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Received for review November 16, 1964. Accepted July 7, 1965. Division of Agricultural and Food Chemistry, 148th Meeting, ACS, Chicago, Ill., September 1964. Work conducted under a grant for the U. S. Department of Agriculture, P. L. 480 (FG-Fr-103-61).

WORLD-WIDE RESEARCH

Recent Studies on the Microbial Degradation of Cotton

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Recent work at the Shirley Institute funded by a grant from the United States Department of Agriculture is summarized. A detailed study of the mechanism of action of the cellulase from *Myrothecium verrucaria* has shown that only relatively few sites in the cotton hair are accessible to the enzymes and that attack proceeds by solubilization of the cotton in the immediate vicinity of these sites. This suggested successful trials in which cotton was protected from degradation at very low levels of substitution with a bulky substituent when care was taken to minimize swelling. Some of the properties of the individual enzymes that contribute to the cellulolytic action of this fungus have been investigated.

The Shirley Institute has long been interested in the microbial degradation of cotton textiles, and this communication summarizes the recent advances made during the tenure of a grant from the United States Department of Agriculture. Microbial degradation causes considerable losses both to the industry and to the consumer, especially under tropical or subtropical conditions. During World War II, the average life of tentage in certain parts of the Far East was reduced by fungal attack to less than six weeks. A great many rot-proofing agents have been made available over the years. Shirlan (salicylanilide) was produced in 1929 and others in common use include metallic naphthenates, organic mercurials, and chlorinated phenolic compounds. However, even today, there is probably no completely satisfactory rot-proofing agent available.

A very large volume of largely *ad hoc* work has already gone into the production of presently available rot-proofing agents, but it seemed that a study of the mechanism of deterioration, by providing a better understanding of it, would form a basis for a more rational approach to the development of improved methods of prevention.

Extracellular Attack by *Myrothecium verrucaria*

It seemed reasonable to suppose that the degradation of cotton was caused by extracellular enzyme systems produced by the organisms. It was, therefore, surprising that cell-free filtrates obtained from microorganisms, which were themselves extremely active, had been found, by all the workers in this field, to have little action on native cotton (*13*), although they were well able to attack cotton that had been swollen (*5*). The differences in rate and extent of attack produced by the organism and by its extracellular extract were well illustrated in the case of *Myrothecium verrucaria*. After a lag phase of about two days, this organism would reduce the strength of a scoured cotton yarn to nothing in an additional two to three days. On the other hand, a filtrate from the same organism, prepared after periods of up to 14 days, or even longer, would at best reduce the strength by about one third even after incubation for many days (Figure 1). If, however, the cotton was swollen by alkali before exposure to the filtrate, both the rate and extent of attack were comparable with those obtained when the organism grew on unswollen cotton.

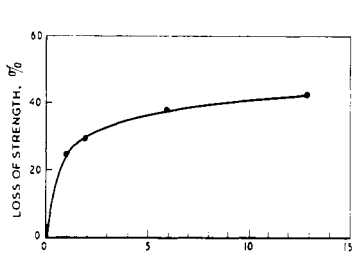

Figure 1. The degradation of cotton yarn by treatment with a culture filtrate from *M. verrucaria*

Most investigators, although aware of the problem posed by these differences, have concentrated their attention on the use of substrates made more accessible than unswollen cotton. On the assumption that the cell-free filtrates contain the extracellular enzymes involved in the cellulose breakdown, this apparent lack of activity is of considerable interest. For present purposes, however, it was pointless to investigate the degradation of easily accessible substrates, and it was desirable to use enzyme preparations with degradative powers similar to the living organisms. The purpose of this work was, first, to try to explain the low activity of the enzyme preparations and, second, to determine how the organism causes cellulolytic degradation with the ultimate objective of seeking fresh methods for its prevention.

Reese suggested (*8*) that hyphal penetration of the cotton hairs was an essential preliminary to the enzyme attack. Past experience with cotton degraded by microorganisms did not support this view. Cotton hairs had often been seen displaying damage typical of attack by microorganisms with no trace of the organism in their immediate vicinity

