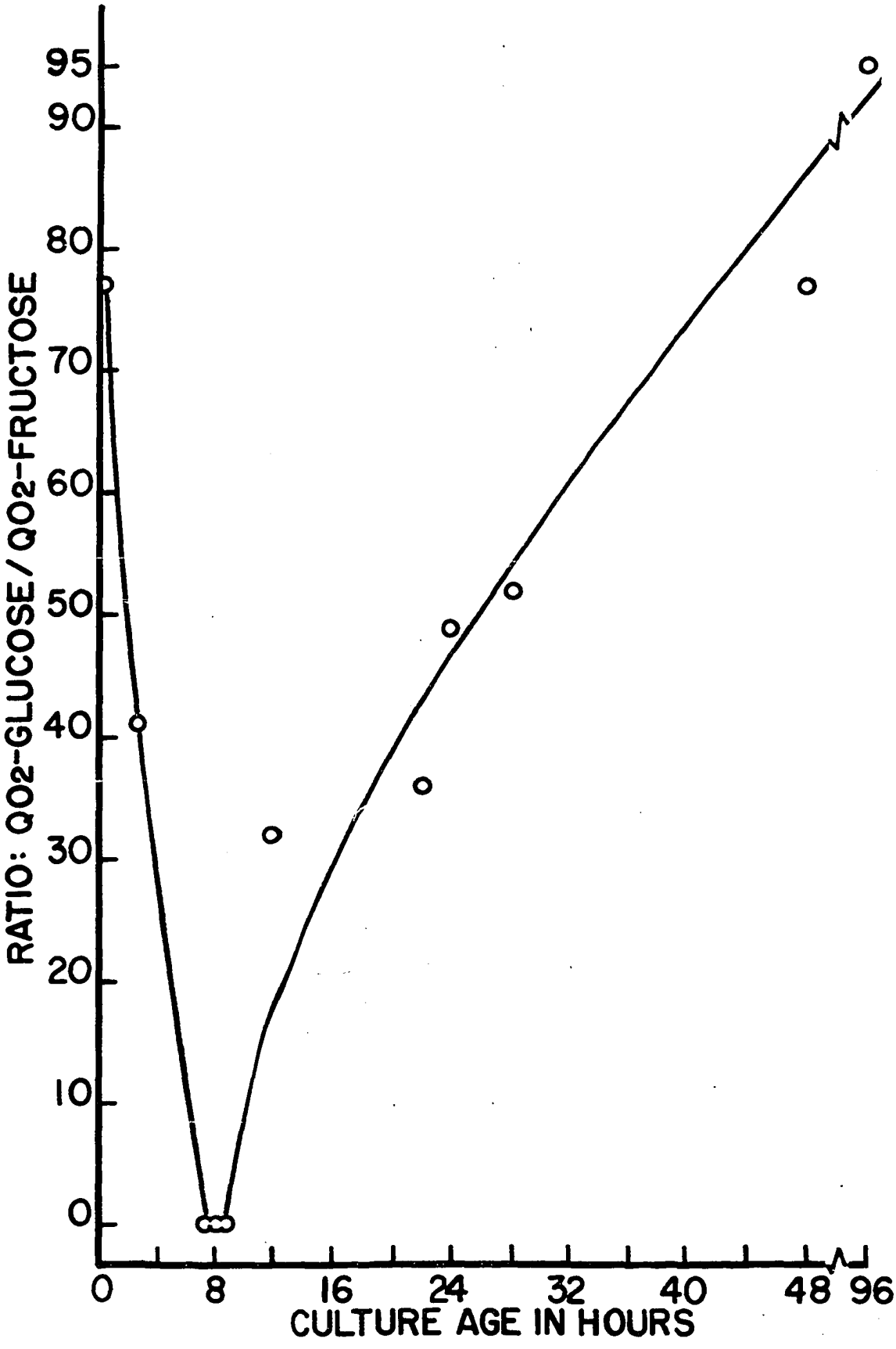
TABLE 5

Comparison of glucose 6-phosphate dehydrogenase activity in cell-free extracts from cultures of various ages

Culture age in hours	mg protein in reaction	Activity*
8	1.8	12.7
22	1.7	11.6
24	2.7	3.9
48	1.6	21.9

*Assay conditions were as described in Figure 1. Dehydrogenase activity was recorded as units of optical density change per minute per mg protein.

