FOREWORD

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ABSTRACT

Cytochemical analyses of the fungus, Aspergillus niger ATCC 6275, have revealed the presence of chitin in the longitudinal and cross walls of the hyphae, chitin in the primary lamellae, and both chitin and cellulose in the secondary lamellae of the walls of conidiophores, vesicles, and conidia. Similar analyses have shown the presence of chitin alone in the walls of the corresponding cells of the fungus, Myrothecium verrucaria ATCC 9095. Adaptations of standard cytochemical procedures to the identification of cell wall materials in the bacterium, Pseudomonas aeruginosa ATCC 13388, have indicated the probable presence of chitin in the primary, and cellulose in the secondary, wall lamellae. Attempts to identify cell wall materials in the bacterium, Escherichia coli K12 10798, have shown definite differences in the make-up of the primary and secondary wall lamellae and the possible presence of cellulose in the secondary deposits. These four organisms are among those which contaminate aviation fuels. Information concerning the nature and location of the resistant substances which make up their cell walls can be used in developing methods of treatment which will lead to their control.

PUBLICATION REVIEW

This technical documentary report has been reviewed and is approved.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

INTRODUCTION

Our primary objective was to develop a new approach to the control of fungi and bacteria which contaminate aircraft fuels. The first step in this approach involves the accumulation of information concerning the chemical make-up of the cell walls of four of the contaminating organisms, with particular emphasis upon such resistant wall substances as chitin and cellulose.

The method of study is necessarily cytochemical, since the data obtained must cover not only the nature of the substances which make up the walls of the cells, but also the localization of these materials in any given cell wall with respect to one another and with respect to the protoplast.

Cytochemical analyses require, in many instances, chemical conversion of the wall materials under investigation. The procedures are carried out in such a way as to bring about these conversions without immediate destruction of the cell wall. The reactions mechanism involved is frequently hydrolysis, and the hydrolytic agents must be applied in such a way that the degredation products can be observed microscopically and identified in the relative arrangement of the unhydrolyzed counterparts in the untreated cell walls. Such cytochemical data enable the experimenter to reconstruct a fairly accurate presentation of the chemical and physical make-up of the cell walls of any given organism in the living state. This information, in turn, indicates the types of reagents which could be expected to attack the known cell wall substances without injury to the medium in which, or upon which, the fungus or the bacterium are growing.

The four common organisms which have been studied cytochemically, in connection with this project, are comparable to some of those which have been found in jet fuels and are identified as follows:

Aspergillus niger ATCC 6275*
Myrothecium verrucaria ATCC 9095
Escherichia coli K12 ATCC 10798
Pseudomonas aeruginosa ATCC 13388

* American Type Culture Collection
2112 M Street, N. W.
Washington, D. C.

PREVIOUS INVESTIGATIONS OF FUNGAL CELL WALLS

In 1811, Braconnot (7) gave the name fongine to an insoluble residue which he obtained by treating fungi with hot alkali. Payen (45), in 1843, determined the presence of nitrogen in this residue and commented upon the failure of the samples to give the reaction characteristic of cellulose with sulphuric acid and iodine. In 1866 de Bary (2) found that true cellulose is present in a number of fungi, but that in most fungi there is a peculiar type of substance which he named pilzzellulose or fungal cellulose. Richter (51) in 1881 reported that fungi do not have a special type of cellulose in their cell walls; that the normal cellulose in fungal membranes is encrusted with other substances, probably protein; and that typical cellulose reactions are obtained in these membranes after maceration in 7 to 8 per cent potassium hydroxide for from two to three weeks to two to three months.

During the last decade of the nineteenth century, the accumulated information concerning the composition of fungal cell walls was subjected to a critical review and important experimental work in this field was initiated. In 1823 Odier (43) had discovered a substance in the wing covers of May beetles which he named chitin. In two series of papers, published by Winterstein (70, 71, 72, 73) and by Gilson (25, 26, 27), between 1893 and 1899, there is found the experimental evidence which led the authors to conclude, independently, that in fungi there is a substance similar to, if not identical with, the chitin which had been identified in various arthropods such as insects, lobsters and crayfish. In 1898 van Wisselingh (74) published the first extensive survey of fungi for the purpose of detecting the presence of chitin in their cell walls, using a technique which had been developed almost a half century earlier. Rouget (53) had discovered in 1859 that arthopod chitin which had been boiled in concentrated potassium hydroxide solution became soluble in dilute organic acids. He described this product as "modified chitin" and found that the modified chitin was colored violet by dilute solutions of iodine and acid, whereas chitin was colored brown. The name chitosan was given to Rouget's modified chitin in 1895, by Hoppe-Seyler (33), and van Wisselingh (74) adapted the chemical procedures for producing modified chitin to a cytochemical method for the identification and localization of chitin in fungal cell walls. This method has become the well known "chitosan reaction". With some slight changes in procedure, and adaptations to the material under investigation, the method used today is essentially that used by van Wisselingh in these first extensive identifications of chitin in the cell walls of fungi.

Van Wisselingh found chitin in the cell walls of higher fungi, almost without exception. In the Bacteriaceae, Peronosporaceae, Saccharomycetaceae and Saprolegniaceae, no chitin was present. Of three Myxomycetes studied, chitin was found in only one. Indicative of the detail with which these cytochemical studies were

made are the statements that in Mucor mucedo, the cell walls of sporangiophores, sporangia, columella and spores contained chitin; in Rhizopus nigricans, a large amount of chitin was in the cell walls of the mycelia, sporangiophores, sporangia, columella and spores, only the wall of the sporangium appearing to contain another substance besides chitin; and, more pertinent to the present report, in Aspergillus glaucus, the hyphae, conidiophores, sterigmata and conidia contained chitin, while in the walls of the perithecia and asci no chitin was found. In the ascospores, a small, biconvex plate of chitin was observed in the walls. In the large number of fungi studied, cellulose was identified only in the walls of certain Peronosporaceae, Saprolegniaceae and in the Myxomycetes. Summarizing his results with respect to the presence of cellulose and chitin, van Wisselingh stated that, in many of the fungi studied, chitin was present in the cell walls; in a smaller number, cellulose was found; in some instances both were absent; and in no single case were chitin and cellulose found together.

Many years later, in 1925, van Wisselingh (76) published a comprehensive review of the existing information concerning the chemical nature of chitin and of its occurrence in the plant kingdom. His own findings, and those of many contemporary workers, were summarized in the statement that chitin had been found to be confined to the higher fungi and Zygomycetes, and to be absent in the Bacteriaceae, Cyanophyceae, Myxomycetes, Algae (with the exception of Geosiphon), Bryophytes, Pteriodophytes and Phanerogams. The efforts which had been made to identify chitin in the cell walls of bacteria were discussed in considerable detail. Viehoever (64), who had obtained positive results with Bacillus alvei, B. subtilis, Sarcina ureae and others, had suggested the possibility that those who had obtained negative results, including van Wisselingh, had failed to adapt the method used in converting chitin to chitosan to the more delicate membranes of the bacterial organism. By heating the bacteria in 50% KOH solution, in an autoclave, at 6 atmospheres pressure for 15 minutes, he had succeeded in identifying chitosan in the bacterial walls. It is of particular interest that in this second and last review by van Wisselingh, he again expressed the earlier viewpoint that chitin and cellulose do not occur in the same fungal cell wall.

The twentieth century has contributed the new and valuable technique of X-ray diffraction to the study of plant cell walls. The resolution of the unit-cell structures of cellulose and chitin have served to point out their differences as well as their similarities. The diffraction data of purified chitin of both plant and animal origin have served to indicate that they are identical, when freed of associated cell wall substances. The structural formulae of chitin and cellulose show that the difference between them is located in one position at which the OH in cellulose is replaced by the NH-Ac group in chitin, the so-called nitrogen-containing carbohydrate. The voluminous literature which has accumulated in

this field of X-ray diffraction analysis of cellulose and of chitin during the past four decades, need be represented by only a limited number of references in the present report: Herzog (31) in 1924; Gonell (28) in 1926; Khouvine (35) in 1932; Farr and Clark (15) in 1932; Meyer and Pankov (40) in 1935; Heyn (32) in 1936; Clark and Smith (11) in 1936 and van Iterson, Meyer and Lotmar (34) in 1936. Noteworthy is the extensive use of the X-ray diffraction technique in the attempts to resolve the controversies concerning the possible presence of chitin in either filamentous or non-filamentous yeasts, or in both: Frey (18) in 1950, Roelofsen and Hoette (52) in 1951.

Chitin, in the natural state in both plants and animals, is commonly admixed with proteins and other substances. In the purified state, it is a colorless solid, insoluble in water, ether, alcohol, and even in concentrated alkali at temperatures of 140° to 180°C. In strong acid solutions it slowly undergoes hydrolysis to glucosamine and acetic acid, in contrast to cellulose which, upon hydrolysis, yields glucose. When treated with saturated potassium hydroxide, at 140° to 180°C, chitin is converted to chitosan. In comparison with cellulose, the products of hydrolytic degredation are as follows:

cellulose	chitin
cellobiose	chitosan
glucose	acetylglucosamine

Concurrently, with the advantages of a better understanding of the properties of both cellulose and chitin, microchemical and cytochemical information concerning the composition and structure of the cell walls of fungi have continued to accumulate.

Thomas (61, 62, 63) in a series of chemical analyses of the hyphae of the Fusaria, Sclerotinia and the Pythiaceae, published from 1928 to 1942, found that chitin and various fatty acids present in fungal cell walls mask the presence of cellulose. The Fusaria which were examined contained small amounts of cellulose and relatively large amounts of chitin in their cell walls. Both cellulose and chitin were likewise found in Sclerotinia and in the Pythiaceae. Callose, pectic material, and protein were also found, in association with the cellulose, chitin and fatty acids, in the mycelia examined. Concurrently, Nabel (42) discovered both cellulose and chitin in the wall of the sporangium of Rhizidiomyces bivellatus, and this result was confirmed by Locquin (36) in 1943. In 1954, Farr (17) reported the presence of both cellulose and chitin in the conidiophores of Aspergillus niger and A. carbonarius, along with protein, callose and an indication of fatty acid in the conidiophore wall of A. carbonarius. More recently, Fuller and

Barshad (19) and Fuller (20) have reported the presence of both cellulose and chitin in the cell walls of Rhizidiomyces sp. The findings of these authors serve to emphasize the importance of cytochemical studies in detecting and localizing cellulose and chitin when they are present in the same cell wall. They also extend into the field of desired information outlined by Thomas (61, p. 547) as follows:

> "No theory is advanced at this time regarding the probable origin and order of development of the cell wall. More detailed study is necessary in following closely the progress of growth before this can be done. More critical microchemical methods would greatly facilitate such an investigation."

The cytochemical approach to the study of fungal cell walls necessarily involves large numbers of cell samples in successive stages of development and also the identification of any given substance in the presence of one or many other substances. It is becoming quite generally recognized, however, that this type of approach represents the one most likely to furnish information which can be used as a foundation for the development of methods of control of fungal growth, when problems of contamination and deterioration are concerned.

CYTOCHEMICAL ANALYSES OF Aspergillus niger ATCC 6275

Aspergillus niger ATCC 6275 (NRRL 334) is a typical black Aspergillus found commonly in routine examinations of soil and moldy materials. It has been chosen as one of the organisms to be analyzed under the terms of this contract because it is considered to be comparable to the black Aspergillus which has been found in jet fuel.

Another black Aspergillus has been under investigation in these laboratories for a number of years, and the results of these analyses are now being prepared for publication. The first culture of this organism was furnished by the late Dr. Charles Thom with the designation Aspergillus niger No. 4707.878. In "A Manual of the Aspergilli", Charles Thom and Kenneth B. Raper, London, Balliere, Tindall and Cox, 1945, pp. 227 and 228, it appears under the designation, Thom No. 4707.878, NRRL No. 67, with the following comment:

"Since strain '67' appears in the industrial fermentation literature as Aspergillus niger and has been consistently distributed under this name over a period of several years, it is not our purpose to challenge this designation, for this binomial is often used in a very general sense to cover any black member of the group. We do wish to emphasize, however, that this strain does not represent the common type of black Aspergillus usually isolated in routine examination of soil and moldy materials in general. The fact that it possesses large spores is of the greatest value in checking its purity and further commends it for use in industrial operations." Because of the size characteristics indicated, falling between the typical A. niger and A. carbonarius*, and since the culture Thom No. 4707.878, NRRL 67, had been contributed by De Fonseca of Rio de Janeiro, the alternate name of Aspergillus fonsecaeus 4707.878, NRRL 67 is suggested by the authors. They point out also that it appears likely that A. fonsecaeus 4707.878, NRRL 67 represents a type of organism which is more abundant in South America than in this country. It is a matter of record, therefore, that this fungus differs with the common Aspergillus niger ATCC 6275 (NRRL 334) in both point of origin and in the sizes of its constituents cells. There is no available evidence, however, concerning either differences or similarities in the chemical make-up of the cell walls in these two strains of black Aspergilli.

The differences in the dimensions of the cells in these two strains may be summarized as follows:

* Personal communication from Dr. Charles Thom.

	"ATCC 6275"	"NRRL 67"
	(size in microns)	
Conidial Heads	300 - 500	300 - 1000
Conidiophores	200 - 400 x 7 - 10	1500 - 2500 x 20 - 30
Vesicles	20 - 75	50 - 75
Sterigmata:		
Primary	20 - 30 x 6 - 8	30 - 45 x 10 - 13
Secondary	6 - 10 x 2 - 3	8 - 14 x 5 - 6.5
Conidia	2.5 - 4	5 - 9

The development of methods for the control of certain types of bacterial and fungal growth, which represents the ultimate object of this research must be based upon the chemical composition of the cell walls of bacteria and fungi which are known to be comparable to those which contaminate aircraft fuels and deteriorate materials. There is no justification, therefore, for the use of cytochemical data which have been obtained from strain NRRL 67 in the development of a control method for strain ATCC 6275. Preliminary studies of Aspergillus funigatus and Aspergillus carbonarius, NRRL 369 have shown, however, that the Aspergilli are likely to have a number of points of likeness in the composition and localization of the substances in their cell walls. This information is valuable in planning the study of any new member of the Aspergillus group, and has been used in selecting the techniques to be applied to strain ATCC 6275 in determining the chemical make-up of its cell walls, at successive stages of development.

Material and Methods

The culture of Aspergillus niger ATCC 6275 was obtained through the courtesy of Dr. Carl Wessel of the Prevention of Deterioration Center, National Research Council, National Academy of Sciences, Washington, D. C. The cultures were grown on Czapek Solution Agar, at room temperature. Growth was abundant and fungal cells from hyphae to conidia, at successive stages of development, have been available for cytochemical analyses. Petri dish cultures have facilitated the harvesting of cells for use in examination of the untreated as well as the treated material.

a. van Wisselingh Color Test:

The three Aspergilli which have been studied previously were found to have large amounts of chitin in the primary layers of the wall of their various types of cells. The use of the van Wisselingh color test for the identification and localization of chitin was, therefore,

indicated. In Aspergillus niger ATCC 6275, treatment of the cells of all types in saturated KOH at 160° for two hours was found to be adequate for the conversion of chitin to chitosan. Following this conversion, treatment of the cells with 95% alcohol for 15 minutes to harden the chitosan, was followed by washing with water to remove the alcohol and then the application of aqueuos I_2KI solution and a trace of 1% H_2SO_4 solution. After a period of 10 to 15 minutes, all chitosan, in the same relative position occupied by the chitin in the untreated cell wall, was colored a deep red-violet color.

b. Payen's Sulphuric Acid-Iodine Reaction for Cellulose:

The secondary lamellae of the cell walls of the various types of cells in A. niger NRRL 67 and in A. carbonarius NRRL 369 were found to be composed, in part, of cellulose, with the exception of the cell walls of the hyphae. The thin, lateral walls of the hyphae appeared to have little or no secondary deposits and they, as well as the thicker cross walls, were found to be composed largely of chitin. By treatment of the other types of cells for a long period of time (2 months to 3 months) with a 72 % solution of H_2SO_4, followed by the addition of I_2KI solution, the presence of and the localization of cellulose in the secondary wall lamellae could be determined. In these preparations the cellulose appears in the form of small granules, deposited on the inner surface of the primary lamella, and surrounded by chitin. The yellow coloration of the continuous phase of the chitinous material, in the secondary deposits, is in sharp contrast to the blue coloration of the particles of cellulose distributed through the chitinous matrix. These results have served to indicate that a hydrolyzing reagent applied to the cell wall would have to pass through not only the chitin of the primary layer, but also through the chitin extending through the entire depth of the secondary deposits, before reaching the protoplast. These identifications and localizations of chitin and cellulose served to explain the delayed reactions to the strong solution of sulphuric acid.

With the exception of this same lengthening of the period of treatment in the solution of 72% sulphuric acid, no changes and adaptations of the H_2SO_4 - I_2KI method for the identification and localization of cellulose have been found to be necessary in the study of the cells of A. niger ATCC 6275.

c. Double Refraction in Polarized Light:

The earlier examinations of the cells of A. niger NRRL 67, and of A. carbonarius NRRL 369, in polarized light, brought out the fact that, in these two Aspergilli, the chitin is weakly birefringent and the cellulose more strongly birefringent. This technique was of particular value in studying the cell walls at successive stages of

development. The progress of the deposition of cellulose in the secondary lamellae could be followed with considerable precision by the gradual increase in intensity of the double refraction in these areas of the cell walls. This same technique has been used upon the developing cell walls in the current studies of A. niger ATCC 6275.

d. Fluorescence in Ultra-violet Light:

In the cells of A. niger NRRL 67 and of A. carbonarius NRRL 369, the fluorescence of cell wall materials had been observed in all types of cells, mounted in 0.1 M sucrose, at successive stages of development. This optical method produced an appearance in the cells which was very nearly the opposite of their appearance in polarized light, since the chitinous areas of the wall produces a more pronounced fluorescence than the cellulose. The fluorescence of cell wall materials in Aspergillus niger ATCC 6275 have been studied in a similar manner.

e. Enzymatic Hydrolysis:

Following the identification and localization of chitin in the cell walls of A. niger NRRL 67 and A. carbonarius NRRL 369, the final confirmation of these findings was obtained by hydrolysis of the chitin with the appropriate enzyme. The enzyme to be used as a cytochemical reagent in this instance was chitinase and it occurs in the juice of the puffball, Calvatia gigantea. The extracted juice was dialyzed in the presence of 0.08 M acetate buffer, at pH 5.0, and applied, without dilution, to the fungal cells. Hydrolysis of the chitin and disruption of the cell walls took place within 8 to 10 hours, at room temperature. The retained potency of the puffball juice which had been stored under toluol, at 5°C, for seven months, is indicated in the effect produced upon conidia from A. niger ATCC 6275, in the present experiments with enzymatic hydrolysis.

f. X-ray Diffraction Analyses of Chitin and Cellulose:

The X-ray diffraction analyses of purified hyphae from A. niger NRRL 67 and A. carbonarius NRRL 369 have been found to produce the sharpest diffraction patterns of chitin. This, in turn, corroborates, the results obtained by means of other techniques, which have shown that there are no secondary deposits of cellulose in the walls of the hyphae. When X-ray diffraction patterns of masses of hyphae with conidiophores, or of conidiophores alone are made, the diffraction lines of both cellulose and chitin appear (17).