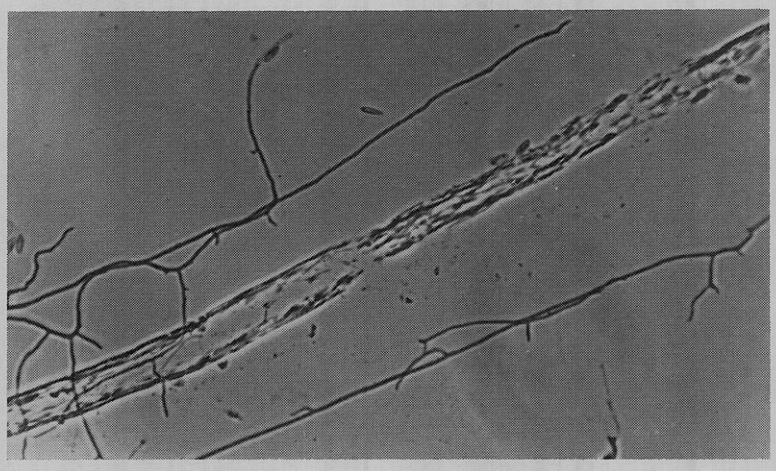

Figure 2. An extensively degraded cotton hair with mycelium of *M. verrucaria* growing nearby, but not in direct contact with the cotton

(Figure 2). There was still the possibility that the mycelium might have grown, died off, and then disappeared so that it could have been in contact with the cotton at an earlier stage. A film obtained by time lapse photography showed conclusively that degradation was possible in the complete absence of local mycelial growth. A series of photographs of a portion of cotton hair mounted on agar with spores of *M. verrucaria* was taken at intervals of 10 minutes over a period of 27 days. This showed clearly that within the field of view obtainable (20× diameter of the cotton hair), the mycelial growth was random in direction with no preference at all for cotton hairs either already or subsequently degraded. Thus, the growth of the organism was unrelated to the position of the cotton, and the degradation of any part of the latter was not dependent on the proximity of mycelium at any stage of its growth.

Although the attack was recognized as extracellular, cell-free filtrates that could produce extensive degradation of native, unswollen cotton still could not be prepared. Changes in such factors as conditions and history of growth, composition of culture media, and age of cultures from which inocula were prepared failed to produce any dramatic increase in the cellulolytic activity of the cell-free filtrates. Attention was turned for a while from the organism and its enzyme system to the substrate, the cotton hairs. At least part of the explanation of the difficulty in degrading unswollen cotton with cell-free filtrates must arise from the structure of the cotton hair itself, because of the marked differences in behavior exhibited by swollen and unswollen cotton. The degradation of cotton was investigated in two stages, by a comparison of the action of cellulases and hydrochloric acid (*10*) and, later, by a detailed study of the attack by cellulases (*12*).

Comparison of Enzyme and Acid Degradation

Degradation by fungi produces much smaller changes in the degree of polymerization of cellulose (as measured by its fluidity in cuprammonium) for a given loss of strength than does acid degradation. Indeed, this difference has been used for many years as one of the criteria for distinguishing between fungal and acid degradation of weakened cotton products (*4*, *9*). The difference has commonly been attributed to the highly localized growth of fungus, which digests whole regions of the substrate, leaving other areas untouched. However, loss of strength, with little change in fluidity, was also a characteristic of cotton degraded by cell-free culture filtrates. The localization of attack in this case could not have been due to uneven growth of the organism, but must have been caused by restriction of the enzyme itself. Whitaker (*16*) used the cellulase from *M. verrucaria* to degrade swollen and partially degraded cellulose. He showed that the changes in both weight- and number-average degree of polymerization in relation to loss of weight were consistent with a theory of random attack on all the β-glucoside linkages. Such pretreatment would, of course, have increased the susceptibility of the cellulose to attack and must have altered the degree of localization.

The relation (Figure 3) between loss of weight of undegraded cotton during hydrolysis and during aftertreatment with alkali—used as an empirical measure of the terminal aldehyde groups produced by the hydrolysis (*1*)—showed that for the production of a given number of terminal aldehyde groups in the insoluble residue, much more substrate was removed during enzymic than during acid attack. Hence, whether or not the acid attack is truly random with respect to all the β-glucoside links, enzymic hydrolysis cannot be.

This evidence, taken alone, suggested a predominantly endwise attack, but a comparison of the weight loss during hydrolysis with the degree of polymerization of the insoluble residue (Figure 4) showed that, for a given loss of weight, the effect on the degree of polymerization produced by enzymic degradation was much greater than that corresponding to endwise attack. Curves 3 (taken from Whitaker) and 4 (acid degradation) are markedly similar but completely different from the curves for unmercerized or even mercerized cotton attacked by cellulase. This suggests that the theory of random attack proposed by Whitaker may apply only to a cellulose substrate sufficiently swollen to be more than usually accessible to large molecules. Walseth (*15*), working with a cellulase from *Aspergillus niger*, put forward a similar view of the limited accessibility of the substrate.