Figure 11. The ratios of QO_2-glucose to QO_2-fructose for cells taken from cultures of various ages.


RATIO: QO2-GLUCOSE / QO2-FRUCTOSE
95
90
80
70
60
50
40
30
20
10
0
0
8
16
24
32
40
48
96
CULTURE AGE IN HOURS

repeatedly frozen and thawed in a dry ice-acetone bath. The addition of acid or base to the extraction solvent led to essentially quantitative removal of the pigment from the cells. The use of base, as indicated in the extraction procedure, did not result in any changes in the visible absorption spectra of the pigment when compared to that obtained by extraction with neutral solvents. In the described procedure, saponification and extraction are carried out in the same step.

In preliminary studies, crude pigment extracted with neutral solvents and partitioned between 90% methanol and petroleum ether by the procedure of Sobin and Stahly (1942), showed the pigments to be epiphasic before, and hypophasic after saponification. By comparison with the behavior of carotenoids (Karrer and Jucker, 1950) this indicates that the pigment may be connected to two or more fatty acids by ester linkages which are broken by the hydrolysis conditions to leave free hydroxyl groups on the pigment, thus increasing its solubility in polar solvents.

Chemical and physical properties of the pigments. During the course of the purification procedure, a number of substances were isolated which were not of primary interest in this study of the pigments. The absorption spectrum of some of these substances was recorded, and little if any

work was done with them.

The red pigment which was separated on the magnesia column and crystallized from methanol was soluble in non-polar solvents such as hexane, benzene and carbon tetrachloride as well as polar solvents such as alcohol and water. The pigment was soluble to the extent of approximately 5% in carbon tetrachloride and was even more soluble in water. Water solutions were basic and upon the addition of HCl, the pigment was precipitated. Comparative infrared spectra of the pigment before and after acid precipitation by careful addition of dilute HCl to a chilled solution of the pigment is shown in Figures 12-A and 12-C, respectively.

Table 6 shows the results of analysis, a summary of chemical tests, and the absorption maxima in a number of solvents for the red pigment. The red pigment reacted slowly with 2:4 dinitrophenylhydrazine to form a yellow, insoluble precipitate which was washed three times with 95% ethanol and its infrared spectrum determined (Fig. 12-D). The precipitate, presumably a phenylhydrazone, had a molecular weight of approximately 264 (Rast) and a melting range of 122-124 C (uncorrected). This information indicated that the phenylhydrazone formed after oxidative cleavage of the pigment into small fractions.

TABLE 6

A summary of some of the properties of the red pigment isolated from Nocardia corallina

Visible Absorption Spectra:

Solvent	Absorption maxima
methanol	465
absolute ethanol	465
normal hexane	470
chloroform	472
carbon tetrachloride	475
benzene	476
carbon disulfide	497

Color Reactions:

Carr-Price Test--Blue; $FeCl_3$ Test positive for phenolic group
Con. H_2SO_4--Bluish-green interfacial ring; slight blue-violet in the acid layer. Upon addition of water, the ether layer became blue-green
Aqueous HCl--Pale blue color in acid layer

Partition between petroleum ether and 90% methanol--
Pigment almost entirely epiphasic before saponification
Pigment almost entirely hyphophasic after saponification

Melting range--186-189 C (uncorrected)

Optical Activity--
No optical activity in benzene solution using the D-line of sodium