Samples of A. niger ATCC 6275 have been purified for X-ray diffraction analysis as follows:

1. mycelium collected when conidiophores are young, before conidia are formed.
2. washed 2 to 4 times with boiling water.
3. treated with 2% HCl, at room temperature for 24 hours.
4. washed, dried and treated successively with alcohol, chloroform and ether.
5. treated with 2% NaOH for 6 hours at room temperature.
6. centrifuged until supernatant liquid was colorless.
7. treated with 1% sodium bisulfite, followed by 2% HCl.
8. washed successively with water, alcohol and ether.

RESULTS

The comparatively small sizes of the cells in Aspergillus niger ATCC 6275 were conspicuous in the earliest microscopical examinations of this fungus. The conidia are characterized not only by their diminutive size, but also by the smooth, spineless surface of the majority of them and the appearance of the black aspergillin, immediately inside of the primary lamella of the conidial wall, at a very early stage of development. These points are illustrated in Figure 1-A. In Figure 1-B, conidia are shown which have incipient spines and ridges in their primary lamellae, and, in Figure 1-C and D, are examples of large conidia with incipient spines and fairly well developed spines respectively. These larger conidia were found very rarely in the many samples of strain ATCC 6275 which were examined. The present cytochemical analyses of conidia were made, therefore, upon cells which in general are very small in size and very dark in color at an early stage of development.

In Figure 2-A, B and C are small conidia which show a positive chitosan reaction. They have been treated with saturated potassium hydroxide for two hours at a temperature of 160°C. The conversion of the chitin to chitosan has been accompanied with little change in cell size, but the color of the conidia is now red-violet instead of black. The color of the aspergillin is destroyed by the treatment with strong alkali, and the red-violet color is the specific reaction of chitosan to I_2KI solution in the presence of a trace of acid. Figure 2-D represents the chitosan reaction in a young conidium of the larger type. Swelling of the primary lamella of the wall has reduced the size of the lumen, but the original size of the cell shows little change.

In Figure 2-E, F and G are shown the reactions of conidia, at different stages of development, to treatment for 30 days with 72% sulphuric acid. Swelling of the wall materials has produced a measurable, but slight, increase in the sizes of the conidia, although the volume of the lumen, in the unpigmented, younger conidia, can be seen to be greatly decreased. The blackness of the aspergillin remains unchanged, and, in the mature conidia, shown in G, it obscures completely any changes in the wall and in the cell lumen. Addition of I_2KI solution to these specimens, at this stage of the treatment with 72 per cent sulphuric acid, did not produce the blue coloration characteristic of cellulose in the cell wall. That cellulose is being deposited in the secondary wall layers, by the protoplast, is indicated, however, by the double refraction of the wall material in the conidia shown in Figure 2, H and I. In the previous cytochemical analyses of A. niger and A. carbonarius (17), the time required for the penetration of the chitin in the primary wall lamella by the 72 per cent sulphuric acid was between two and three months. Only then was the blue cellulose, surrounded by the yellow, partially hydrolyzed chitin, in clear evidence and in sharp distinction. At the end of an equivalent period of time, the addition of I_2KI solution

to the cells of A. niger ATCC 6275 brought out the presence of cellulose in the secondary lamellae of the conidiophores, vesicles and conidia. The lack of any blue coloration in the hyphae which had been subjected to the same treatment again indicated the absence of cellulose in these cells. The presence and localization of both cellulose and chitin have been found to be similar, therefore, in A. niger NRRL 67, A. carbonarius NRRL 369 and in A. niger ATCC 6275. The physical state of the cellulose in these diminutive conidia will require more detailed study before it can be described. If it is in the particulate form, the particles, in turn, surrounded by chitin, the particle size will be smaller than those found in A. niger and A. carbonarius.

The fluorescence of the chitinous primary lamella in the walls of cells from A. niger ATCC 6275 is shown in Figure 3-A to E. The fluorescence of the thin walls of cells in early stages of development can be easily detected, and as the thickness of the wall increases, the fluorescence increases in brightness. This optical behavior is identical with that observed in both of the strains of Aspergillus niger which have been studied previously. The possibility of the presence of callose in the regions of most active growth, and the effect which it might have upon the degree of fluorescence in these areas, has not been established conclusively in any of the Aspergilli examined to date.

The progressive increase in the amount of black aspergillin in the walls of the developing conidia, renders the detection of the fluorescence in any conidia, except the youngest ones, very difficult. This is illustrated in Figure 4. The lateral walls, cross walls and growing tips of the hyphae are seen to be fluorescing brightly, while the fluorescence of only one very young conidium, slightly right of center, can be detected with ease. Closer scrutiny of this photomicrograph, however, reveals the presence of numerous conidia in the walls of which the fluorescence is almost completely quenched by the aspergillin. While observing the fluorescence in these specimens, it was noted that the blackening conidia became clearly visible when they were resting upon a thick mat of fluorescing hyphae. This appearance is shown in Figure 5. One large conidium and several smaller ones, in various stages of darkening with aspergillin, are well defined.

The three stages of conidiophore and vesicle development shown in Figure 6 serve to indicate the value of polarized light in following the deposition of materials which make up the secondary lamellae of the conidiophore walls. The highly doubly refractive cellulose, formed in the protoplasm, is deposited gradually from base to tip of the lengthening conidiophore. The double refraction of the secondary lamellae in young conidia, shown in Figure 2-H and I, is eventually masked by the increasing amounts of aspergillin in the conidial walls, as shown in the fruiting heads of B and C, Figure 6. The formation of small amounts of aspergillin in the conidiophore wall near to the vesicle sometimes produces a detectable, but less

complete masking of the double refraction, as indicated in Figure 6-C. The significance of these phenomena in polarized light is emphasized by the fact that the appearance of these cell walls of Aspergillus niger ATCC 6275, in polarized light, during the period of secondary wall formation, is identical with that observed previously in strain NRRL 67 and in A. carbonarius NRRL 369, at comparable stages of development. These identifications of cellulose in the secondary lamellae of the cell walls are corroborated by the report from Dr. G. L. Clark that X-ray diffraction rings, characteristic of both cellulose and of chitin, are present in the diffraction patterns from these samples made at DePauw University.

The use of appropriate enzymes, as cytochemical reagents, is usually the last step in a series of cytochemical analyses of a plant cell wall. Once the nature and location of the cell wall materials have been determined, the most precise check on the accuracy of the accumulated data is in the attempt to hydrolyze the known substance or substances with their respective enzymes. The primary lamellae of the walls of the various types of cells in Aspergillus niger ATCC 6275 have been found to be chitinous. With the exception of the hyphae, where no secondary deposits are in evidence, the secondary deposits in the conidiophores, vesicles and conidia are composed of a continuous matrix of chitin surrounding intermittent deposits of cellulose. Upon this basis, the enzyme, chitinase, could be expected to bring about the comparatively rapid breakdown of these cell walls through the hydrolysis of the chitin. The effect of the liquid from the puffball, Calvatia gigantea, which contains a powerful chitinase as well as a cellulase, upon the conidia of A. niger ATCC 6275, is illustrated in Figure 7. The puffball juice which was used in these experiments had been in storage at 5°C, under toluol, for seven months. Although the puffball juice has been found to lose approximately 50 per cent of its potency as a hydrolytic agent in one year, the effect upon the conidia, shown in Figure 7 can be seen to have been prompt and fairly drastic. All of the photomicrographs were made at various intervals during a twelve hour period of treatment with the chitinase. When conidia were treated with a 0.5 mg./ml. solution of the chitinase-containing Worthington Lysozyme, the effect upon the conidia was even more drastic, as shown in Figure 8. These results serve to confirm the present conception of the nature and location of resistant substances in the cell walls of Aspergillus niger ATCC 6275, based upon other optical and chemical methods of cell wall analysis

SUMMARY

The resistance of many types of fungi to treatment with strong chemical reagents was found, during the last decade of the nineteenth century, to be due to the presence of chitin in their cell walls. Cytochemical analyses of the hyphae, conidiophores, vesicles, sterigmata and conidia of Aspergillus niger ATCC 6275, have resulted in the location of chitin in the primary lamellae,

and the location of cellulose, apparently surrounded by chitin, in the secondary lamellae of the cell walls, with the exception of the hyphae in the walls of which no cellulose has been found.

The nature and location of cell wall substances in A. niger ATCC 6275 are similar to the nature and location of cell wall substances in the two black Aspergilli which have been studied previously, A. niger NRRL 67 and A. carbonarius NRRL 369.

In the course of these analyses, the traditional resistance of chitin to hydrolysis by concentrated acid and saturated alkaline solutions has been demonstrated, along with the comparatively rapid breakdown of the chitinous cell walls by the chitinase-containing juice of the giant puffball, Calvata gigantea, and by a 0.5 mg./ml. solution of the chitinase-containing Worthington Lysozyme.

Figure 1

Conidia of Aspergillus niger ATCC 6275. Untreated.

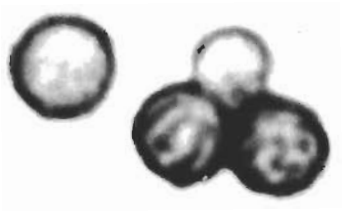
A. Conidia, two developing and one mature, typical of those produced most abundantly in these cultures. The surfaces are spineless, and black coloring matter (aspergillin) appears inside the primary lamella of the cell wall at an early stage of development.

B. Mature conidia, with incipient spines and ridges, are found less frequently than those with smooth surfaces.

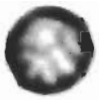
C. Eight of the smaller conidia and one large, slightly spinulose conidium of a size found rarely in these cultures.

D. Two young conidia, and one large, mature conidium with well developed spines of a type found very rarely in these cultures.

A



3.5 μ



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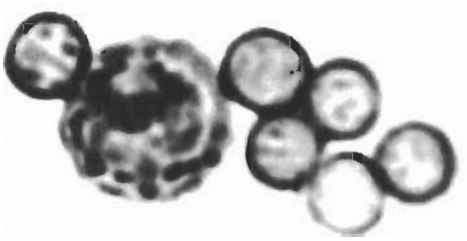

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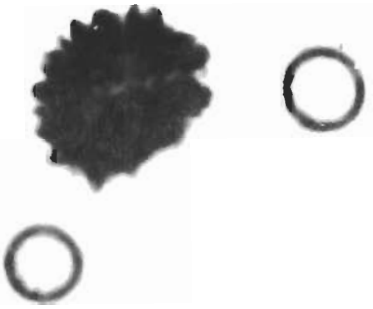


D

3.5 μ

Figure 2

Identifications of Chitin and Cellulose
in
Aspergillus niger ATCC 6275

A, B and C. Conversion of chitin to chitosan, in the walls of smaller conidia, by treatment with saturated potassium hydroxide at 150°C for 3 hours, produces little change in the original sizes of the young (A), developing (B) and mature (C) conidia. Black walls, decolorized during the conversion of chitin to chitosan, become red-violet in color upon addition of a trace of iodine-potassium iodide solution.

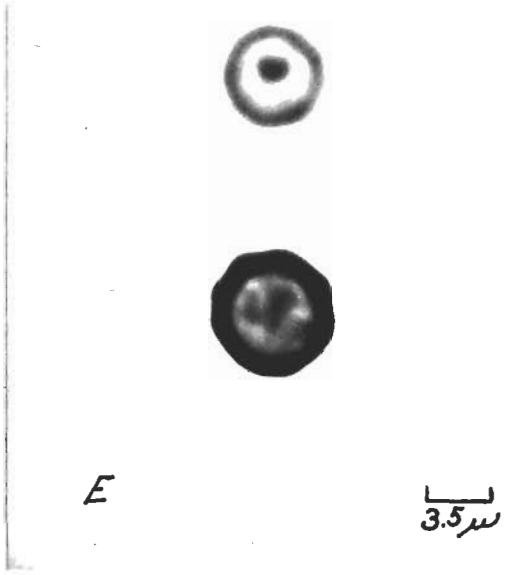
D. Chitosan reaction in the wall of a very young conidium of the larger type. Swelling of the wall material reduces the volume of the lumen with little change in the original size of the conidium.

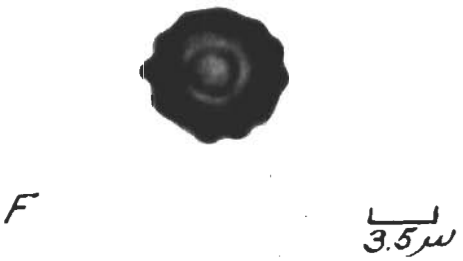
Identification of Cellulose (Sulphuric Acid-Iodine Reaction)

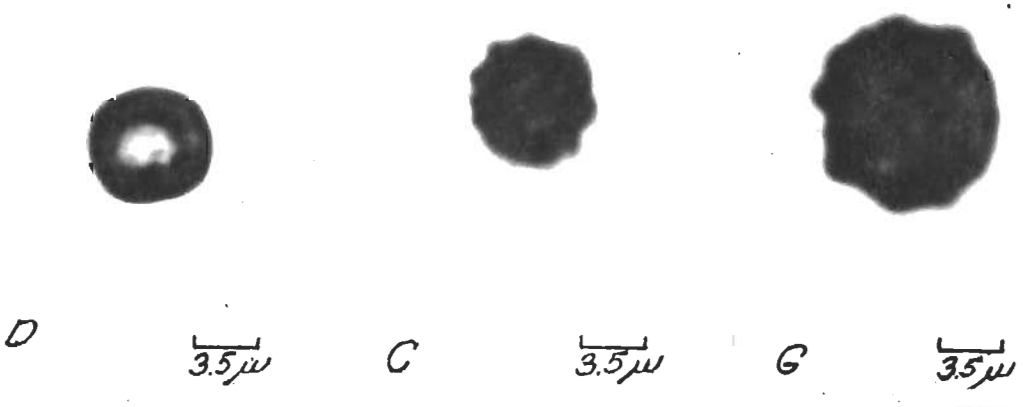
E, F and G. Conidia at four successive stages of development, after treatment with 72% sulphuric acid for 30 days. A slight enlargement is produced by the treatment with acid, most pronounced in the very young conidia (E, above). The black aspergillin is not visibly altered by the strong acid. Addition of iodine-potassium iodide solution to the specimens, after treatment with sulphuric acid for one month. This reaction can be expected after a period of treatment of two to three months.

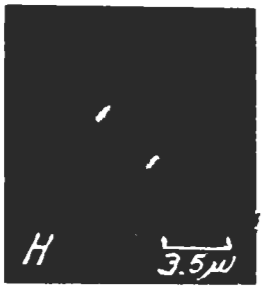
H. Double refraction of secondary deposits in the wall of a young conidium indicates the presence of cellulose.

I. Double refraction in a developing conidium with heavier secondary deposits.









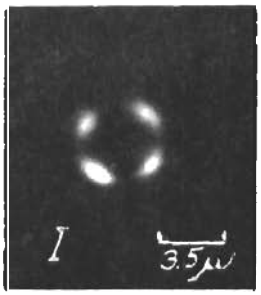


Figure 3

Localization of Chitin in Aspergillus niger ATCC 6275
by means of Fluorescence

A, B, C, and D	Successive stages of development of conidiophore, vesicle and conidia showing accumulations of fluorescent material. The fluorescence correlates with the localizations of chitin by other methods.
E	Fluorescence at two stages of development of very young conidia.

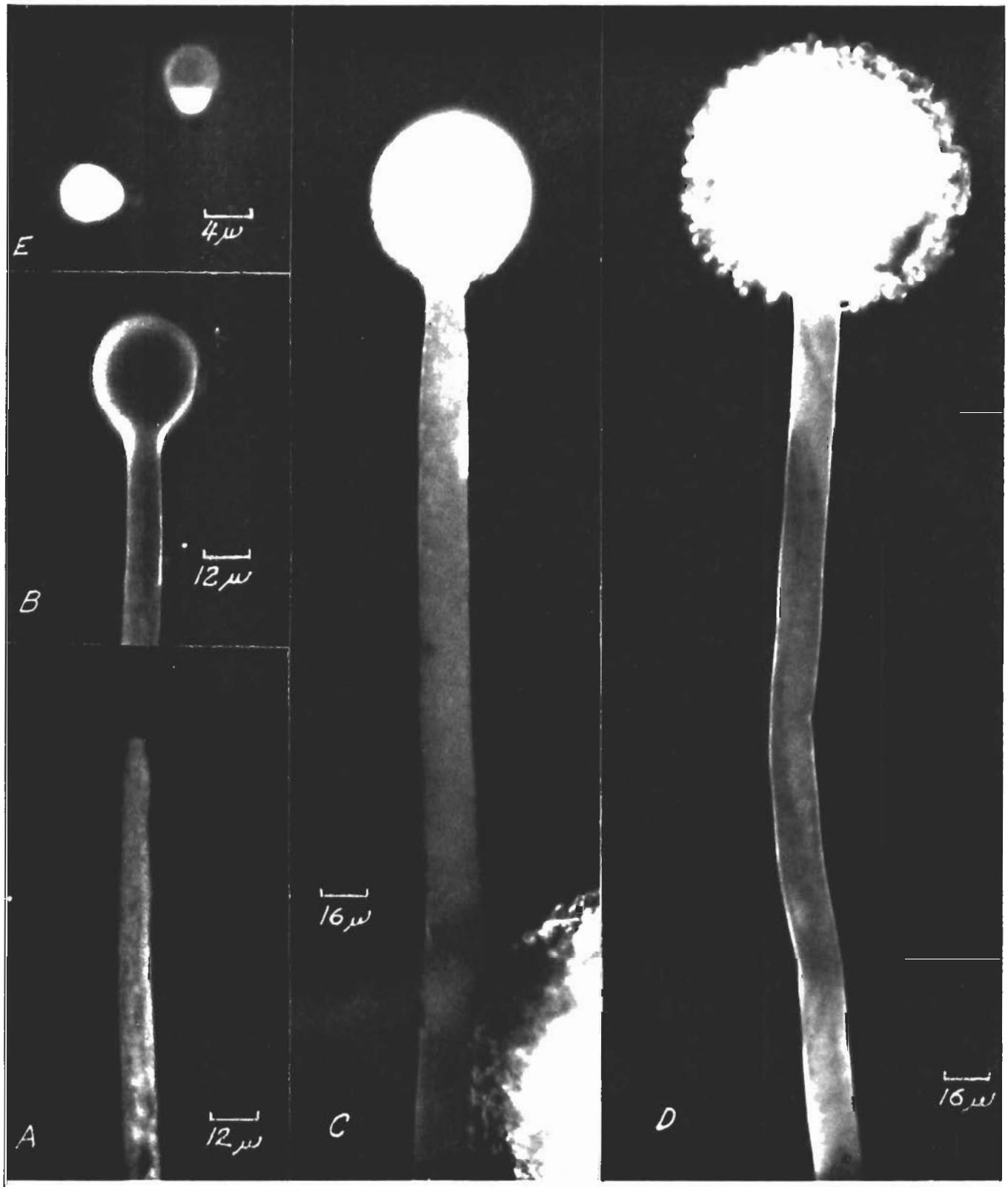


Figure 4

Localization of Chitin in Aspergillus niger ATCC 6275
by means of Fluorescence

A mass of developing hyphae showing fluorescent lateral walls, with heavier accumulations of fluorescent materials in the growing tips and cross walls (lower left). Of the many conidia scattered throughout the mass, only one, very young and highly fluorescent, is conspicuous (right of center). In the older, barely visible conidia the fluorescence is quenched by the accumulations of black aspergillin.

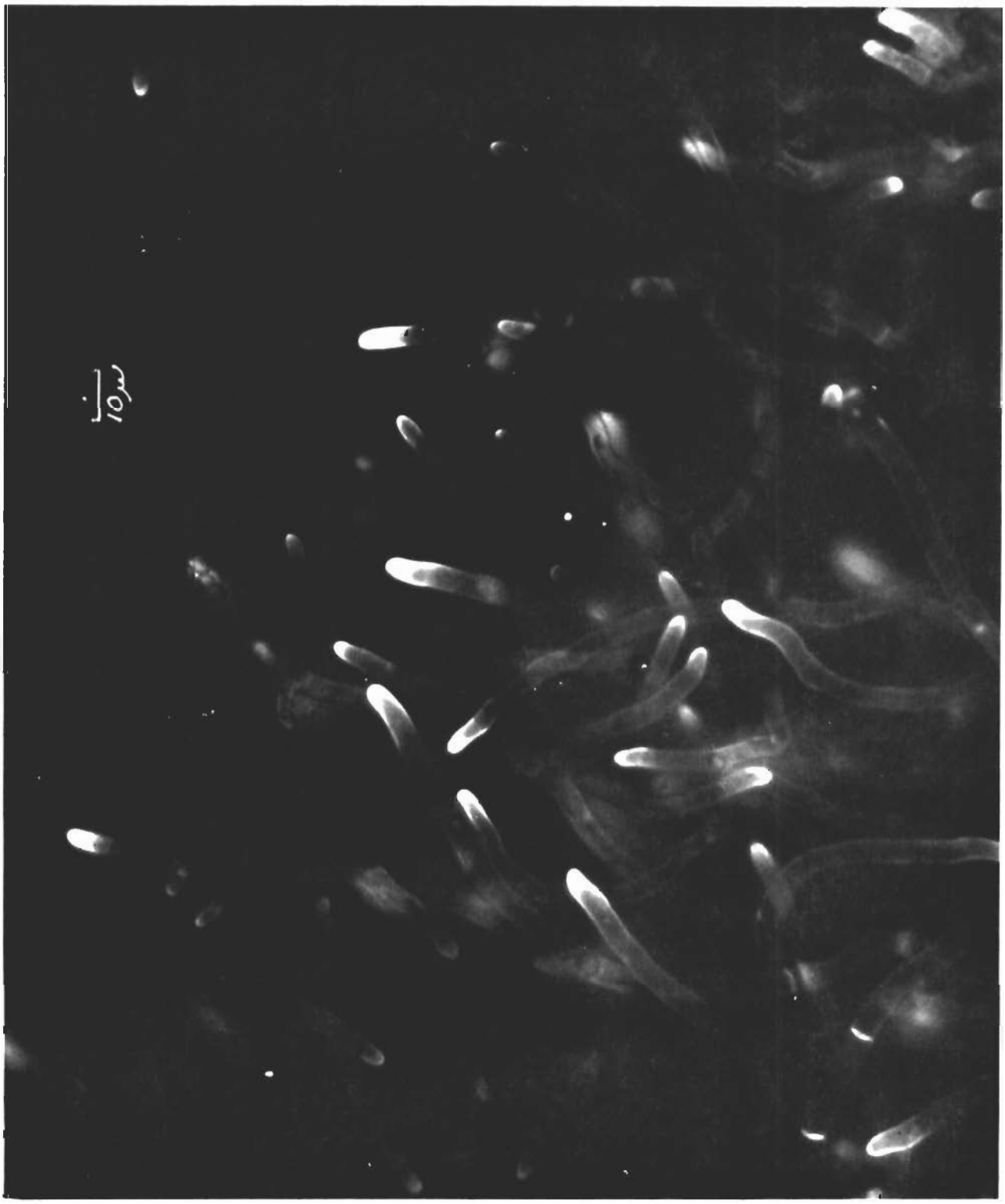


Figure 5

Quenching of Fluorescence in Aspergillus niger ATCC 6275 by Aspergillin

Mature conidia, almost invisible in Figure 4, because of the quenching of their fluorescence by accumulations of black aspergillin in their walls, are rendered visible by a thick mat of fjuorescing hyphae. One large, spineless conidium and many smaller conidia in various stages of darkening can be seen.

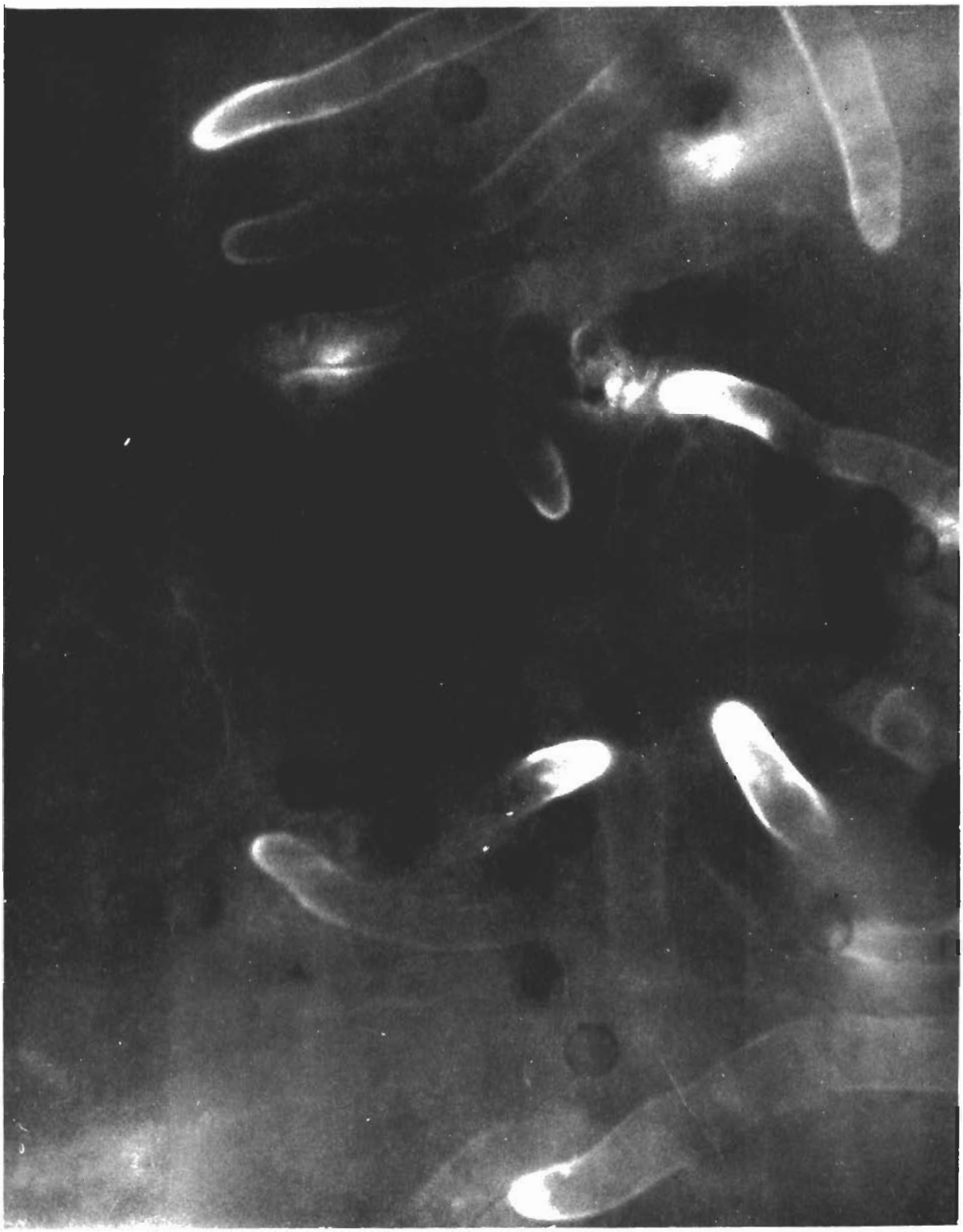


Figure 6

Double Refraction of Secondary Wall Lamellae of
Aspergillus niger ATCC 6275 in Polarized Light

A. Deposition of secondary lamellae in the wall of a young conidiophore takes place gradually from base to tip, and can be followed by the pronounced double refraction in polarized light.

B. Later stage of development showing progressive deposition of secondary lamellae in a conidiophore. Double refraction of older conidia in outer regions of the fruiting head is masked by accumulations of black aspergillin in their walls, in contrast to the pronounced double refraction of the younger conidia toward the center.

C. Mature conidiophore and fruiting head. Secondary deposits of doubly refractive material in the conidiophore wall are completed, and the outer mass of darkened conidia have been shed.

Figure 7

Reactions of Conidia of Aspergillus niger ATCC 6275 to Juice of the Puffball, Calvatia gigantea

Mature conidia of Aspergillus niger ATCC 6275, after treatment with the chitinase-containing liquid from the sporophore of Calvatia gigantea, for a period of 10 to 12 hours.

A. Two conidia with disrupted primary walls; one conidium with abnormal germ tubes.

B and C. Four of the less reactive conidia show the translucent appearance of the conidia produced quickly, upon contact with the enzyme.

D, E, F and G. Five of the more reactive conidia.

H and I. A conidium photographed 10 hours (H) and twelve hours (I) after treatment with sporophore juice.

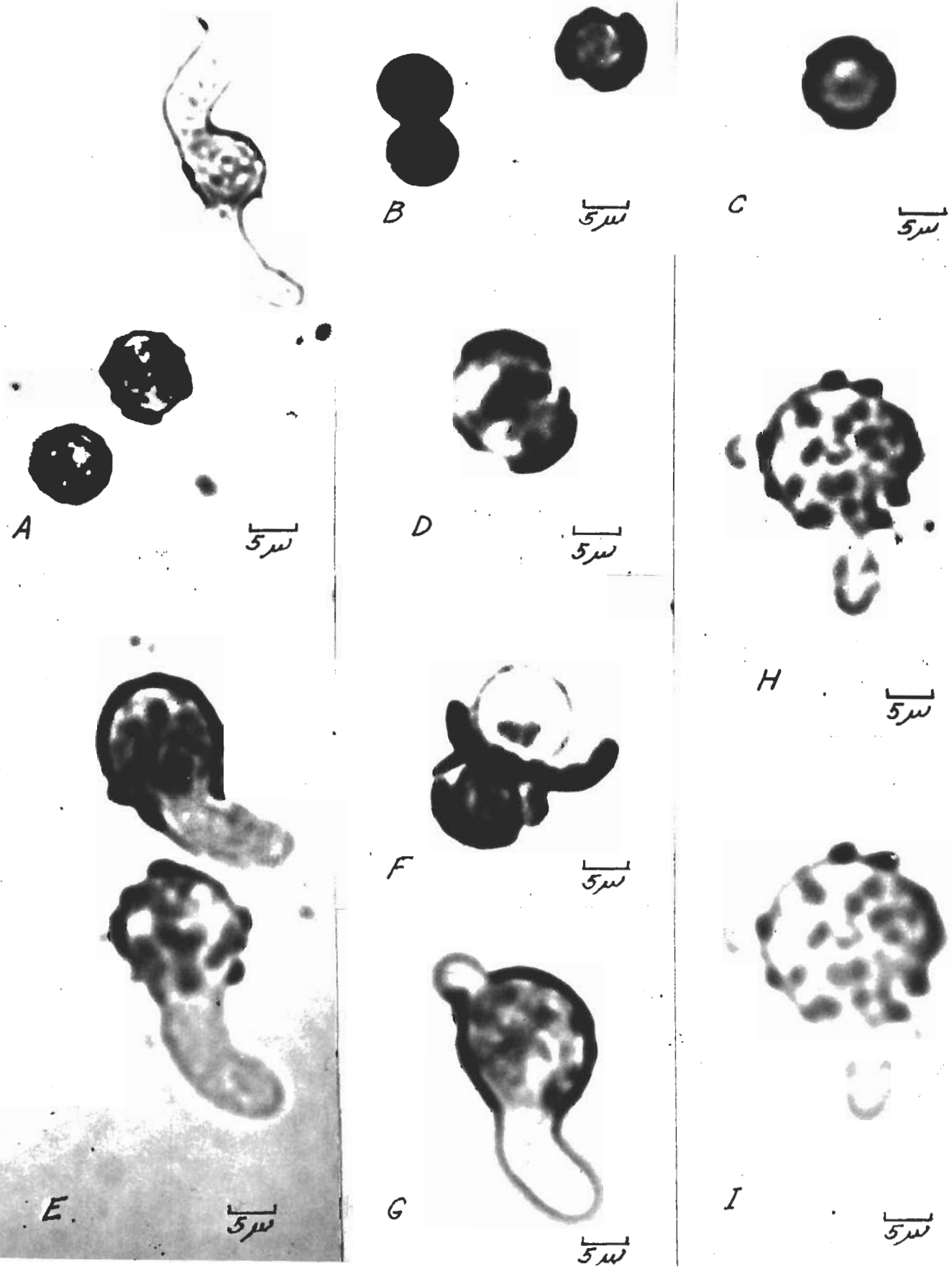


Figure 8

Reactions of Conidia of Aspergillus niger ATCC 6275
to a Solution of Worthington Lysozyme

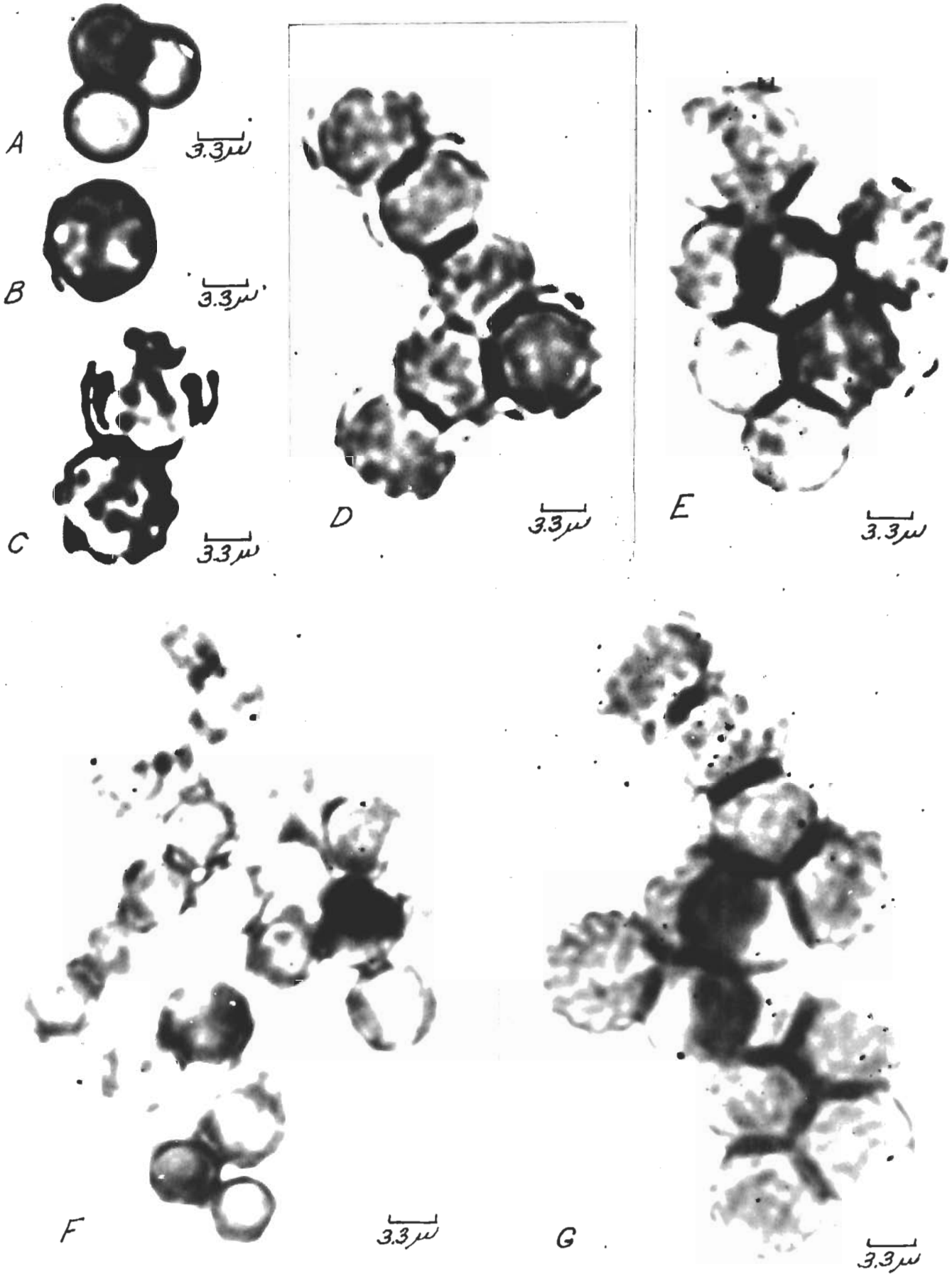
A. Spineless conidia of the smaller type become transparent at an early stage of reaction to the enzyme.

B. Early stage of hydrolysis in the wall of a conidium of the larger type.

C. Later stage of hydrolysis in the walls of two spinulose conidia.

D, E, and G. Late stages of hydrolysis in groups of spinulose conidia.

H. Early stages of reaction of a group of young, spineless conidia indicate the drastic effect of the enzyme solution on the thinner, chitinous walls.



CYTOCHEMICAL ANALYSES OF *Myrothecium verrucaria* ATCC 9095

Myrothecium verrucaria, the second organism to be analyzed cytochemically, is comparable to a fungus which has been found in contaminated jet fuels and is also an active agent in the deterioration of cellulosic materials.

In 1942 Greathouse, Klemme and Barker (29) reported upon the extraordinary activity of the fungus *Metarrhizium sp.* in the decomposition of cotton fabric in certain pure culture experiments. Two years later this same fungus was described by Pope (48) as a new species of *Metarrhizium*, namely *M. glutinosum*. The various cells which make up the tissue of the fungus were characterized in considerable detail by this author. The mycelial mat grown on filter paper is pure white with olive green to olivaceous black conidial masses on tufts of mycelium. The conidiophores, up to 75 microns long, are erect and septate, penicillately branched above, forming a palisade layer in tufts, upon which dark, glutinous masses of conidia are produced. The conidial chains are formed directly on the finger-like conidiophores. When mature they are elongate-ovoid in shape, 6 - 9.6 microns in length and 1.5 - 3.9 microns at their largest diameter. Their color is a dusky olive green and they are covered with a colorless, glutinous material which holds them together in comparatively large masses and prevents their air-borne distribution. Chlamydospores are produced in bulbous terminal portions of the hyphae and can be found embedded in the mycelium, near to the substrate. They are brown in color, very nearly spherical in shape and 7.4 - 9 microns in diameter. In a later report, Pope (49) includes photomicrographs of the surface of a colony with glutinous masses of conidia and also conidiophores and conidia at higher magnification.