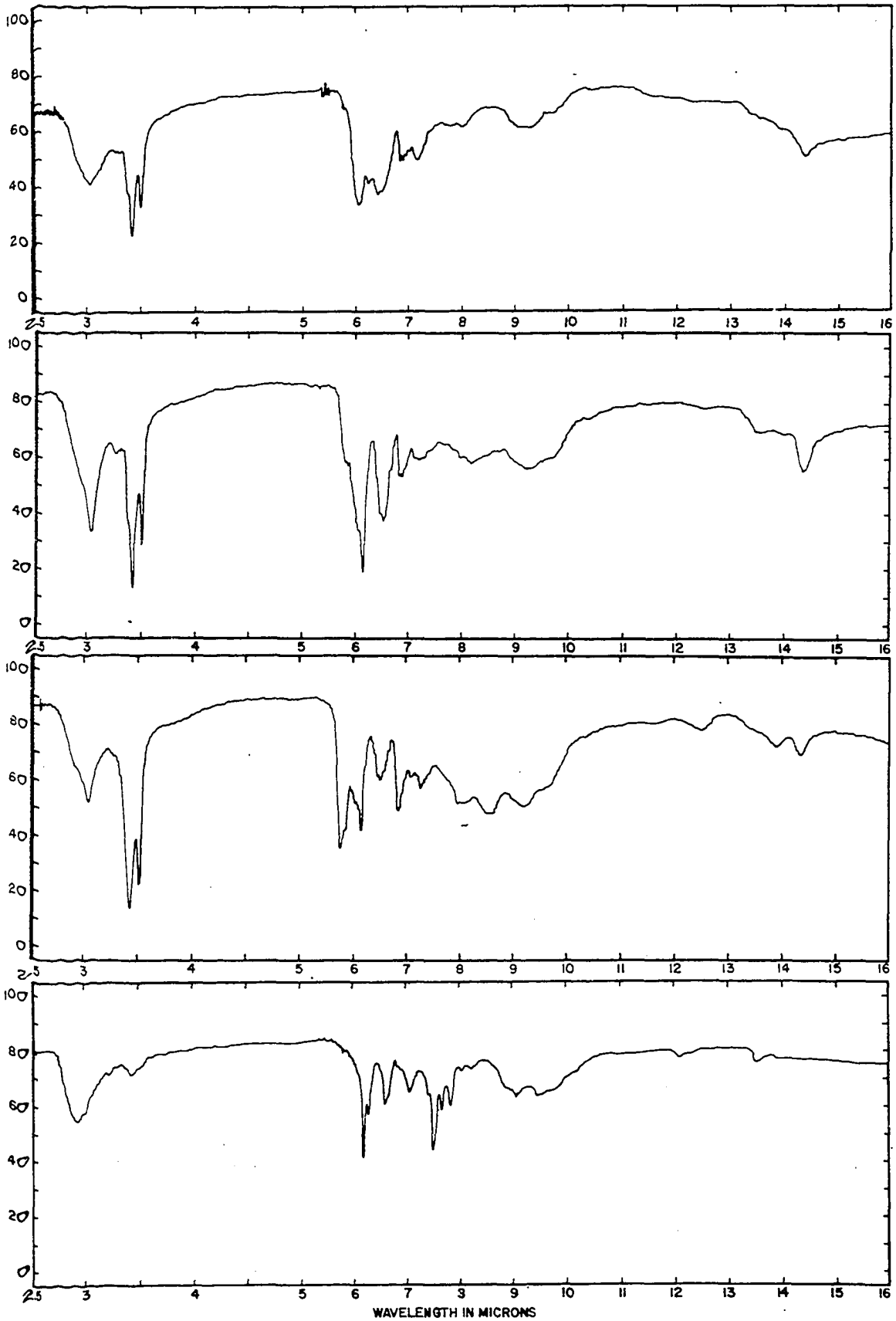
Tightly adsorbed to magnesia column (Fischer, Sea Sorb 43 and Hyflo Super Cel, 1:1) from benzene solution