By this time, *Metarrhizium glutinosum* was listed as USDA isolate 1334.2. In 1946 White (68) confirmed its cellulolytic activity, and its particular value in deterioration studies in the laboratory. There seemed to be considerable evidence that it is not an important factor in the deterioration of cotton fabrics and related military and industrial materials in the field, although it is widely distributed on plant debris in North America, Europe and the tropics, (68), (69).

Following a detailed comparison of the characteristics of *Metarrhizium glutinosum*, USDA 1334.2 and the English fungus, *Myrothecium verrucaria*, known in the literature for almost 150 years, White and Downing (69) concluded that they were identical. In a paper entitled "The Identity of '*Metarrhizium glutinosum*'", published in 1947, they suggested that the name be changed to *Myrothecium verrucaria* Ditmar ex Fries. The former *Metarrhizium glutinosum* USDA 1334.2 is now recognized as *Myrothecium verrucaria* USDA 1334.2, or, as specified in this project, *Myrothecium verrucaria*, ATCC 9095.

A number of studies of the nutrient requirements of M. verrucaria, and also of biologically active produces which are derived from it, suggest the importance of a correlation between these physiological findings and a detailed cytochemical analysis of the organism. Mandels and Norton (37) found that the conidia will germinate only if N, P, K and S are present and that the omission of Mg results in increased germination; that although young spores can germinate soon after they are formed, some spores retain their vitality for a year or more; and that with adequate nutrients 100% germination can be obtained in 3 hours. Brian and McGown (8) found an antifungal substance, glutinosin, in the cultures of M. verrucaria. Extracted with glutinosin, but remaining in the mother liquor after crystallization of the glutinosin, was a material causing a dermatitis similar to that produced by poison ivy. Cytochemical analyses of fungi grown with different nutrient materials are quite likely to provide interesting information. It is also possible that the following of the secretion of biologically active substances with a precise technique such as that of Interference Microscopy could add new and unique information concerning the synthesis and behavior of these materials. The objectives of this research project, and the available time in which to meet these requirements, however, make necessary the concentration of effort upon the identification of cell wall materials, such as chitin and cellulose, which would offer resistance to fungicides and bactericides. The materials and methods used in the present study have been selected, therefore, with this purpose alone in mind.

Materials and Methods

A culture of Myrothecium verrucaria ATCC 9095 was provided through the courtesy of Dr. Carl Wessel and Dr. Walter Bejuki of the Prevention of Deterioration Center, National Academy of Sciences, Washington, D. C. Pertinent references to the accumulated literature on Metarrhizium glutinosum and Myrothecium verrucaria, and reviews of the literature in which these findings were correlated, were prepared by Dr. Bejuki and Miss Virginia White, Librarian. This expert assistance in assembling background information concerning the organism to be analyzed was an important factor in the completion of this study.

The cultures were grown on Czapek Solution Agar, to which a strip of sterile filter paper has been added. Advice concerning a suitable method of culture, normal rate of growth of the fungus and the expected rate of deterioration of the cellulosic fibers in the filter paper was provided by Dr. A. E. Prince. Petri dish cultures, held at room temperature, have facilitated the harvesting of the cells for the examination of the untreated as well as the treated material.

a. van Wisselingh Color Test for the Identification of Chitin:

The use of the van Wisselingh color test for the identification and localization of chitin was indicated by the known occurrence of this material in the cell walls of many fungi. The procedure involves treatment of the cells in saturated KOH at 160° - 180°C for periods of time, varying with the type of organism, from one-half hour to three hours. The hyphae, conidiophores, conidia and chlamydospores of M. verrucaria were treated at 160° for a period of two hours. This length of time was found to be adequate for the conversion of chitin to chitosan, but shorter periods of time were not used. Following this conversion, treatment of the cells with 95% alcohol for 15 minutes to harden the chitosan, washing with water to remove the alchol, application of aqueous I_2KI solution and a trace of 1% H_2SO_4, brought about the red-violet color of the chitosan in the same relative position occupied by the chitin in the untreated cell wall.

b. Payen's Sulphuric Acid-Iodine Method - Identification of Cellulose:

The sulphuric acid-iodine test for the identification and localization of cellulose in plant cell walls consists in the treatment of the specimen with 72% H_2SO_4 and I_2KI solution. In the presence of these two reagents the cellulose is swollen, but not immediately hydrolyzed, and takes on a distinct blue coloration. In higher plant cells, where the cellulose is frequently confined to the secondary lamellae, the non cellulosic substances which constitute the primary lamellae and surround the cellulose in the secondary lamellae are penetrated rapidly by the acid reagent, and the blue coloration of the cellulose takes place in a short time. In fungal cell walls containing both chitin and cellulose, the chitin has been found to occupy the relative position of the non cellulosic material in the walls of the higher plant cells, with the cellulose surrounded by chitinous substance in the secondary lamellae. Since the chitin of the fungal cell wall is extremely resistant to strong acid solutions, Payen's method must be adapted to the slow penetration of the 72% H_2SO_4 by extending the period of treatment of the fungal cell for from three weeks to three months. If frequent examination of the specimen during the period of treatment fails to reveal the typical cellulose reaction, and if the chitin in the cell wall reaches a late stage of hydrolysis during this same period, the absence of cellulose in the wall is confirmed with a high degree of certainty.

c. Double Refraction in Polarized Light:

In the fungal cells analyzed so far by the author, the chitin has been found to show weak double refraction in polarized light. When cellulose has been present, it has shown the characteristic strong double refraction. As information concerning the make up of

fungal cell walls accumulates, the value of this optical method in the detection and localization of these wall substances is increasing. Preliminary examinations of the specimens in polarized light serve to indicate whether either chitin or cellulose, or both, are present, and the results obtained either confirm or disagree with the results obtained by means of other cytochemical methods, thus contributing to the accuracy of the final conclusions.

d. Fluorescence in Ultra-violet Light:

The chitinous lamellae of the fungal cell walls has been found to be stronger than the flucrescence of the cellulose. Some studies of insect chitin have led the investigators to conclude that the fluorescence has been due in large measure to admixed protein. The results are by no means conclusive, however, and the technique of fluorescence has proved to be most valuable in identifying and localizing the lamellae rich in chitin, and in contrasting this optical behavior with that of cellulose, when both are present in fungal cell walls. The value of this technique, as in the case of that of double refraction in polarized light, is increasing as the knowledge of the chemical make up of fungal cell walls increases.

e. Enzymatic Hydrolysis:

Following the identifications of cell wall materials by means of other cytochemical techniques, the action of the corresponding enzymes upon the cell wall serves to check the accuracy of the previous conclusions concerning both the identity of the materials and their locations in the wall with respect to the other substances. The enzymes used in the study of M. verrucaria were the chitinase-containing Worthington Lysozyme in a concentration of 0.5 mg./ml. and juice from the puffball, Calvatia gigantea.

RESULTS

In the earliest period of study of the cultures of Myrothecium verrucaria, difficulty in preparing the specimens for microscopical examination of the untreated material was encountered because of the extreme thinness and delicacy of the walls of the hyphae and conidiophores and the presence of the large amounts of glutinous material around the conidia. When a water mount of fungal tissue containing all of these types of cells was observed, after attempting to make the necessarily thin specimen for examination at high magnifications, the hyphae and conidiophores were frequently damaged and the conidia held together in clumps by the sticky material on their surfaces. A lack of sharpness in the images of the conidia is due to the glutinous material which did not dissolve in the water but was very nearly invisible in the ordinary transmitted light. The presence of the olive green coloring matter in the walls of the older conidia overcame this haziness to some extent. Figure 9, A and B, present different aspects of the same set of conditions, and these are described in the accompanying legends.

At this point, the decision was made as to whether the glutinous material should be removed, if possible, before the various cytochemical procedures were used upon the cell walls. It was concluded that this associated gel is such an intimate part of the normal culture that unextracted samples, through the reactions of this glutinous material to the reagents used, might provide necessary information for the effective use of fungicides later on. All of the information obtained for this report was derived, therefore, from samples which had had no form of pretreatment before observations of double refraction and of fluorescence were made, as well as the observations of the reactions to reagents used in the identification of chitin and cellulose.

The faint double refraction characteristic of chitin is found in the walls of the hyphae, conidiophores, conidia and chlamydospores of Myrothecium verrucaria ATCC 9095. This faintness is extreme in the hyphae and conidiophores because of the thinness of their walls. The reality of this phenomenon of double refraction in a hyphal mat is shown, at low magnification, in Figure 10-C.

The slightly thicker walls of the conidia are brighter in polarized light but, as the conidia mature, the presence of the coloring matter in their walls tends to mask the double refraction. There is, therefore, a stage in the development of a conidium at which this property of double refraction of the wall material can be caught most effectively. This stage is the one at which the wall has attained considerable thickness and before the coloring matter has effectively masked the brightness. Figure 10, A and B, with their accompanying legends illustrate and explain the results with conidia

as they appeared, in both lateral and end views, in polarized light. These conidia were mounted in 0.1 M sucrose solution, the viscosity of which helped to reduce any motion of the small conidia during the necessarily long exposure.

The comparatively strong fluorescence of chitin is in evidence in the walls of the hyphae, conidiophores, conidia and chlamydospores of M. verrucaria ATCC 9095. The fluorescing walls of hyphae and conidia are shown in Figure 11, A. A slight fluorescence of the glutinous material, which clings to all of the chitinous surfaces with which it is associated in the original cultures, and with which it comes into contact during the manipulation of the specimen at the time of mounting, is also in evidence. In Figure 11-B, lateral and end views of the conidia show degrees of intensity in keeping with the thickness of the fluorescing substance which is producing the image at any given point. These mounts were also made in 0.1 M sucrose solution which decreased the motion and also does not fluoresce.

The results of the test for chitin, by means of the chitosan reaction, were positive in the walls of the hyphae, conidiophores, conidia and chlamydospores of M. verrucaria ATCC 9095. The treatment with saturated KOH at 160°C for a period of two hours appeared to either destroy or decolorize the olive green material in the conidial walls. All darkening of the walls in any of the cells shown in Figure 12, A and B, is due to the red-violet color of the chitosan in the same relative positions occupied by the chitin in the untreated walls.

A surprising result in this particular cytochemical test was the indication that the glutinous material had not been removed by the drastic treatment. The conidia were still held together in masses similar to those in the untreated material, and a sharp focus was, if anything, more difficult to obtain than in the fresh material because of the surrounding haze. The walls of the hyphae, conidiophores and conidia are thin; those of the chlamydospores thick, and consequently a deeper red-violet in color. No clue as to the chemical make up of the glutinous material which appeared to persist has been obtained. If it is chitinous, there is no evidence of conversion to chitosan by even a pale red-violet coloration. Although the possibility of extra-cellular cellulose cannot be excluded, a special adaptation of the Payen sulphuric acid-iodine test will be needed to establish this point. Whatever it is, it does not seem to be destroyed by strong alkali at high temperatures; does not prevent the penetration of the reagents which convert the chitin to chitosan and identify and localize chitosan in the cell walls, and, as will be seen later, does seem to be removed by 72% sulphuric acid and does not prevent the hydrolysis of the chitinous wall material by chitinase. These observations have brought up several possibilities which can be clarified by further study, if desired.

The hyphae, conidiophores, conidia and chlamydospores of M. verrucaria ATCC 9095 were under treatment with 72% sulphuric acid and iodine-potassium iodide solution for more than three months, and have been observed at weekly intervals during that period of time. The specimens shown in Figure 12, C and D, were photographed after treatment for two months. At no time, either before or since that time has there been any indication of a blue coloration in the walls which would reveal the presence of cellulose. The photomicrographs shown were taken at a time when the chitinous walls, though highly swollen, were sharply defined. The glutinous material which persisted during the conversion to chitosan is apparently more sensitive to the treatment with strong acid. The darkening, in these black-and-white illustrations, around and within the protoplast, is due to the brown coloration of the proteins; that at the conidial surface is due to the olive green coloring matter which is not decolorized by the acid treatment.

The reaction of the conidial walls of M. verrucaria ATCC 9095 to the 0.5 mg./ml. solution of chitinase-containing Worthington lysozyme was prompt and pronounced. Within the 10 hour period of treatment, all of the stages of hydrolysis of the wall material shown in Figure 13, A, B, C and D, could be found. The coloring matter in the walls of the conidia was not destroyed by the enzyme and its presence seems to help, in some measure, to distinguish between the glutinous material, which appears to remain to some extent, and the swelling wall material which is being hydrolyzed. More detailed studies, by means of this cytochemical technique, could be expected to add to our knowledge of the nature and behavior of this glutinous substance.

Figure 14, A, B, C and D, presents various stages of reaction of conidia to a full strength solution of dialyzed puffball juice (Calvatia gigantea). Compare the appearance with the appearance of the lysozyme-treated conidia in Figure 13.

SUMMARY

Analyses of hyphae, conidiophores and conidia of Myrothecium verrucaria ATCC 9095 indicate that their comparatively thin walls, as well as the thicker walls of the chlamydospores are chitinous. Cellulose has not been found, to date, in any of the cells of this fungus. The glutinous substance which surrounds the conidia presents some difficulty in the preparation of the samples for microscopical examination. It also tends to diminish the sharpness of the image of the conidium in ordinary light and to obscure, to some extent, both the fluorescence and double refraction of the conidial wall. There is some evidence that this colorless gel was altered little by the treatments with certain strong chemical reagents, although it does not prevent the reactions of these reagents with the chitin in the conidial wall.

The traditional resistance of chitin to hydrolysis by concentrated acid and saturated alkaline solutions is clearly in evidence in the behavior of the cell walls of M. verrucaria. Hydrolysis of the chitinous walls by the chitinase-containing Worthington Lysozyme, as well as the juice of the puffball, Calvatia gigantea, has been found to take place with the characteristic speed.

Figure 9

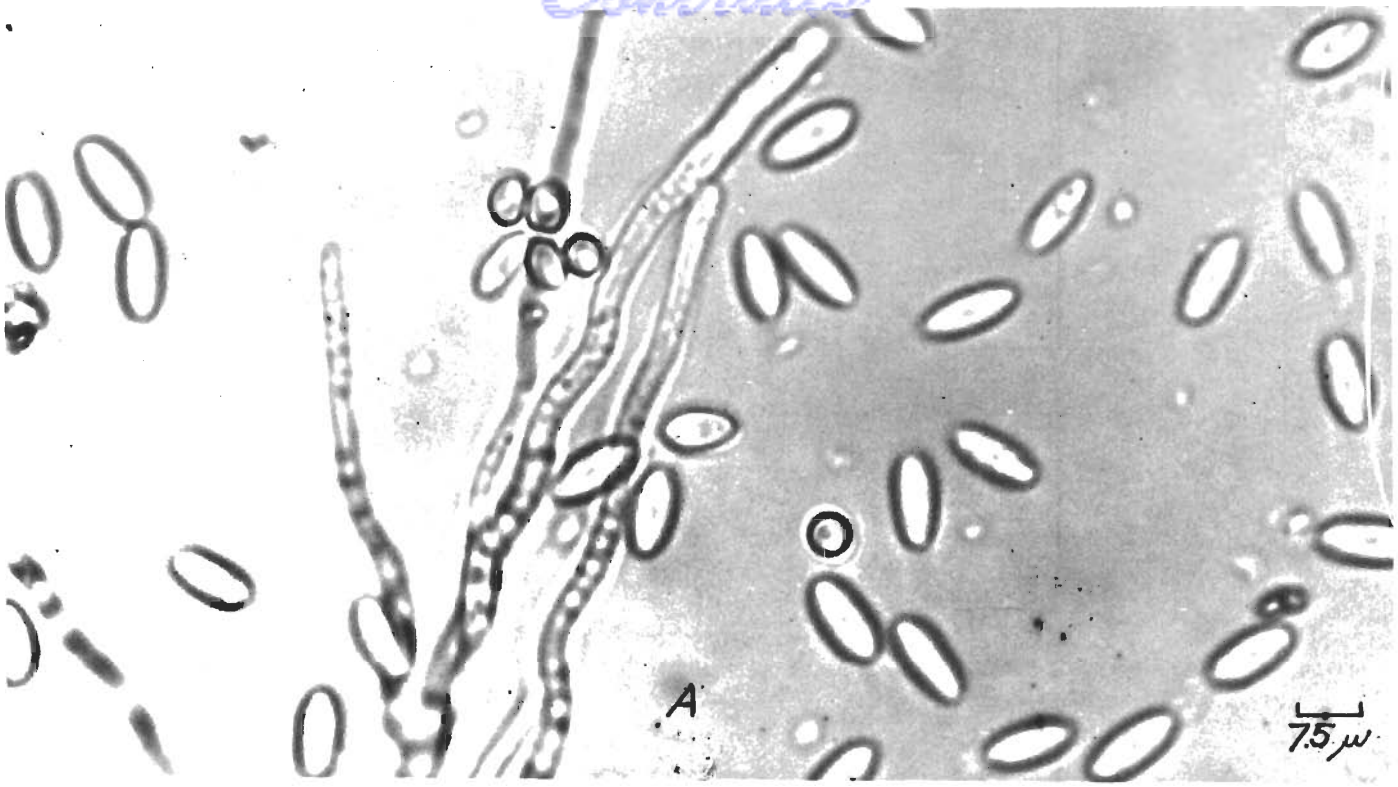
Hyphae and Conidia of Myrothecium verrucaria ATCC 9095

Untreated

A. Tips of actively growing hyphae surrounded by maturing conidia. The ellipsoidal shapes represent lateral views and the round shapes represent end views of the conidia. A few conidia are tipped to positions intermediate between those which appear in the lateral and end views.

In this photomicrograph there is less clumping of the conidia than was observed in most specimens examined, but the lack of sharpness in definition indicates the presence of the colorless gel which covers them.

B. With an objective of greater resolving power, details of protoplasmic structure and the thinness of the conidial walls are brought out more clearly. The haze of clear gel which tends to hold the conidia together, is also in evidence.



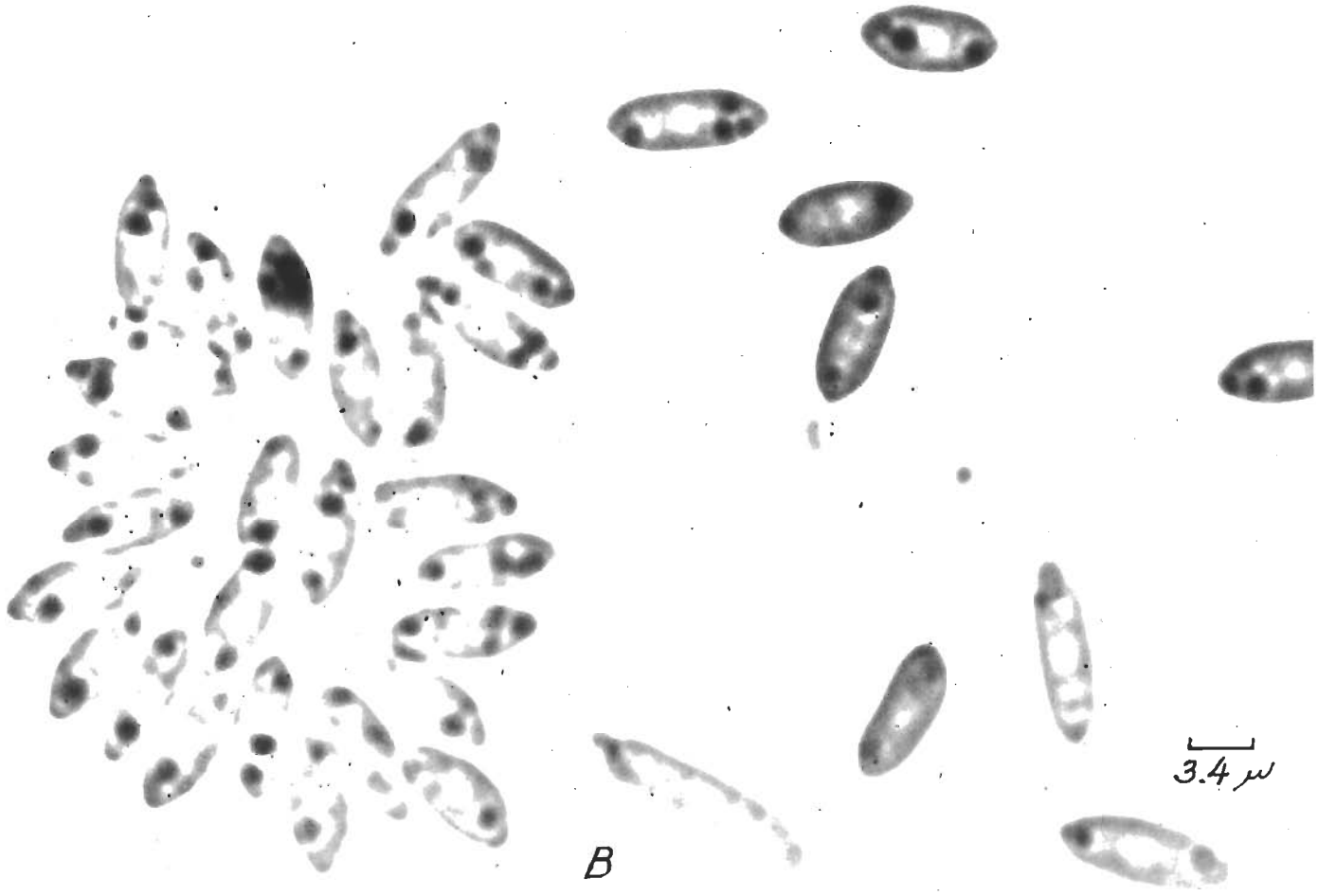


Figure 10

Double-refraction of Hyphae and Conidia of M. verrucaria ATCC 9095 in Polarized Light

A. Double refraction of the chitinous walls of the conidia in various positions with respect to the direction of the beam of polarized light. A large number of the conidia are in sharp focus in lateral view. End views of other conidia in this same field are out of focus and may be seen in Figure 10, B.

B. End views of many conidia which were out of focus in Figure 10, A show comparatively great brightness due to the depth of the wall material through which the polarized light passes. The "dark cross" appearance, similar to that in the starch grain, indicates that the long axes of the anisotropic molecules are parallel to the wall surface.

C. The low intensity of the double refraction in the thin, chitinous walls of the hyphae makes photographic recording of the phenomenon difficult except when masses of hyphae at low magnification are used.

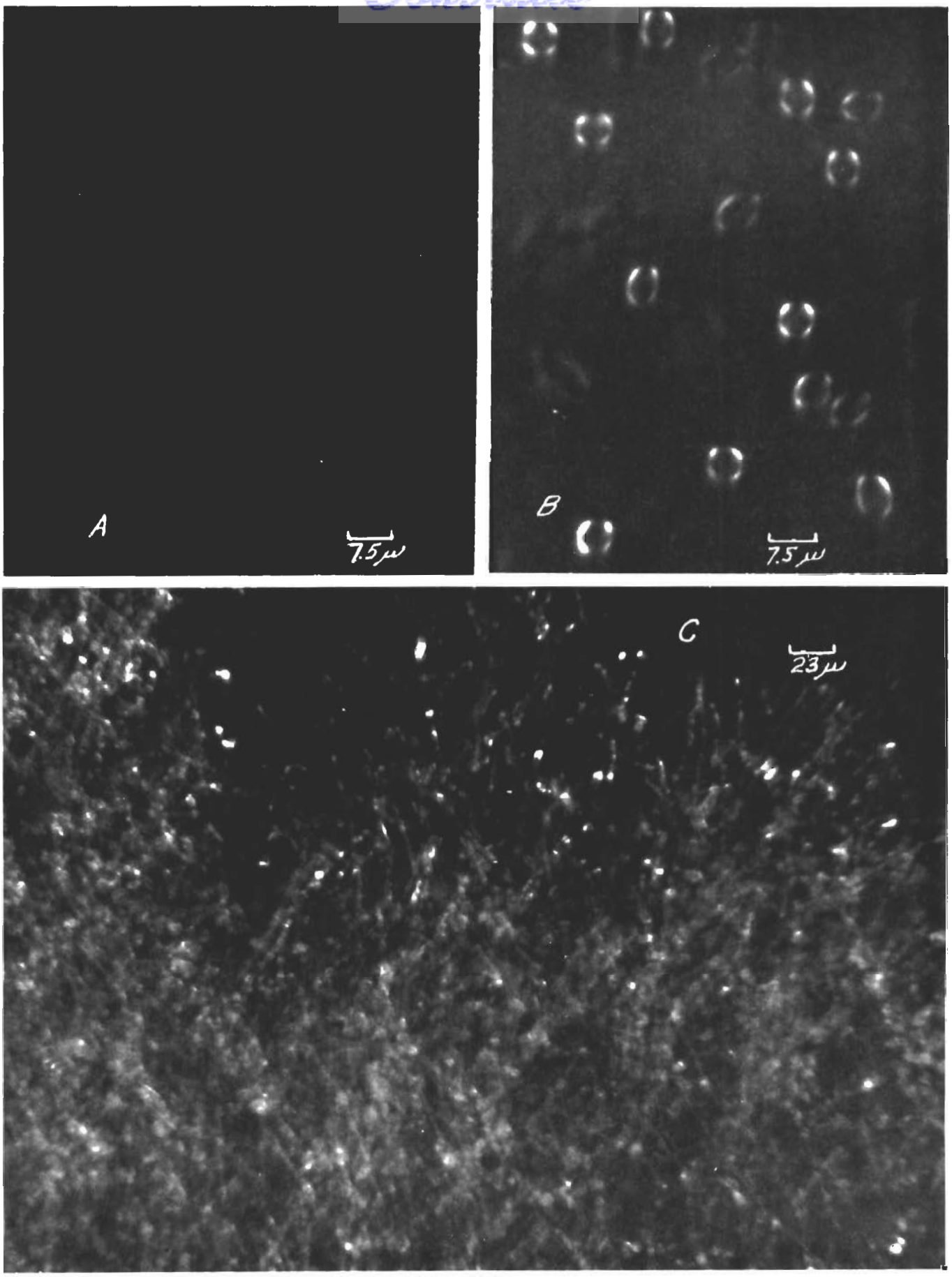
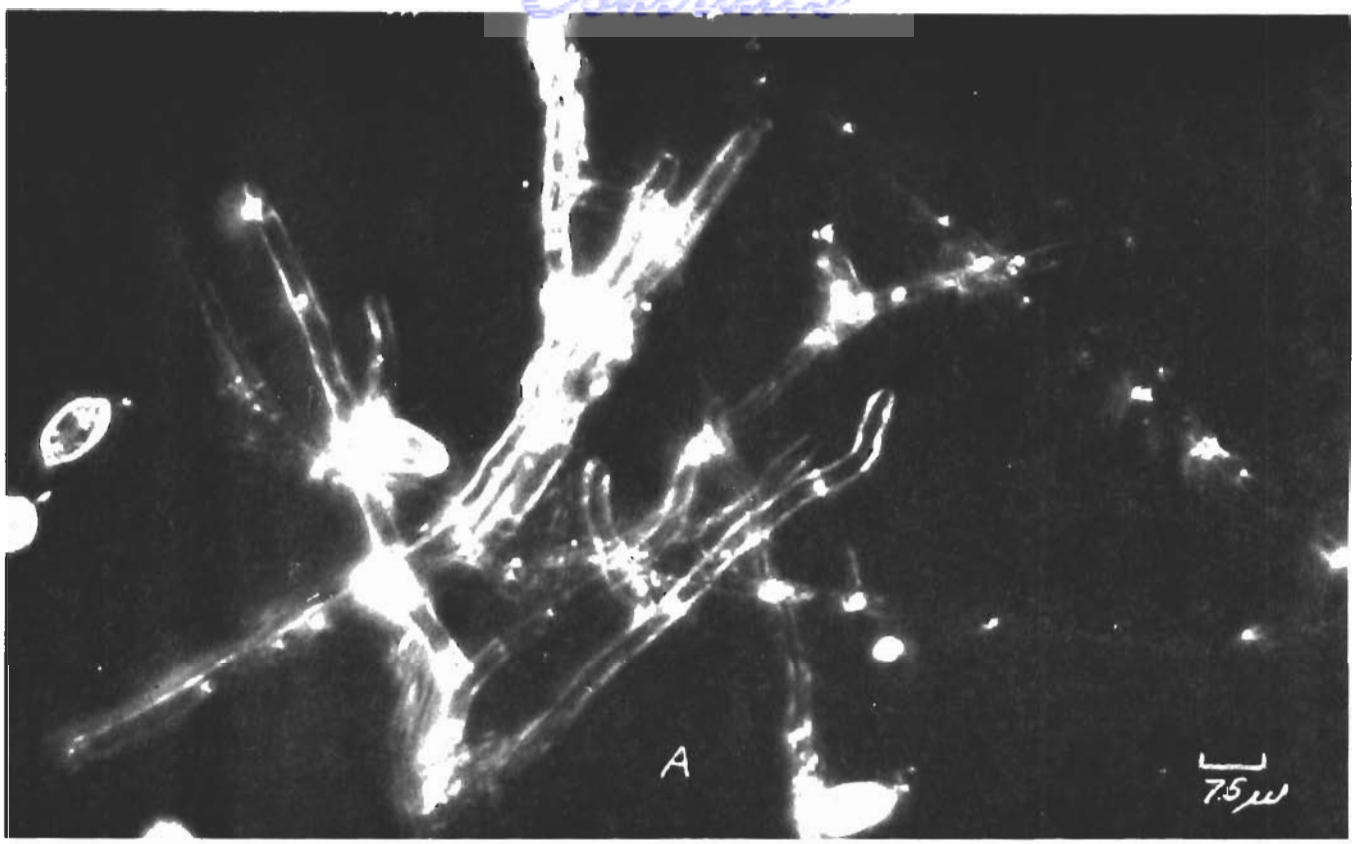


Figure 11

Fluorescence of Hyphae and Conidia of M. verrucaria
ATCC 9095 in Ultra-violet Light

A. Fluorescence in the thin, chitinous walls of the hyphae and somewhat thicker walls of the conidia is clearly in evidence, along with a faint fluorescence of the surrounding gel.

B. Fluorescence of the chitinous walls of the conidia in both lateral and end views can be seen, along with the fluorescence of some protoplasmic contents. There is also a faint fluorescence in the colorless gel which surrounds the conidia.



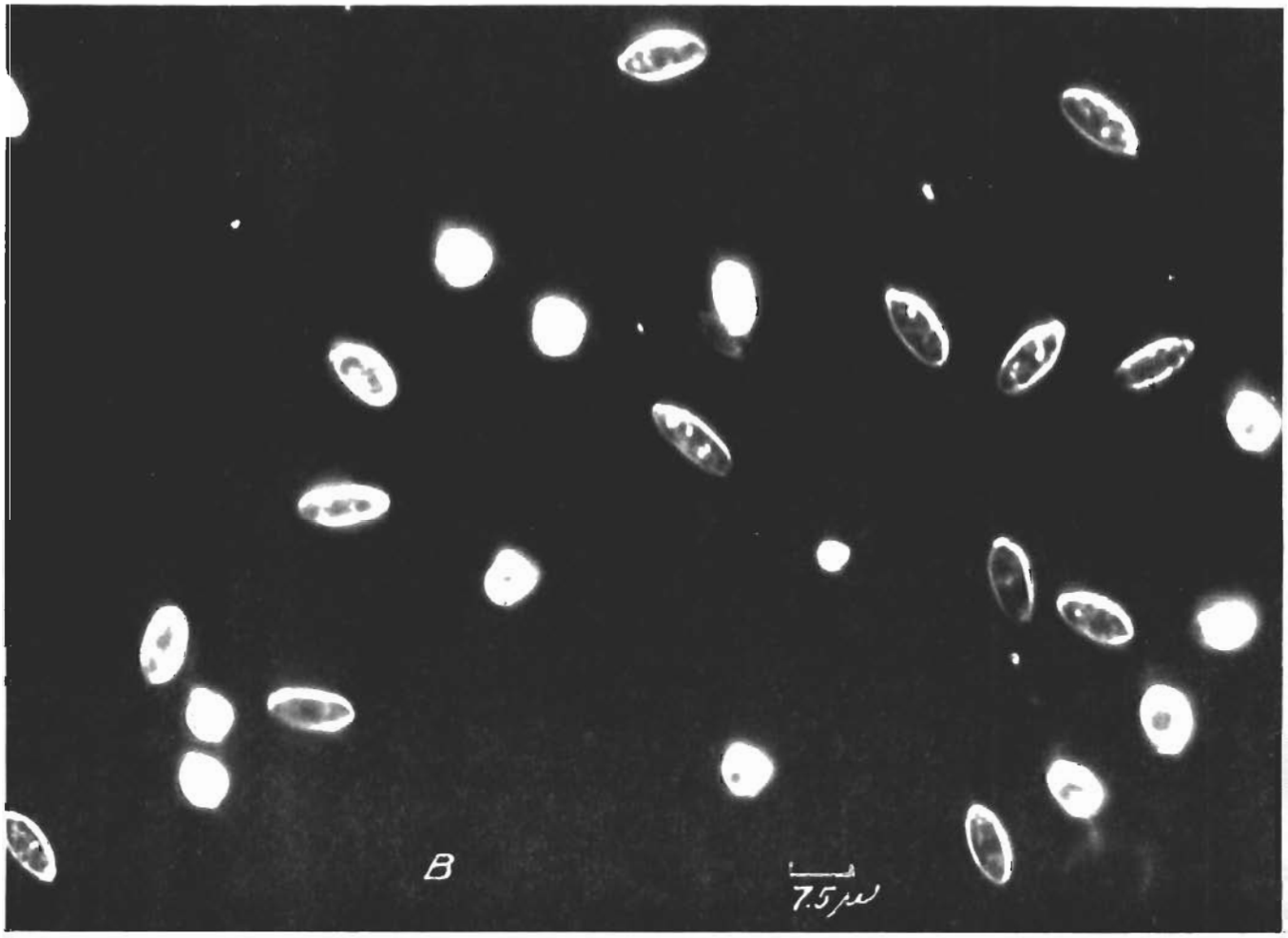


Figure 12

Identification of Chitin in Walls of Conidia and Hyphae of M. verrucaria ATCC 9095 and Negative Results for Cellulose

A. Conversion of chitin to chitosan in the walls of mature conidia by treatment with saturated potassium hydroxide at 160°C for two hours. Upon addition of a trace of iodine-potassium iodide solution, the thin cell walls become red-violet in color. A sharp focus is prevented by the uncolored gel which surrounds the conidia and tends to prevent their separation.

B. Chitosan reaction in the lateral walls and cross walls of hyphae.

C. Treatment with 72% H_2SO_4 and a trace of I_2KI solution brought about swelling and gradual hydrolysis of the thin chitinous walls of both conidia and hyphae. This specimen, photographed after a period of two months, has shown no evidence of the blue coloration characteristic of cellulose. The darkening around and within the protoplast in both conidia and hyphae is due to the brown coloration of the proteins. The reacting chitinous wall material is pale yellow, the color characteristic of chitin when treated with strong sulphuric acid and iodine.

D. Reactions of conidia at various stages of development to 72% H_2SO_4 and I_2KI solution is the same as those of the conidia shown and described in A.

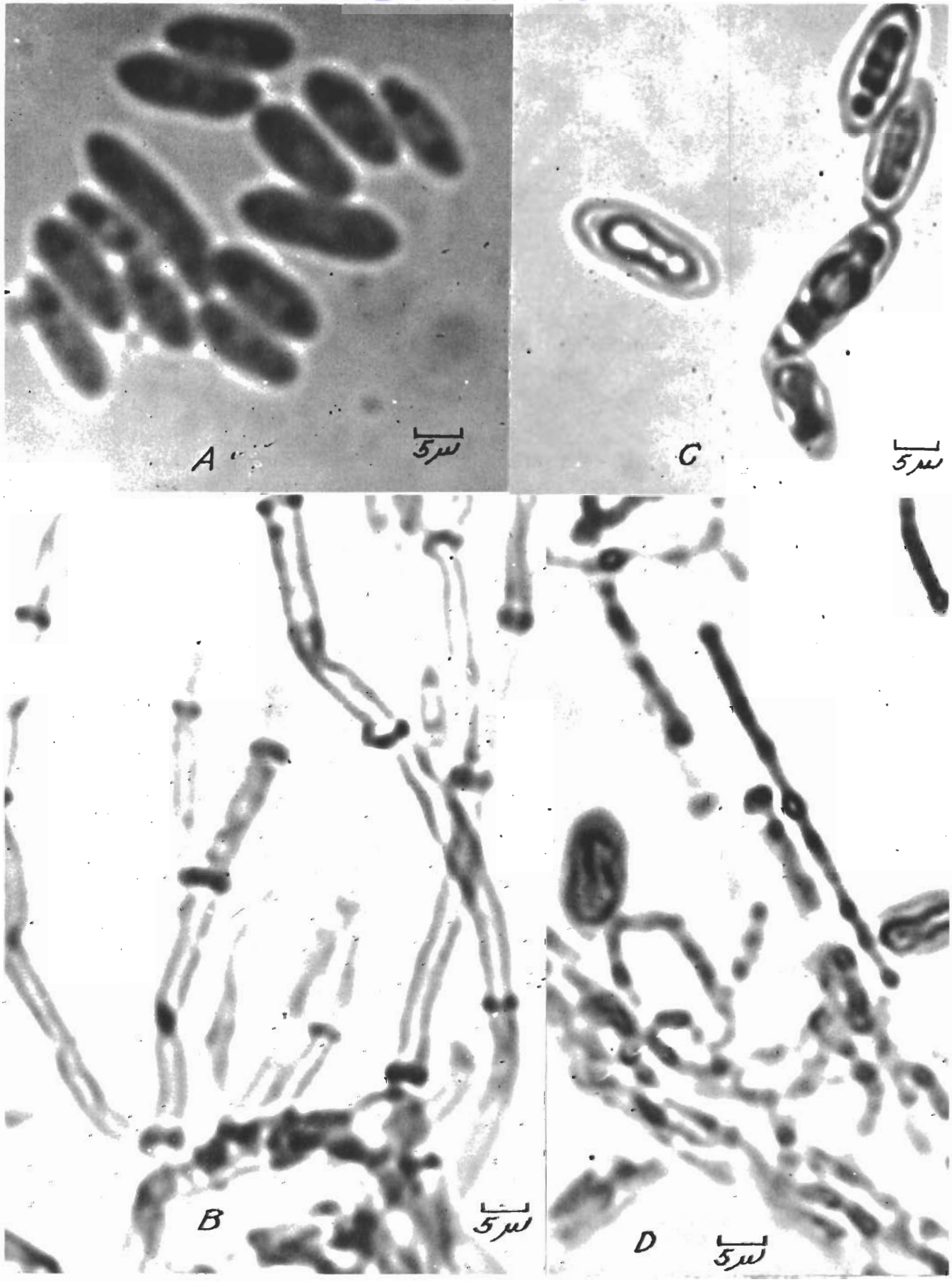


Figure 13

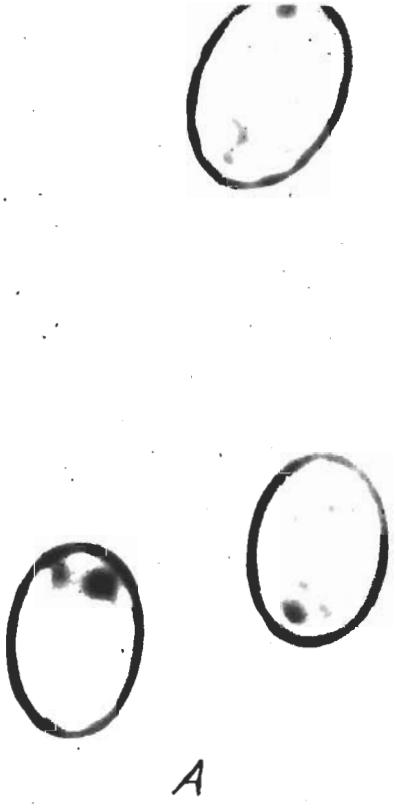
Reactions of Conidia of M. verrucaria ATCC 9095 to a 0.5 mg./ml. Solution of Worthington Lysozyme

A. A common type of reaction of a thin chitinous wall to the enzyme is an over-all stretching and consequent cell enlargement. In these three cells, the shape has become more nearly spherical.