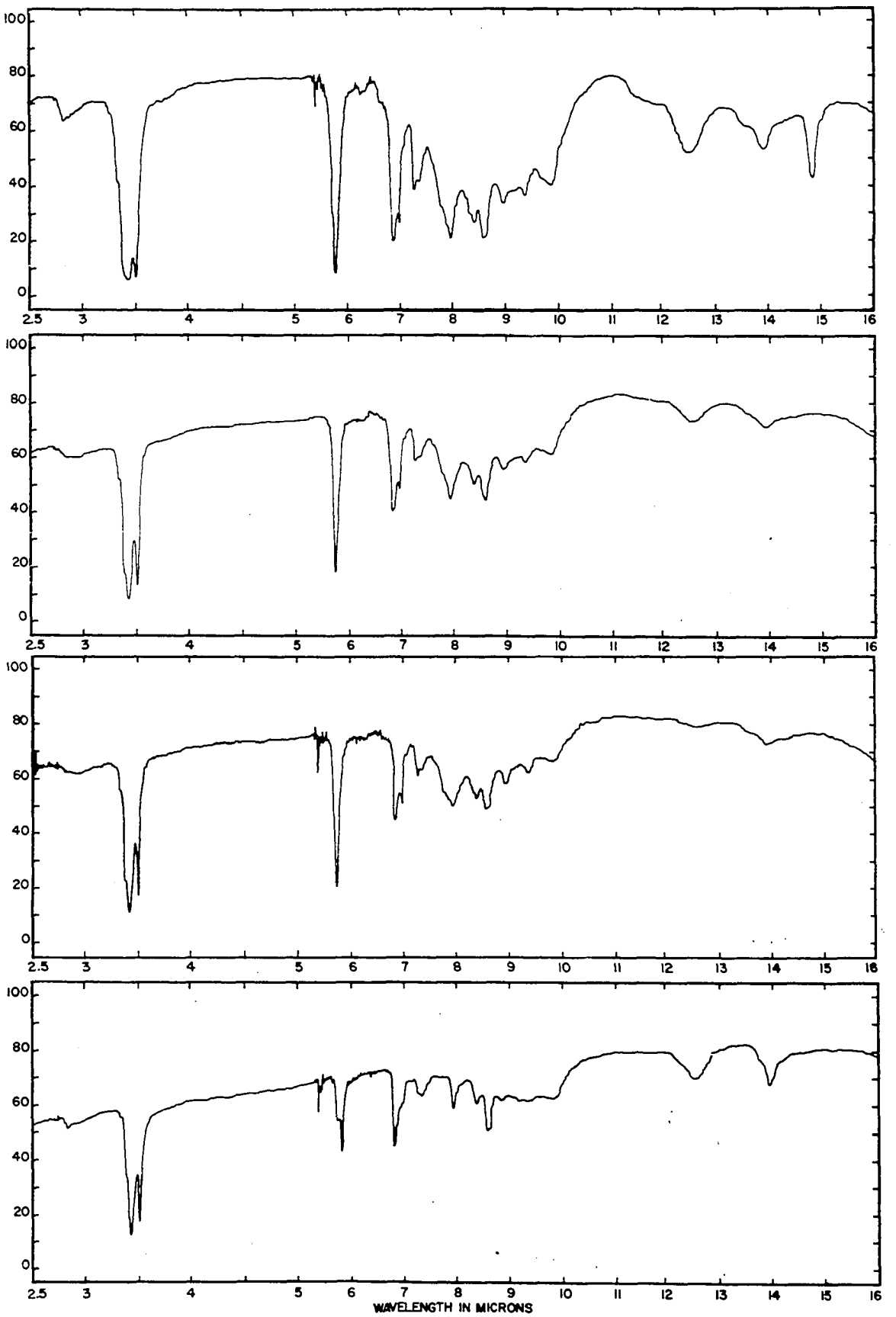
The pigment is relatively stable in air before and after saponification

Results of chemical analysis: C, 62.6; H, 9.0; N, 5.0, and C-methyl group 3.7. Molecular weight was 893.

Figure 12. Infrared spectra of the red pigment isolated from Nocardia corallina. The spectra, in order from the top down, were obtained from: (1) the crystalline, red pigment after extraction with saponification conditions, and separation by column chromatography, (2) the red pigment after precipitation by addition of strong HCl, (3) the red pigment after precipitation by careful addition of 0.1 N HCl with chilling, and (4) the product formed when the red pigment was reacted with 2:4 dinitrophenylhydrazine.