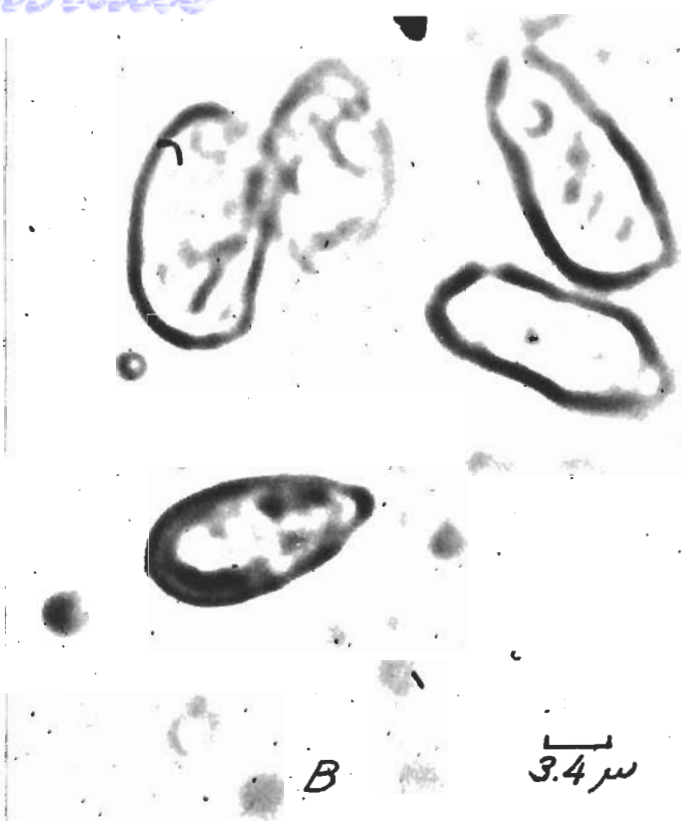
B. Hydrolysis of the wall material at a more advanced stage than that shown in A has taken place with less change in cell shape during enlargement.

C. Presence of the colorless gel around the conidium is more evident than in A and B. The enzyme has apparently passed through the gel to attack the cell membrane.

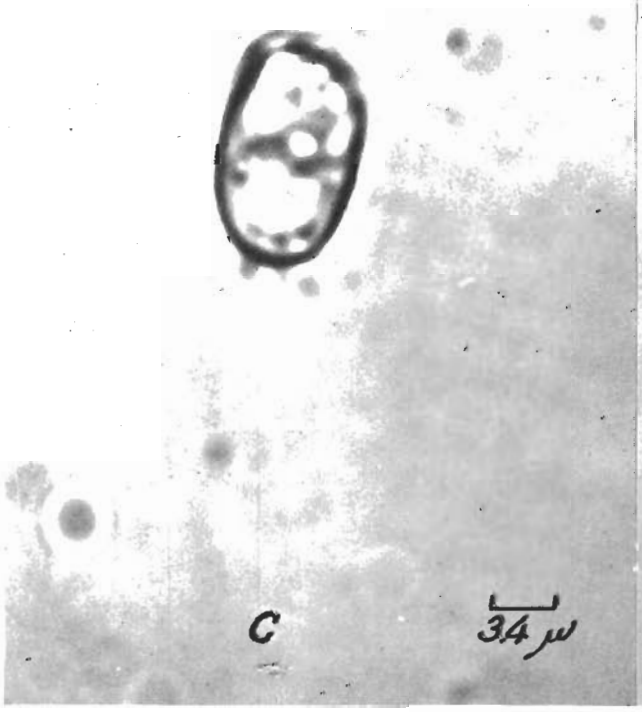
D. Late stage of reaction of chitinous wall and protoplasmic material.



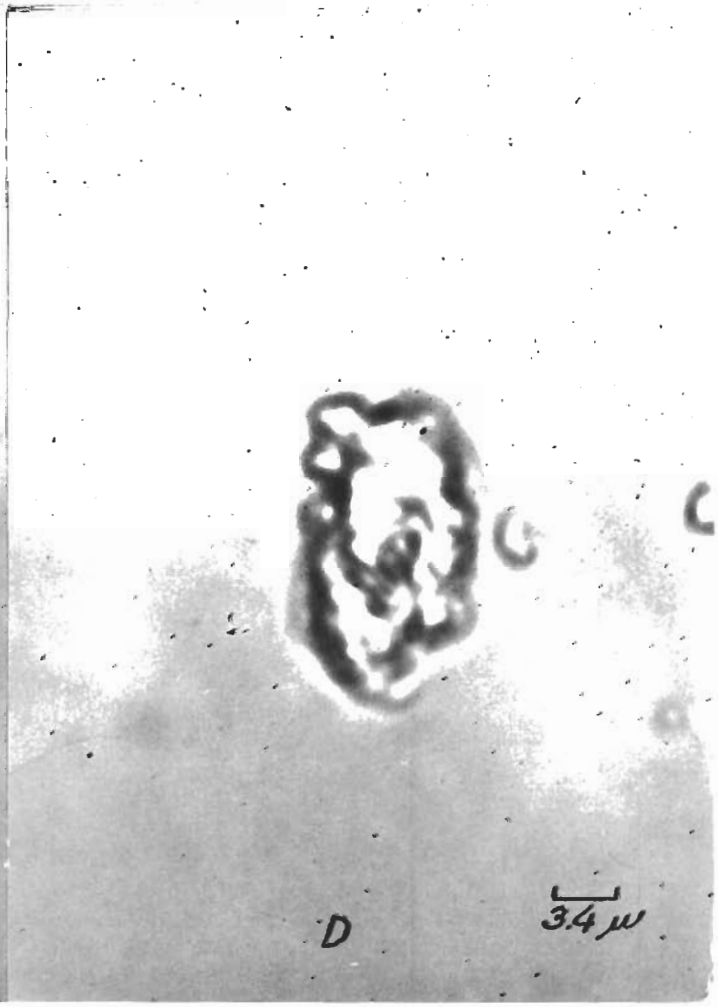
A



B



C



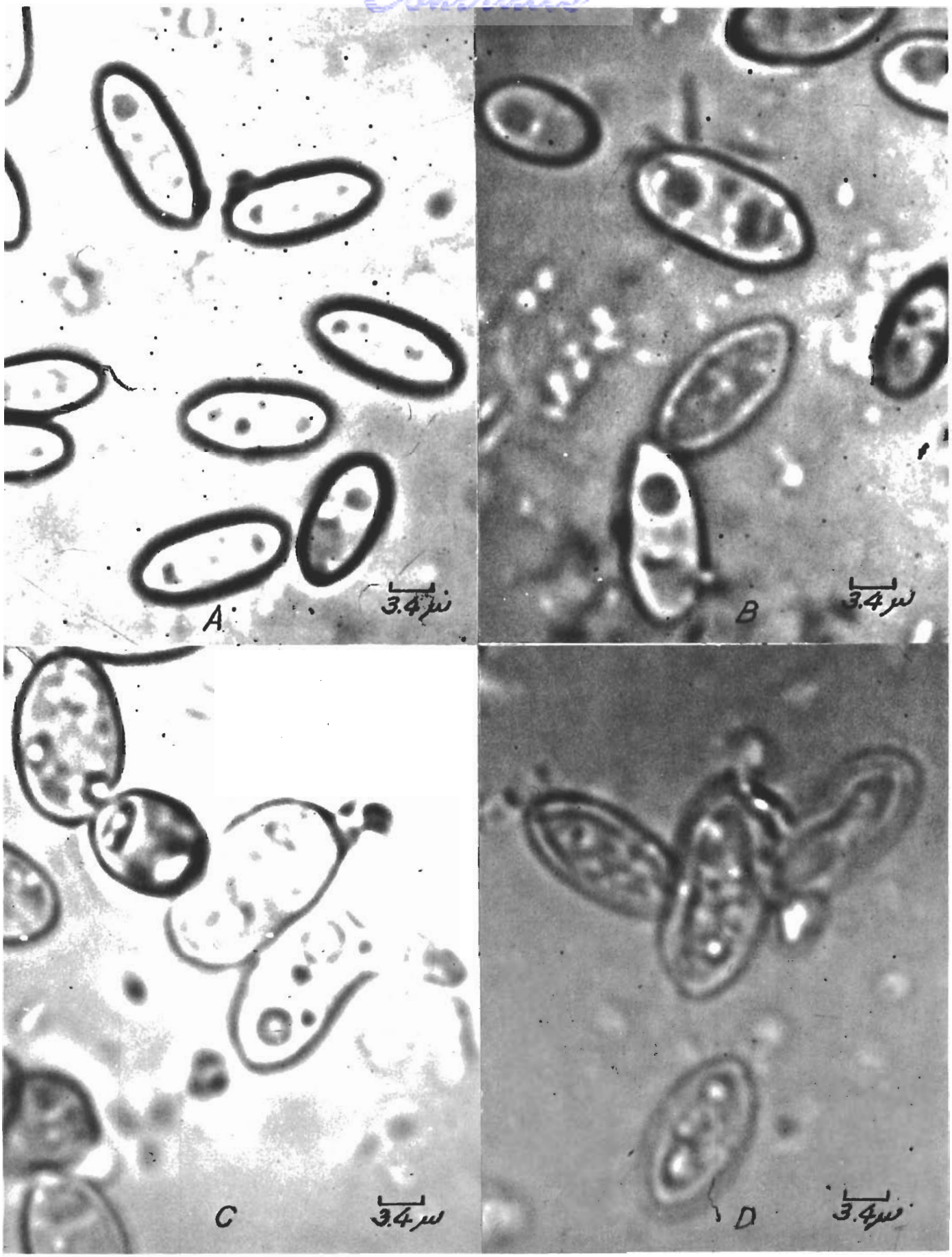
D

Figure 14

Reactions of Conidia of M. verrucaria ATCC 9095 to Juice of the Puffball, Calvatia gigantea

A, B. Earlier stages of reaction to a full strength solution of dialyzed puffball juice.

C, D. Later stages of reaction to a full strength solution of dialyzed puffball juice.



EARLIER INVESTIGATIONS OF BACTERIAL CELL WALLS

In 1898 van Wisselingh (75) had examined the cell walls of Bacillus Megaterium De Bary, Bacillus anthracoides Trev., Bacillus mesentericus vulgatus Fluegge, Bacillus violaceus Schroeter, and Bacillus pulcher Beijerinck, for the presence of chitin, and had obtained negative results with every type of bacterial organism studied. After treatment with concentrated potassium hydroxide, at 160°C, a procedure which normally converts chitin to chitosan, the chitosan could not be found. In the residues from the bacteria after treatment at 300°C in glycerine, a method used to remove the non-chitinous membrane materials, no chitin was present.

Ruppel (54), in 1898-1899, expressed the opinion that the tubercle bacillus, Mycobacterium tuberculosis,contained a substance closely related to either keratin or chitin, but the chitosan reaction was not employed in an attempt to identify it. Emmerling (14), in 1899, stated that he had found a chitin-like substance in a bacterium which was infecting the fruit of Sorbus aucuparia. Through treatment of the bacterial substance with concentrated hydrochloric acid, he claimed to have obtained a conversion to hydroxylglucosamine, but no attempt to identify chitosan during the degredation process was made. During the following year, Helbing (30) reported an indication of the presence of chitin in the wall of Mycobacterium tuberculosis by means of a staining reaction, but the chitosan reaction was not employed in his experiments. In the same year, Aronson (1) found no chitin in the walls of Bacillus diphtheriae.

In 1907, Garbowski (22) found no chitin in his microchemical studies of the walls of Bacillus luteus and Bacillus tumescens. In 1909 Burri and Allemann (9) reported that lactic acid bacteria involved in the souring of milk were surrounded by a slime which was colored violet by treatment with iodine and weak sulphuric acid. Upon the basis of this reaction they expressed the opinion that the material was chitin-like. Although the red-violet coloration of chitosan, when treated with solutions of iodine-potassium iodide and weak sulphuric acid, is accepted as indicative of chitin in the unconverted cell walls, the procedures used with this bacterial slime are not comparable to the method used in the well known chitosan reaction, and the results are not conclusive with respect to the chitinous nature of the slime. During the same year, Wester (66) obtained negative results during his efforts to find chitin in the wall of Staphlococcus aureus. Concurrently Panzer (44) assumed the presence of chitin in the wall of Mycobacterium tuberculosis, although the chitosan reaction had not been used in the course of his experiments, and Beijerinck (3) reported the presence of a nitrogen-containing substance similar to chitin in the walls of Streptococcus hollandicus.

Viehoever (64), during the following year, reported the presence of chitin in the cell walls of Bacillus alvei and Bacillus

subtilis upon the basis of his results with a modified chitosan reaction. In discussing the negative results which had been obtained by van Wisselingh in his attempts to identify chitin in the walls of bacteria, he suggested the possibility that van Wisselingh had not adapted the method used in converting the chitin to chitosan in the fungi to the more delicate membranes of the bacterial organisms. By heating the bacteria in 50% KOH solution, in an autoclave, at 6 atmospheres pressure, for 15 minutes, Viehoever had obtained a blue-violet coloration in the walls after treatment with the iodine-potassium iodide and weak sulphuric acid solutions. The negative results of van Wisselingh were considered to be due to too prolonged treatment in the strong alkaline solution; those of Wester and Garbowski to too brief treatment with the same solution.

Tamura (60), using macrochemical methods, found no evidence of the presence of chitin in the walls of Mycobacterium lacticum and Bacillus diphtheriae.

In 1916, van Wisselingh (75) reported the absence of chitin in the cell walls of Bacillus prodigiosus (Kiliensis), red and white mutants, Aerobacter viscosus, Bacterium aceti, Bacterium melanogenum, Bacterium ranceus, Bacterium xylinum, Mycobacterium album X, Mycobacterium album II, Mycobacterium album III, Mycobacterium hyalinum, Mycobacterium lacticola, Mycobacterium luteum, Mycobacterium phlei, Mycobacterium roseum, Mycobacterium rubrum, Sarcina flava, Streptethrix chromogens, Streptethrix odorifera, and in the cell walls of the bacteria taken from the root nodules of Baptisia leucophaea, Lathyrus latifolius, Melilotus officinalis and Trilolium pratense.

In 1921 von Wettstein (67), in an effort to clarify the results of Viehoever, studied the reactions of the cell membranes of Bacillus alvei, Bacillus asteroporus, Bacillus probatus, Bacillus robur, Bacillus sphaericus, Bacillus subtilis, Bacillus tumescens, Sarcina ureae Beijerinck, and the sulphur bacillus, Beggiatoa alba. "Conversions to chitosan" were attempted over a wide range of concentrations of potassium hydroxide, temperatures and lengths of periods of treatment. The results with respect to the chitosan reaction were in every instance negative. Although some colorations in the treated cell walls, from rose to dark red-brown, were obtained through the action of potassium hydroxide solutions, and became clearer after the addition of weak sulphuric acid solution, the iodine potassium-iodide solution was not involved in the color reaction. Von Wettstein concluded that the coloration obtained was not characteristic of chitosan and that it represented a reaction between an unknown cell substance and the potassium hydroxide.

In 1925 van Wisselingh (76) reviewed his studies of bacterial cell walls which had extended over more than a quarter of a century, and restated his conclusions that the many attempts to identify chitin had been negative. Viehoever's findings and interpretations were

discussed. His belief that, in carrying out the chitosan reactions, van Wisselingh had treated the organisms for too long, and Wester and Garbowski for too short, periods of time was examined critically in the light of more recent results with variations in time, concentration of alkali and temperature. Van Wisselingh's considered opinion was that the key to the explanation of the difference in the results could be found in Viehoever's own assertion that "Die Farbung nicht immer rein war, die Farbention nicht immer rein violet war." In this connection, van Wisselingh pointed out that, when properly carried out, the chitosan reaction is precise and unmistakable. His over-all conclusion, therefore, was that true chitin probably was not present in the walls of any of the bacteria which had been analyzed, although there seemed to be no conclusive evidence as to what the wall material might be.

The uncertainties which continue to be evident in discussions of the structure and composition of bacterial cell walls can be exemplified by observations of and interpretations of the structure and composition of Acetobacter xylinus. In 1934, Farr and Eckerson (16) described their cytochemical findings with respect to the membrane-like aggregations of this bacterium as follows:

> p. page 196. "The entire 'membrane' has been found to consist of bacterial organisms with no true intercellular substance. The single bacterium is composed of a protoplast surrounded by a cellulose membrane which, in turn, is covered with a layer of pectic substance. This pectic coating is not thick enough to obscure the double refraction of the cellulose membrane in polarized light."

The double refraction of the cellulose in the wall of the bacterium, the blue coloration of this cellulose when treated with 72% sulphuric acid and iodine and the pectic reactions of the outer, or primary, layer of the bacterial wall to ruthenium red stain were illustrated in both black-and-white and color at a magnification of 2700 diameters.

In contrast to these findings, Mühlethaler (41), in 1949, having studied these same cellulose-forming bacteria, reported that they produce an amorphous mass of a precursor of cellulose which accumulates in the culture media at some distance from the surface of the bacterial cell. It then differentiates into a mass of long cellulose fibrils of about 25 mu in diameter as observed in the electron microscope. As the culture ages, most of the amorphous material disappears and the resulting fibrils become so intertwined as to build a rigid membrane. These very different descriptions of the structure and composition of the "membrane" formed by Acteobecter xylinus in a nutrient medium have not been resolved. Subsequent X-ray diffraction analyses of the membranes have not clarified the picture,

since these data could be interpreted upon the basis of either type of membrane structure. Electron microscopical studies of the bacterial "membranes", lacking chemical identifications of the fibrillar structures described, have failed to provide conclusive evidence as to whether the cellulose arises intracellularly, a product of protoplasmic synthesis, or extracellularly, in the nutrient medium.