Figure 13. Infrared spectra of the yellow pigment and a related compound isolated from Nocardia corallina. The spectra, in order from the top down, were obtained from: (1) the yellow pigment after separation from the red pigment by column chromatography, (2) the yellow pigment after rechromatigraphing and removing a purple component with a high absorption in the ultra violet region, and (3) the yellow pigment further purified by the removal of the white, low melting point oil shown in (4).


100
80
60
40
20
0
2.5
3
4
5
6
7
8
9
10
11
12
13
14
15
16
WAVELENGTH IN MICRONS


100
80
60
40
20
0
2.5
3
4
5
6
7
8
9
10
11
12
13
14
15
16
WAVELENGTH IN MICRONS

Summary of the properties of the yellow pigment.

Less work has been done with the yellow component of the pigment, but the infrared spectra (Fig. 13a-c) indicate similarities to the red pigment. Figure 13-d shows the infrared spectra of a white, low melting point (approximately 25 C), waxy material which was separated from the yellow pigment as a methanol insoluble precipitate. This material was also insoluble in acetone. Its abosrption spectra indicates similarities to the spectra of the pigments.

CHAPTER IV

DISCUSSION

Morphogenesis

Evidence presented in this paper indicates that fragmentation does not occur as a sudden, en masse transformation from essentially coenocytic hyphae to bacillary cells by the simultaneous production of multiple cross-walls. Rather, fragmentation begins with the production of a single cross-wall per hypha and results in separation into two hyphae which continue to elongate. The incidence of cross-wall formation increases during the period of hyphal growth and results in a significant increase in cell numbers. Multiple cross-walls form in the last stages of the hyphal phase and under some conditions, the hyphae fragment into rather uniform cultures of bacillary cells.

Webb (1956) published evidence which shows that cross-wall formation precedes fragmentation, and some evidence was presented to show that cell plates are formed

between dividing nuclei. Hagedorn's published electron micrographs (1959a) show that cell-wall material is deposited inward by a centripetal growth of cell-wall material but do not establish the mechanism which orients the position where the cross-walls form. If cross-wall formation does occur only between dividing nuclei, as the work of both Webb and Clark (1956) and Hagedorn (1959a and 1959b) indicates, then fragmentation of hyphae results in the formation of several binucleate cells and two uninucleate cells from each hypha. The continuation of multiplication by nuclear division accompanied by cross-wall formation would, within a few generations, produce a culture which was predominantly uninucleate since the number of binucleate cells would remain constant, and the number of uninucleate cells would increase at a greater than logarithmic rate.

Any acceptable theory for the growth cycle must propose a mechanism for the production of uninucleate coccoids from multinucleate hyphae. In the previously proposed life cycle (Webb, 1956), these cells were postulated to arise by nuclear fusion of binucleate bacillary cells. On the basis of radiation sensitivity determinations and cytological observations, the bacillary cells were reported to be haploid.

Theories should be subjected to experimental tests

and their validity can be established only by such procedures. Certainly, the usefulness of any theory or hypothesis is measured by the extent to which observations are predicted or explained by its application. This study substantiates, in part, the previously proposed life cycle. However, certain discrepancies were noted which need to be considered in relation to the overall growth pattern. Plate counts, turbidity curves, observations of stained slides, and photographs of growing cultures, indicate that much of the increase in mass and numbers occurs during the hyphae phase by fragmentation or by hyphal division.

In addition, the cytological evidence for fusion is subject to other interpretations. The structures described as fusion tubules are observed only infrequently, and frequency of occurrence is not correlated with the stage of growth when the bacillary cells predominate. These structures are most often observed during the hyphae stage, before reduction division was proposed to occur. Since branching of hyphae does occur, and during the early stages of formation the branches are small in diameter, it appears that such structures might appear to connect two separate cells because of their orientation on the slide. The cytological evidence for fusion of the two nuclei within a single bacillary cell

could as easily be interpreted as an advanced stage of nuclear division. The two processes, an early stage of nuclear division and a late stage of nuclear fusion, might be very similar in appearance.