CURRENT CONCEPTIONS OF BACTERIAL STRUCTURE AND COMPOSITION

Knowledge of the composition and structure of the bacteria is being increased rapidly by advances in organic chemical techniques and by the high resolutions obtainable in the electron microscope. The current conceptions of these diminutive and ubiquitous organisms are therefore mixtures of past and present results, both general and specific in nature.

Many bacteria have been found to have the structure of a typical plant cell, consisting of the protoplast, cytoplasmic membrane and the cell wall. A so-called "outside cell wall" may be in the form of either a rigid capsule or a loose slime. Special appendages, or flagellae, are present on most of the bacteria which are capable of locomotion. These flagellae appear to arise in the cytoplasmic membrane and extend through the cell wall and also the capsule or slime coating, when present. Endospores, resistant structures capable of withstanding many unfavorable conditions for life and growth of the organism, are produced within the cells of some bacteria.

Cell walls in the bacteria are usually strong and rigid, and their thickness is reported (10) to range from 10 to 25 mu. The walls of gram-negative forms are, in general, thinner than those of the gram-positive forms. The detailed studies of bacterial cell wall structure, which have been made by means of electron microscopy, have sometimes required the isolation of the cell walls or cell wall fragments by either mechanical disruption, or enzymatic digestion, or thin sectioning. In such specimens, the walls of gram-positive bacteria appear to be homogenous in structure, while those of the gram-negative forms, in thin sections, show multilayers.

Not all bacteria have all three layers (cell wall, capsule and loose slime). When capsules do occur, they are usually thick and viscous, some appearing to have structure, others amorphous. Carpenter (10) illustrates this characteristic in heavily encapsulated streptococcus chains by a photomicrograph in which the bacteria are simply mounted in india ink. The resistant, unstained capsules stand out sharply against the black background. The loose slime, when present, may be similar in composition to the capsule, both being comparatively rich in carbohydrates, but less resistant and more soluble because of its physical state. The loose slime can be removed without harm to the organism, and is replaced by the cell. Capsules are highly protective, even enabling the bacterium to resist phagocytosis. If the capsule is removed, however, the bacterium is readily ingested by the phagocyte.

Although water constitutes 75 to 85 per cent of the bacterial cell, the list of additional chemical compounds reads like the lists from many types of higher plant cells. Proteins make up approximately 50 per cent of the dry weight, carbohydrates 10 to 30

per cent. Lipids are usually found in amounts varying from 10 to 15 per cent, but can reach 40 per cent when grown upon certain nutrient media. A list of additional chemical constituents may include sugars, organic acids, nucleotides, phosphate esters, amino acids, vitamins, enzymes and coenzymes. Bacterial carbohydrates are reported to be located primarily in the cell walls and capsules; lipids in the cell wall and cytoplasmic membrane. Bacterial proteins occur in the protoplasts, cytoplasmic membranes and cell walls. Flagellae, when present, are reported to be composed of a fibrous, elastic protein substance which resembles both the keratin of the animal skin and myosin of the animal muscle. Most of the known amino acids have been detected in bacterial proteins. One amino acid, diaminopimelic acid (DPA) has been found only in the bacteria and blue-green algae. This amino acid is described by Carpenter (10), page 123, as "...part of a macromolecular polypeptide structure also containing muramic acid which is essential for the rigidity of the cell wall."

Determinations of the constituents of mechanically isolated cell walls have shown that both the gram-positive and gram-negative bacteria contain diaminopimelic acid and muramic acid. The gram-negative forms contain most of the known amino acids, while the gram-positive bacteria have shown only a few of the principal amino acids, no sulfur-containing amino acids and no aromatic amino acids. This difference in the amino acid content of the cell walls of gram-positive and gram-negative bacteria is balanced by the 35 to 60 per cent polysaccharide content in the gram-positive forms as compared with the 15 to 20 per cent in the gram-negative forms. Lipids are reported to be as low as 0 to 2 per cent in the gram-positive bacteria and higher (15 to 20 per cent) in the gram-negative forms (10, page 125).

McCarty (39), in 1952, Ghuysen (23), in 1957, and Strange (59), in 1959, reported upon a number of enzyme systems which bring about the breakdown of bacterial cell walls. In 1959, Salton (56) studied the degredation products from the walls of Bacillus megaterium, Sarcina lutea and Micrococcus lysodeikticus, after treatment with lysozyme. In the M. lysodeikticus samples about half of the original walls of the bacteria became diffusible after treatment with lysozyme. The most definite chemical identification was that of a material containing glucosamine and muramic acid. This material was also found in the hydrolytic products from the lysozyme-treated walls of B. megaterium and Sarcina lutea. Of particular interest was the finding that both amino groups of the amino sugars were acetylated.

Berger and Weiser (6), in 1957, reported that acetylglucosamine is produced by the action of lysozyme upon chitin. Concurrently a paper by Repaske (50), dealing with the lysis of gram-negative bacteria by lysozyme, and two papers by Perkins (47, a and b) confirmed the work of Salton (56) and of Berger and Weiser with respect to the action of lysozyme upon bacterial cell walls. In

1960, Ghuysen and Salton (24) reported that isolated disaccharides from the cell walls of M. lysodeikticus, when digested with egg white lysozyme, broke down into the free N-acetylamino sugars, N-acetylglucosamine and N-acetylmuramin acid. Although the effects of lysozyme upon bacterial cell walls are far from being completely understood, a pattern is beginning to emerge, and results of enzymatic hydrolysis such as these will be extremely valuable in the development of an accurate picture of the composition and structure of any given bacterial cell wall.

Salton (57) has raised the question, still unanswered, concerning the location of the pigment in pigmented bacteria. Some pigmented forms give isolated wall fractions devoid of pigment, while in others the pigment compounds persist during isolation. Salton suggests that especially in the photosynthetic bacteria, which contain both carotenoids and photosynthetic pigments, the appearance of these substances in the cell wall isolates may be an artifact of isolation procedures.

Pseudomonas aeruginosa, of the genus Pseudomonas, is a rod-shaped, gram-negative bacterium, with a rigid wall, and with polar flagellae used as a means of locomotion. It is usually found in limited numbers in soil or water, produces a bluish or greenish-brown water-soluble pigment, has a tendency to form a pellicle, is proteolytic, and is reported to be pathogenic for both man and animals (4, 5). Synonyms for P. aeruginosa are Bacillus pycocyaneus and Pseudomonas pycocyanea.

In a study of the chemical composition of the walls of a number of gram-negative bacteria, Weidel and Primosigh (65) found a complete range of amino acids, substantial amounts of lipid and frequently monosaccharide constituents. The total lipid and amino sugar content for P. aeruginosa and E. coli are given as follows:

Cell Walls	% Dry Wt. of Cell	
	Total Lipid	Amino Sugars
P. aeruginosa	11	2.1 - 2.7
E. coli	22	3.0

Davis, et al (13) of the School of Aviation Medicine, Randolph AFB, Texas, have found that pigmentation can be a useful criterion when the organisms are cultured upon suitable media. On Sabouraud Maltose Agar, recommended by Martineau and Forget (38), pigment was produced in 95 per cent of the cultures and this method has proved to be a valuable one for the early detection of P. aeruginosa in clinical practice.

Concurrently Gaby and Hadley (21) have presented a practical laboratory method of identification based upon the cytochrome oxidase concentration present in the cells of P. aeruginosa. Either tube cultures or agar plates can be used and a blue color is produced upon

the addition of a mixture of a-napthol solution and p-aminodimethylanaline oxalate.

Schneierson, Amsterdam and Perlman (58) have found that adequate oxygen in the culture tube or petri dish is a prime requisite for the production of pigment in P. aeruginosa, since pigment was absent in tightly stoppered tubes. Chloramphenicol and erythromycin also inhibited the formation of pigment. The authors observed, however, that the suppression of color by these antibiotics was not a simple matter of oxygenation. The antibiotics exert their pigment-depressing effect by interfering with pigment biosynthesis in the cell, and once this mechanism is disturbed it is not restored in successive cultures supplied with O_2.

Two important reports on Pseudomonas aeruginosa were prepared by Walter M. Bejuki (4, 5) of the Prevention of Deterioration Center, and were presented, respectively, to the ASTM Committee D 20, on March 10, 1960, in Cincinnati, Ohio, and June 30, 1960, in Atlantic City, New Jersey. The emphasis in these reports is placed upon the inadvisability of using P. aeruginosa as a test organism for the deterioration of materials because of its reported pathogenicity. In the course of developing this main thesis, much important background information concerning P. aeruginosa is presented. Although widely distributed in nature, its role in natural microbiological deterioration is now in the process of being evaluated. Many strains of Pseudomonas, however, have been found among the organisms which contaminate aircraft fuels.

Escherichia coli is one of the common colon bacilli belonging to the genus Escherichia. It is rod-shaped (0.4 - 0.7 x 1.0 - 3.0 microns), gram negative, grows luxuriantly on ordinary nutrient agar and broth and grows more rapidly at 30 - 37°C than at room temperature. It has no flagellae. Chromatin bodies, or nuclei, are prominent in the cell, occupying 15 - 25 per cent of the protoplasmic volume. E. coli is found rarely outside the intestinal tract of man and animals, except in water and soil which have been contaminated with human and animal excreta. It is primarily non-pathogenic, living on the body wastes of the intestine. Only when it spreads to the bladder and kidneys does it behave, at least temporarily, as a parasite.

The use of E. coli in sanitary water and food analyses has brought about extensive studies of its morphological characteristics as well as its growth habits and cultural needs. This information, with numerous illustrations, is found in most of the current text books and reference books on bacteriology and microbiology. E. coli has served as the experimental material for many chemical studies of gram-negative bacteria and also for numerous electron microscopical studies of bacterial cell walls.

CYTOCHEMICAL ANALYSES of *Pseudomonas aeruginosa* and *Escherichia coli*

A culture of *Pseudomonas aeruginosa* ATCC 13388 was obtained through the courtesy of Dr. Walter Bejuki of the Prevention of Deterioration Center of the National Academy of Sciences, Washington, D. C. The culture of *Escherichia coli* K 12, ATCC 10798 was purchased from the American Type Culture Collection, 2112 M Street, N. W., Washington, D. C.

Both of the organisms were grown on Nutrient Difco-Bacto Agar and also on Sabouraud's Maltose Agar, in petri dishes 6 cms. in diameter. The cultures of *P. aeruginosa* were held at room temperature and those of *E. coli* at approximately 32°C. Inoculations of a sufficient number of plates for one type of analysis of each of the two bacteria were made at one time. This procedure permitted direct comparisons of the reactions of the bacteria to the same treatment. As soon as any given analysis and comparison was completed, all unused cultures in this lot of material were destroyed. This procedure provided fresh, actively growing organisms, as well as mature bacterial cells, for every type of analysis. Pigment formation was somewhat more pronounced in the cultures of *P. aeruginosa* grown upon Sabouraud's Maltose Agar than in those grown upon Nutrient Difco-Bacto Agar, but no cultural difficulties were encountered in either type of culture medium. Cultures of *P. aeruginosa* grown at 32°C developed more rapidly than those which were held at room temperature, but no other differences were noted.

The methods of analysis used with these bacteria were the standard cytochemical procedures, with certain adaptations to bacterial organisms indicated by the results of earlier workers. Living bacteria were used for the observations in ordinary light, polarized light and the studies of fluorescence in ultra violet light. In preparing the specimens for analysis by these optical methods, the slightly viscous 0.1 M sucrose solution, used as a mounting medium for studies of fluorescence, helped to reduce the motion of the small organisms without interfering with the precision of the methods. Fluorescence phenomena were studied with both light and dark-field condensers and both before and after treatment with 0.005 per cent analine blue solution in M/15 K_2HPO_4, according to the method of Currier (12).

Both the van Wisselingh chitosan reaction, for the identification of chitin, and the Payen Sulphuric acid-iodine method, for the identification of cellulose, involve drastic treatment of cell wall materials and immediate death of the organisms. In the light of the results obtained by previous workers in cytochemical studies of bacterial cell walls (56, 57, 64, 74 and 76), negative results in attempts to identify chitin could not be considered to be final until

numerous lengths of treatment in saturated KOH at 180°C over a range of time periods from 10 minutes to 3 hours had been employed. In some measure this same type of caution needed to be exercised in carrying out the sulphuric acid-iodine method of Payen for the identification of cellulose. Only after the treatment of the bacteria for different lengths of time, in standard concentrations of reagents and at standard temperatures, had failed to produce significant results, could changes in concentrations and temperatures be justified. Upon the basis of present data, alterations in concentrations and temperatures appear to be unnecessary.

Enzymatic hydrolysis of the bacterial cell wall materials have been attempted with 0.5 mg./ml. Worthington Lysozyme and with 0.5 mg./ml. Cellase 1000, made by the Wallerstein Company. The lysozyme is considered to be both a chitinase and a B-glucosaminidase, and the Cellase 1000, a highly potent cellulase.

Results

The cytochemical studies of Pseudomonas aeruginosa and Escherichia coli have been carried on concurrently. Although there have been differences in the types and degrees of reactions of the two organisms to the reagents used, two experimental factors which seem to apply to both of them can be brought out at once:

1. In attempting to identify chitin in the cell walls of both organisms, the initial treatments of 3 hours, followed by treatments of 2 hours and then 1 hour at 180°C in saturated KOH were all of too long duration. Centrifuged residues were small in volume and contained fragments of questionable identity. No entire organisms of either type were found. Swollen fragments, brown after the addition of the solution of I_2KI, could have represented reacting cell wall material which had been considered to be chitosan by Viehoever (64) and believed to be non-chitinous in nature by van Wisselingh (75). In any event, they could not be related to the original bacterial cell wall materials in these experiments.

2. The prolonged treatment in 72% H_2SO_4, amounting to 2 to 3 months in some fungi, has been found to be necessary to penetrate the chitinous primary lamellae of the fungal cell walls and bring out the presence of cellulose in the secondary lamellae. When the cells of P. aeruginosa and E. coli were treated in this reagent for 2 months and 3 months, respectively, no recognizable traces of the organisms could be found in centrifuged residues. The period of time for the treatment was first reduced to 1 month, then 2 weeks, 1 week, 4 days, and finally 2 days before useful specimens were obtained. Further work needs to be done in order to determine the optimum period of time for treatment for each one of the two organisms.

van Wisselingh Method for the Identification of Chitin

In Figure 15-B and Figure 16-B, it can be seen that even a 50-minute period of treatment in saturated KOH at 180°C is too prolonged for cytochemical differentiations of cell wall materials in both P. aeruginosa and E. coli. The highly swollen organisms shown are representative of the comparatively few which withstood the treatment at all. More significant results were obtained in samples which were treated for 20 minutes, and these specimens were subjected to detailed examinations. Bacterial cell walls which were brown or greyish-brown in color were frequently in evidence. Such reactions were observed by van Wisselingh (75) and were not considered to be characteristic of chitosan. Further study of the samples of P. aeruginosa brought out the evidence that these brown to greyish-brown

colorations were usually in walls of larger, mature bacteria which probably had been heavily pigmented at the time of treatment. The degree to which this pigment had been affected by the treatment with saturated KOH at 180°C could not be determined from this one set of samples. The possibility that some residual pigment may have served to prevent the development of the clear red-violet color characteristic of chitosan cannot be excluded until additional information has been obtained. Fewer of the brown cell walls were found in the sample of E. coli. The wall coloration in these bacterial cells, however, was deep yellow, not red-violet as in the typical chitosan reaction. In many of the samples of P. aeruginosa, the color of the primary wall material in many small, possibly younger, bacteria, varied from bright red to brick red. In no instance was the red-violet coloration of chitosan observed in the specimens of either P. aeruginosa or E. coli.

A series of samples representing conversions of P. aeruginosa organisms with different degrees of original pigmentation and periods of treatment in saturated KOH at 180°C from 10 to 20 minutes, could be expected to explain more fully the nature of these observed colorations in wall material from brown to bright red. Upon the basis of the van Wisselingh chitosan reaction, evidence of the presence of chitin in the primary wall lamella of P. aeruginosa is not yet conclusive. In E. coli, the evidence, at present, is negative.

Payen Method for the Identification of Cellulose

Figure 15-C and Figure 16-C bring out the striking contrast in the reactions of P. aeruginosa and E. coli, respectively, to treatment with 72 per cent H_2SO_4 for two days. In general, the P. aeruginosa organisms were highly reactive and the E. coli organisms comparatively resistant. There is usually some individual variation in reactions of the cells in any given sample, however, and it was in mounts from these same preparations that certain organisms showed definite cytochemical differentiations. In both P. aeruginosa and E. coli the results clearly indicate differences between the chemical makeup of the primary and secondary lamellae. The blue coloration of the material in the secondary lamella of P. aeruginosa is characteristic of cellulose in the presence of 72 per cent H_2SO_4 and I_2KI solution. It is similar in color and localization to the cellulose in the wall of Acetobacter xylinus described earlier by Farr and Eckerson (16). That such similarity between these two bacteria should exist need not be surprising, since both are members of the Pseudomonaceae. The bluish-purple color of the secondary wall material in E. coli is not definitely characteristic of cellulose. There is a possibility, however, that it represents a mixture of cellulose and a proteinaceous material which would be reddish brown when treated with these same reagents.

These results indicate that a period of treatment of less than two days in 72 per cent H_2SO_4 can be expected to provide a greater number of bacterial cells which are suitable for cytochemical differentiations.

The substance or substances in the primary lamellae of both P. aeruginosa and E. coli is deep yellow in color after treatment with 72 per cent H_2SO_4 and I_2KI. In plant cells in general, this yellow coloration of wall substance, in the presence of these reagents, is characteristic of pectic materials, proteins and chitin, and will not serve to identify any given wall substance unless confirmed by other techniques. The immediate value of this reaction, taken alone, is that the differences in chemical makeup of primary and secondary wall substances is clearly differentiated in both of the bacterial organisms. Future analyses and identifications can follow these separate lines, with the procedures and techniques indicated.

Primary Fluorescence and Double Refraction

The presence of the phenomena of both fluorescence in ultra violet light, and double refraction, in polarized light, in individual bacteria, is difficult to observe because of their diminutive size and the small amount of reacting material. The specimens of P. aeruginosa and E. coli were examined both individually, at magnifications of 2000 to 3000 diameters, and also in aggregates of organisms, at 200 to 400 diameters. For the purpose of illustrating the general findings with respect to primary fluorescence and double refraction, clusters of bacteria, in which the individual organisms are not resolved, were photographed at 400 diameters and are shown in Figure 17 A, B, C and D.