The primary evidence to support the proposed ploidy change was the increased radiation sensitivity of the binucleate bacillary cells. The increase in radiation sensitivity of these cells was explained on the basis that the nuclei were haploid, using the unpaired defect theory of Tobias (1952). Applying this theory, it was proposed that these haploid bacillary cells would be more sensitive to radiation because genetic damage would not have to be paired to be lethal. The unpaired defect theory does not appear to be sufficient, however, to substantiate a haploid condition for these bacillary cells since they are binucleate and the next proposed step in the life cycle (fusion) would bring together a double set of genetic material within one nucleus again. It can be seen that even if these cells are haploid, they contain a double set of genetic material since they are binucleate. In order for the theory to explain the increased sensitivity it appears necessary to make the additional assumption that genetic fusion was prevented in a sufficient number of instances to account for the increased sensitivity.

Evidence has increased which indicates that lethal radiation damage may occur through means which do not envolve genetic mechanisms. For example, Lucke and Sarachek (1953) reported that evidence indicated a complicated interrelationship of genetic and non-genetic effects may be envolved. In view of these facts, it is suggested that the proposed ploidy change for this organism should be reinvestigated.

Metabolism

The data presented indicate that cell-free extracts of Nocardia corallina contain enzymes for the conversion of ribose 5-phosphate into ketopentose, heptulose and hexose as well as TPN specific dehydrogenases for glucose 6-phosphate and gluconic acid 6-phosphate. The findings are consistent with the operation of a pentose pathway, but the importance of this pathway in the overall metabolism was not established.

During germination, specific activity of glucose 6-phosphate dehydrogenase decreases while both endogenous and exogenous QO_2 due to fructose or caproate increases to a maximum. Old coccoids have a much lower endogenous metabolism and QO_2 values for both fructose and caproate are also lower than in hyphae. The ratio of QO_2-glucose to QO_2-fructose approaches one in 4-day coccoids. It appears that either or

both the pentose cycle and the Kreb's cycle activities reach a maximum during hyphal development. During this time glucose utilization decreases to zero. Since fructose is utilized, it appears that these cells are impermeable to glucose; or if permeable, then glucose is either not phosphorylated, or not converted to fructose 6-phosphate.

The significance of this difference between the stimulation of oxygen uptake by glucose and fructose can not be fully evaluated at this time. However, glucose stimulates oxygen uptake to the maximum extent in old coccoids which have a relatively high dehydrogenase activity, low endogenous metabolism, and are reported to synthesize and store fat in discrete bodies within the cell (Clark and Aldridge, 1960). Fat synthesis is known to occur by a process which requires reduced TPN (Guyton, 1961a), and two molecules of reduced TPN are produced for each molecule of CO_2 released by the pentose cycle. Recent work (Siperstein and Fagen, 1957; Winegrad and Renold, 1958a, 1958b; Siperstein and Fagen, 1958a, 1958b; and Siperstein, 1959) supports the hypothesis that the availability of reduced TPN may limit the rate of lipogenesis. Future experiments are planned to determine if pentose cycle activity is correlated with fat synthesis and the growth cycle of Nocardia corallina.

The 7-9 hour old hyphae show certain similarities to cells from diabetic animals in which lack of insulin prevents utilization of glucose, either because glucose does not penetrate the membrane, is not phosphorylated, or both (Guyton, 1961b). In the diabetic, there is increased fat utilization and decreased fat synthesis, and evidence indicates that the decreased fat synthesis may result because of lack of reduced TPN from low pentose cycle activity. The inability of 7-9 hour old hyphae to oxidize glucose also appears to occur at an early step in the metabolism of glucose, and appears to envolve either entrance, or phosphorylation of glucose or conversion of glucose 6-phosphate to fructose 6-phosphate. In addition, these cells (7-9 hour old hyphae) show a high ability to oxidize fatty acid and appear to be utilizing stored fat.

Pigmentation

The preliminary assumptions that the pigment might be a carotenoid are not substantiated by the chemical and physical data accumulated for the red pigment. Less information is available about the yellow pigment; however, from a consideration of the data available, it appears that it may have a similar structure. Although the red pigment does give

a blue color with the Carr-Price test, this may be misleading because the test is not specific for carotenes (Karrer and Jucker, 1950). Both the red and yellow components show only a single peak in the visible region of the spectrum; carotenoids, with the exception of those which contain cross-conjugated carbonyls, have two or three maxima in the visible region (Karrer and Jucker, 1950). Although the infrared and phenylhydrazone test indicate that the carbonyl group is present, there is no color change in acid solution which would indicate that the carbonyl is not conjugated with the chromophore (Karrer and Jucker, 1950). The molecular weight, nitrogen content, and lack of trans double bond absorption in the 965 cm^{-1} region (Bellamy, 1958) would appear to eliminate the possibility that this pigment belongs to the carotenoid group.

Because of the high molecular weight of the red pigment it is difficult to make strict interpretations from the data available. The infrared spectra together with the chemical analysis and physical data indicate that the molecule includes a non-polar part composed of carbon and hydrogen and a polar part envolving nitrogen and carbon-oxygen bonds. A search of the literature has not revealed any pigment class to which this compound can be shown to be definitely related.

The strong similarities between the infrared spectra of the yellow and the red component indicate similarities in structure. A pigment with a similar visible absorption spectrum was reported by Turian (1950) from Mycobacterium phlei, but the pigment was not chemically identified.

CHAPTER V

SUMMARY AND CONCLUSIONS

Morphogenesis

Evidence was presented to indicate that the growth cycle of Nocardia corallina can be divided into four phases, primarily on the basis of a changing ratio of rate of cross-wall formation to growth and nuclear division. These phases are: I. Germination to produce long, essentially coenocytic hyphae; II. a period when the relative frequency of cross-wall formation increases, resulting in the formation of hyphae of various lengths which continue to elongate from the tips; III. a phase when cross-wall formation occurs with virtually each nuclear division, resulting in the production of bacillary and coccoid forms, with coccoid forms predominating during the last stage of this phase and; IV. a terminal phase composed of resting cells, many of which appear to be binucleate.

The incidence of cross-wall formation was found to be stimulated by factors which accumulated transiently in the growth medium during phase III. The accumulation of the factors coincided with the phase of rapid cross-wall formation and was missing from cultures in phase I or IV. The rate of accumulation of the factor appeared to be a function of population density. Growth in broth resulted in an extension of phase II and a delay in phase III, possibly by dilution of the factor.

The previously proposed life cycle (Webb, 1956) was discussed in relation to data presented in this paper, and alternate interpretations were suggested for some of the data previously interpreted as evidence for a ploidy change.

Metabolism

Cell-free extracts of Nocardia corallina were shown to contain enzymes for the conversion of ribose 5-phosphate into intermediates of the pentose cycle, as well as TPN specific dehydrogenases for glucose 6-phosphate and gluconate 6-phosphate. These data are consistent with the operation of a pentose cycle in the organism but the extent of operation of the pathway was not established.

Differences in the metabolism of glucose and fructose

were discussed in relation to the life cycle. It was proposed that the inability to use glucose by 7-9 hour hyphae may be due to impermeability or lack of phosphorylating ability in these cells. It was suggested that pentose cycle activity may be correlated with fat metabolism although insufficient data are available to substantiate this proposal at this time.

Pigmentation

Procedures for the isolation and purification of the pigments were outlined. The pigment appeared to contain only two major colored components, which were separated as a yellow oil and a red crystalline compound. It was not possible to identify the pigments with any known compounds on the basis of the spectral and chemical analyses performed. However, substantial evidence was presented to indicate that the pigments are not carotenoids. Analysis of the red pigment showed a molecular weight of 893 with 62.59% carbon, 8.99% hydrogen, 5.01% nitrogen, and 3.72% C-methyl groups. Infrared spectra and other evidence indicated the presence of a carbonyl group which was not conjugated with the chromophore; carbon-hydrogen and nitrogen-hydrogen stretching and possibly hydroxyl groups.

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