In Figure 17-A, clusters of P. aeruginosa, and a single large crystal of unknown composition, were photographed in ordinary light. Figure 17-B shows the same field in ultra violet light. The bacteria are conspicuously fluorescent and the crystal non-fluorescent, with the exception of a small amount of material concentrated at one end of the crystal. Figure 17-C, of the same field, in polarized light, shows that the crystal is highly doubly refractive, and the bacteria either non-doubly refractive or that the dark bacterial pigment has masked the double refraction. Observations, to date, indicate that in P. aeruginosa we have primary fluorescence. Whether this is due to the cell wall materials, or to the pigment, or both, has not yet been determined. Examination of a number of samples of P. aeruginosa, which had been extracted with warm water for partial removal of the pigment, clearly showed an increase in the brightness of the fluorescence. This same treatment, however, appeared to produce no increase in the intensity of the double refraction, as shown in Figure 17-C. This phase of the work should be continued, with series of samples from which increasing amounts of pigment will have been extracted. Examinations of these samples, with low magnifications

of groups of bacteria and high magnifications of individual organisms, will help to clarify the interpretations of both fluorescence and double refraction in P. aeruginosa.

The use of fluorescence phenomena in studying strains of Pseudomonas, which had been isolated from fuel tanks, has been reported recently by Iverson*. A continuation of the present studies, in connection with the cytochemical aspects of fluorescence, should furnish data which could be correlated specifically with these findings.

Primary fluorescence was not observed in the samples of E. coli, either individually or in masses. It could be induced through treatment with aniline blue, according to the method used by Currier (12) in his studies of callose, but data of this type are of little use in our present very limited state of knowledge of bacterial cell walls. Figure 17-D clearly illustrates the double refraction of clusters of E. coli at a magnification of 400 diameters. Figure 17-E shows the double refraction of a single bacterium at 3000 diameters. This single bacterium can be seen to have moved in a diagonal direction during the 35 minute exposure which was necessary to make the photographic recording. The double refraction in the original and final positions, as well as in the path of movement, however, is shown.

The studies, to date, have indicated that in P. aeruginosa, we have the phenomenon of fluorescence, and the absence of double refraction, and in E. coli, double refraction without fluorescence. Final conclusions should not be made concerning the absence of double refraction in the one and the absence of fluorescence in the other until a wider range of optical conditions, especially of different exciter wave lengths in fluorescence, have been employed.

Reactions to Lysozyme and Cellase 1000

Figure 15-D shows that the treatments of P. aeruginosa with a 0.5 mg./ml. solution of Cellase 1000 at 25°C for a period of 10 hours, produced some swelling of the organism and of the pellicle, but no pronounced hydrolysis of the wall material. The effect of the enzyme upon the E. coli organisms under the same experimental conditions can be seen in Figure 16-D to be even less pronounced.

* Iverson, Warren. Preliminary report concerning the isolation, screening and identification of microorganisms from fuel tank bottoms. 7th Progress Report (in preparation). U. S. Quartermaster Engineering Command, Chemical and Plastics Division. Presented at the 10th Conference on Prevention of Microbiological Deterioration of Military Materiel, Natick, Mass., Nov. 28, 1961.

Treatment of P. aeruginosa with 0.5 mg./ml. Worthington Lysozyme solution, at 25°C, produced the drastic dissolution of the bacterial cell walls shown in Figure 15-E. In sharp contrast to the reaction of P. aeruginosa is the limited reaction of E. coli to Worthington Lysozyme shown in Figure 16-E. There is definite swelling of the bacterial wall material in E. coli, and evidence of lysis of wall material at the surface, but the degree of hydrolysis during the 10 hour period, is far less than that in P. aeruginosa. In this connection, it is of interest to consider the findings of Repaske (50), who reported that neither lysozyme nor Versene will by themselves effectively lyse gram-negative bacteria, yet the combination of the two caused rapid lysis. Combinations of lysozyme and Versene have not been used in these experiments. The reaction of P. aeruginosa to lysozyme alone may represent an exception to the Repaske rule of behavior in gram-negative bacteria.

SUMMARY

The cytochemical studies of the two bacteria, Pseudomonas aeruginosa ATCC 13388 and Escherichia coli K 12, ATCC 10798, have been carried out against a background of large numbers of cytochemical studies of bacterial cell walls made during the late nineteenth and early twentieth centuries, along with more recent electron microscopical studies of structural details and organic chemical identifications of degredation products of bacterial cell walls.

Previous cytochemical studies, which should provide not only the nature but also the location of the wall substances, have produced results of such contradictory nature that the value of these techniques in this field of research cannot be considered to be established.

The twofold purpose of this investigation has been to estimate, if possible, the relative usefulness of the cytochemical approach to bacterial cell wall analysis, as well as to determine the chemical makeup of the walls of the two gram-negative bacteria under investigation. Following suitable adaptations of standard cytochemical methods to the diminutive cells chemical differentiations between the primary and secondary wall lamellae have been brought out, and chemical identifications of certain wall substances are well underway. Upon the basis of these findings, it is believed that cytochemical analyses can be expected to contribute precise, unique and useful information in the field of bacterial cell wall analysis. The preliminary results indicate, more specifically, the probable presence of cellulose in the secondary wall deposits of P. aeruginosa and the possible presence of cellulose mixed with another substance in the secondary deposits of the wall of E. coli. The enzymatic reactions suggest the possibility of a chitinous substance in the primary layer of the wall of P. aeruginosa, but are less definite with respect to the nature of the primary layer of the wall of E. coli.

Figure 15

Pseudomonas aeruginosa ATCC 9095. Untreated Control and Reactions to 72 per cent H_2SO_4, Saturated KOH Cellase 1000 and Worthington Lysozyme

A. Untreated bacteria, photographed in ordinary transmitted light.

B. Bacteria after treatment with saturated KOH for 50 minutes at 180°C are highly swollen and well on the way to cellular destruction. Treatment too prolonged for cytochemical analysis.

C. Bacteria after treatment with 72 per cent H_2SO_4 for two days show almost complete hydrolysis of the wall material. Treatment too prolonged for cytochemical analysis.

D. Bacteria after treatment with 0.5 mg./ml. Cellase 1000 at 25°C for 10 hours. Hydrolysis of the pellicle material at surface, and swelling of the organisms are in evidence, but the effect of the enzyme treatment is not pronounced.

E. Bacteria after treatment with 0.5 mg./ml. Worthington Lysozyme for 10 hours at 25°C are in last stages of destruction.

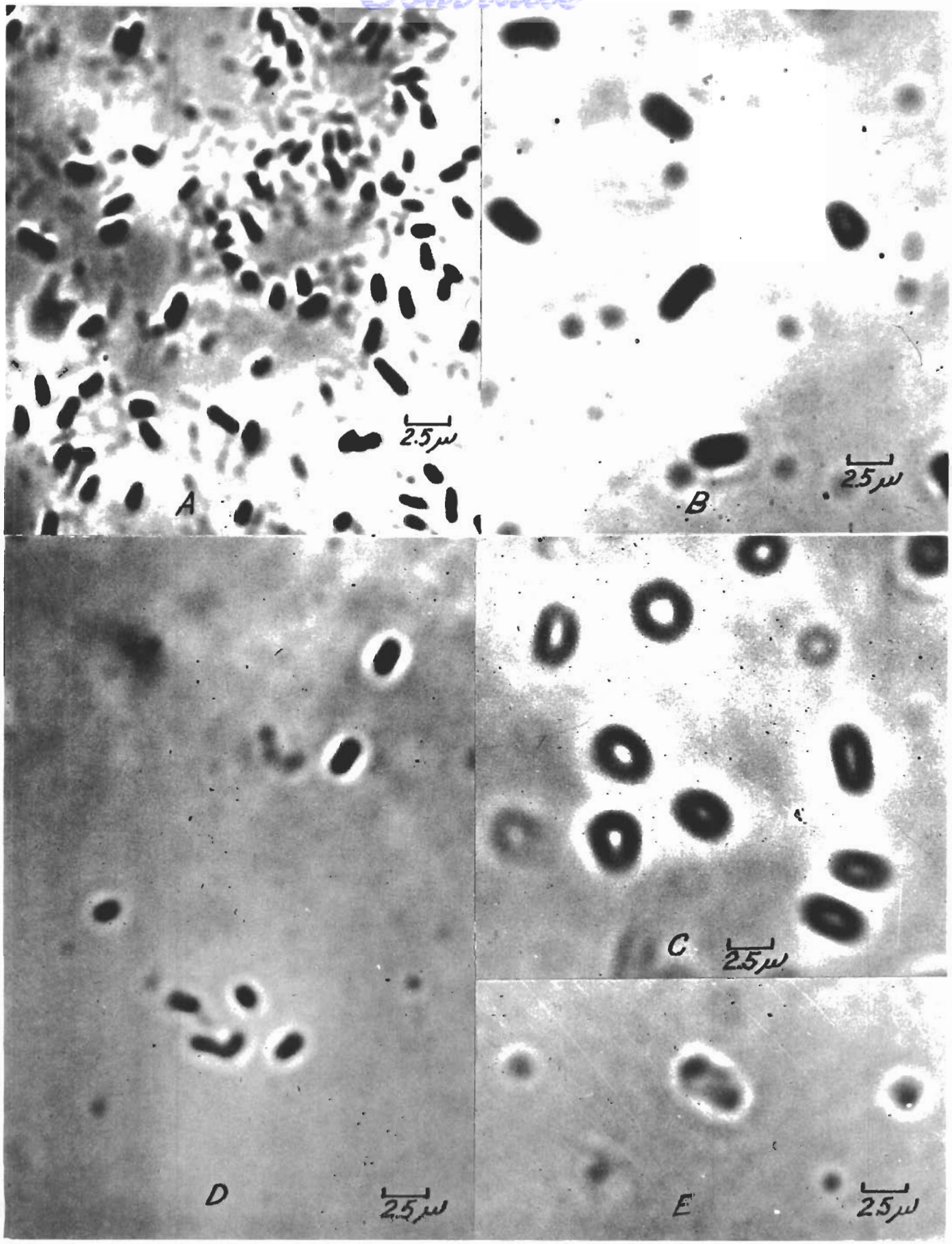


Figure 16

Escherichia coli K 12, ATCC 10798. Untreated Control and Reactions to 72 per cent H_2SO_4, Saturated KOH, Cellase 1000 and Worthington Lysozyme

A. Untreated bacteria, photographed in ordinary, transmitted light.

B. The highly swollen bacteria shown represent the few organisms which escaped destruction during the treatment with saturated KOH for 50 minutes at 180°C. This treatment proved to be too prolonged for cytochemical analyses of cell wall materials.

C. Bacteria after a 2 day treatment in 72 per cent H_2SO_4. This length of treatment in the acid was more nearly correct for subsequent cytochemical differentiation.

D. Bacteria after treatment with 0.5 mg./ml. solution of Cellase 1000, for 10 hours, show considerable resistance to this enzyme.

E. Bacteria after treatment with 0.5 mg./ml. Worthington Lysozyme, at 25°C, for 10 hours, are in early stages of enzymatic hydrolysis, but are much more resistant to this enzyme than was Pseudomonas aeruginosa.

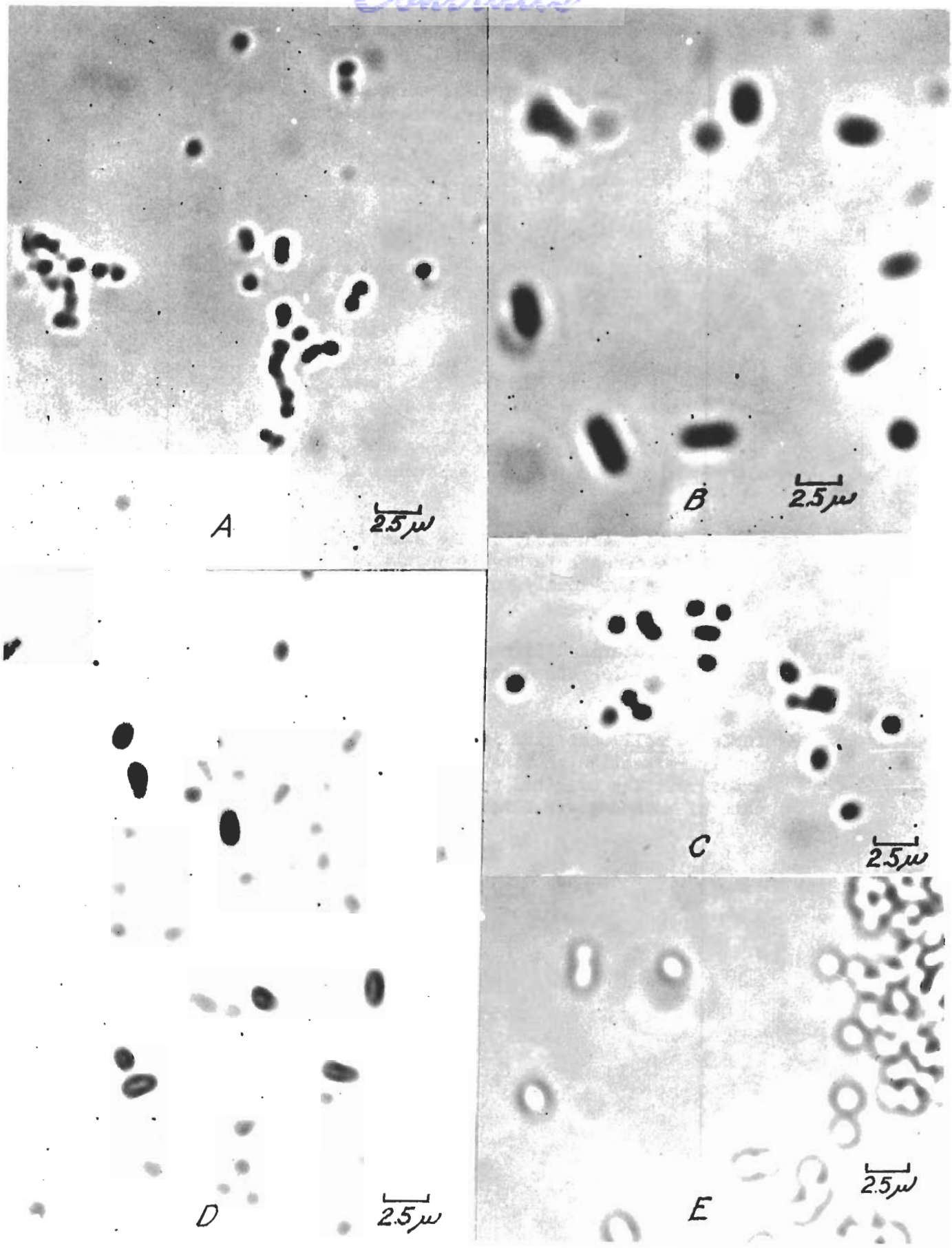


Figure 17

Pseudomonas aeruginosa ATCC 9095. Untreated Control and Reactions to Polarized and Ultra-violet Light

Escherichia coli K 12 ATCC 10798. Double Refraction of Clusters of Bacteria and Single Bacterium, in Polarized Light

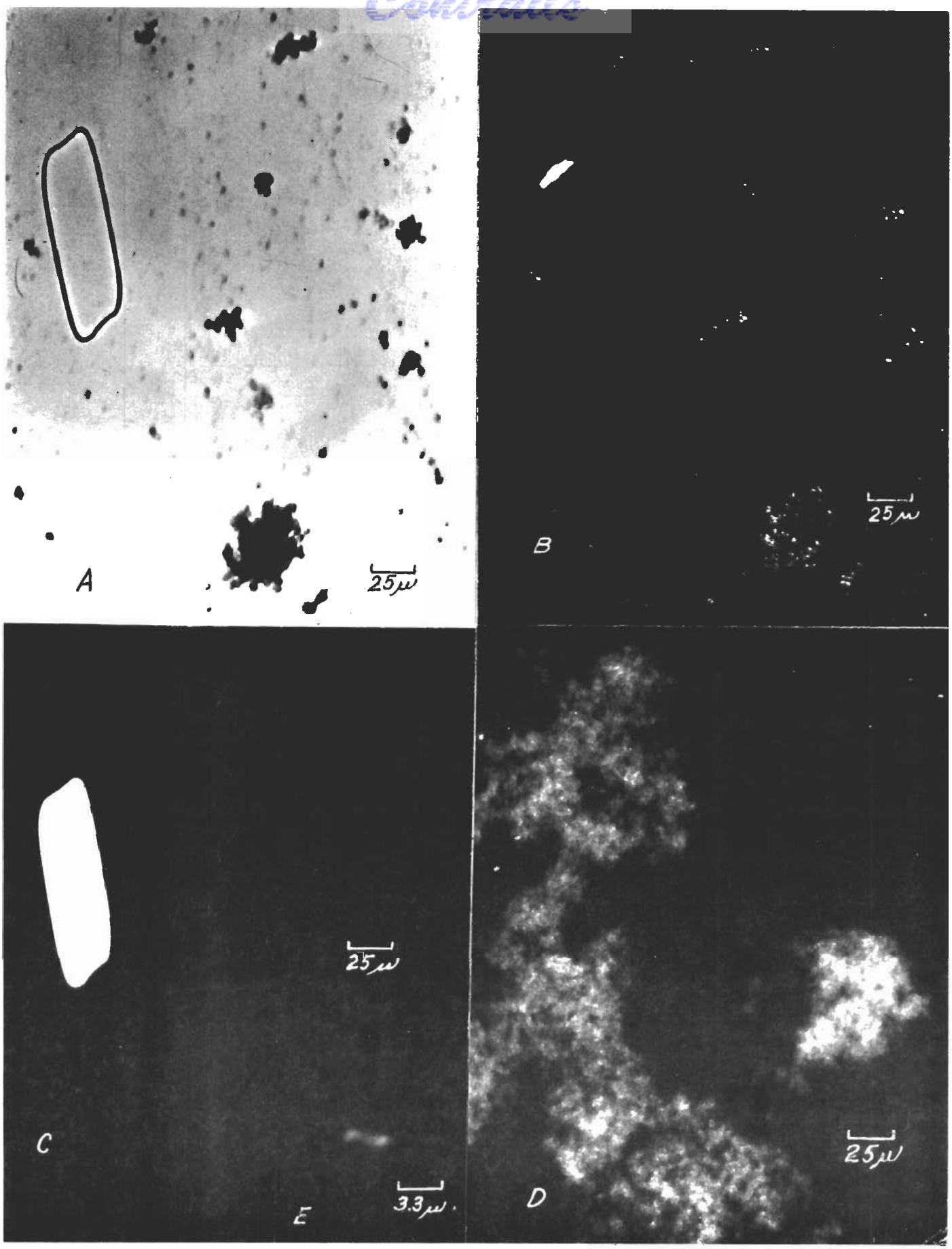
A. P. aeruginosa. Clusters of bacteria and a single crystal of unknown composition in ordinary light.

B. Fluorescence of the same bacterial clusters shown in A, using Zeiss Exciter Filters BG 12 (I and II) transmitting in the range of 300-500 mu, and a Wratten gelatin Barrier Filter No. 8K2. The bacterial clusters are weakly fluorescent, the crystal non-fluorescent except for some accumulated, highly fluorescent substance at one end.

C. In a polarized light study of the same field shown in A and B, the single crystal is highly doubly refractive, the clusters of bacteria non-doubly refractive.

D. E. coli. In polarized light, the clusters of bacteria show definite double refraction.

E. A single E. coli bacterium in polarized light is clearly doubly refractive, but has moved during the 35 minute exposure. The original position, final position and path of movement are clearly shown.



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