Present results indicate that the attack of cellulase probably takes place initially at a relatively few sites in the cotton hair, followed by cleavage of a number of linkages in the immediate vicinity of these sites to give rise to soluble products. The actual location of the sites would be governed by the ability of the enzyme to enter the cotton structure. It is generally accepted that chemical reagents may be limited to the so-called amorphous regions where the cellulose chains are disordered and more weakly bound. A bulky enzyme reagent would be even more restricted in its initial attack, needing larger interstices in the structure than exist in the amorphous regions. Such interstices might be provided by the spaces between the fibrils (*7*). Having entered such spaces, the enzyme might then be able to attack both crystalline and amorphous regions in its immediate vicinity. The ready helical splitting of enzymically degraded cotton hairs provides some evidence for the localization of the initial attack.

Although the resistance of fibrous unswollen cotton to enzymic attack was thus understandable, it was still not apparent why the whole organism could degrade the cotton rapidly, whereas the derived filtrate could not. Hyphal penetration was not an essential prerequisite of attack. Conceivably, however, the living organism could build up local concentrations of enzyme. Experiments in which the concentration of the enzyme solution was increased up to 300-fold by vacuum evaporation increased the loss in strength by only one half. It appeared at this stage that some enzyme species or system present in the growing organism and necessary for the extensive breakdown of unswollen cotton might be lacking in the extracellular extract, and that its repeated renewal (or the repeated removal of an inhibitory factor) might lead to more extensive attack (*12*). Repeated 24-hour treat-

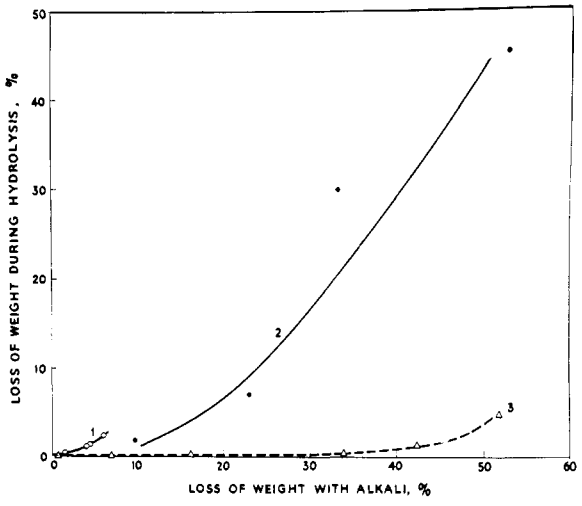

Figure 3. Degradation of (1) unmercerized and (2) mercerized cotton with *M. verrucaria* cellulase

Curve 3 is a comparative curve showing attack of 6N hydrochloric acid on unmercerized cotton
By courtesy of the *Biochemical Journal* **79**, 562 (1961)

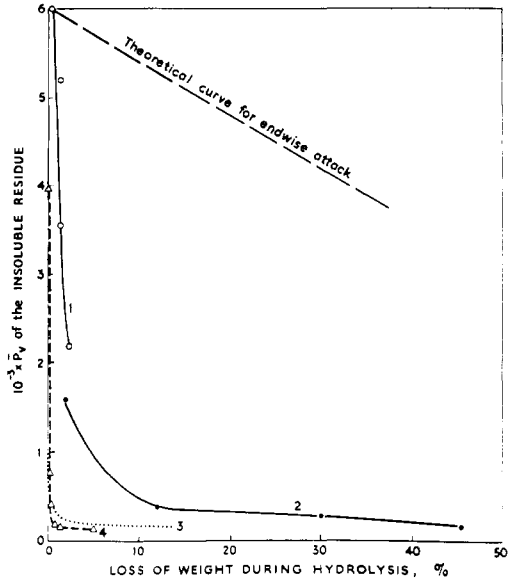

Figure 4. Comparison of degradation of cotton by *M. verrucaria* cellulase (curves 1, 2, and 3) and by 6N hydrochloric acid (curve 4)

Curves 1 and 4 relate to unmercerized cotton, curve 2 to mercerized cotton, and curve 3 to phosphoric acid–swollen cellulose
By courtesy of the *Biochemical Journal* **79**, 562 (1961)

ments of cotton with fresh portions of a cell-free culture filtrate from *M. verrucaria* (Figure 5) supported this explanation because it led to degradation comparable with that produced by the intact microorganism. The complete loss of strength was accompanied by a 30% loss of weight but by only a very small change in degree of polymerization (4100 to 3250). If a 75% loss of strength of unswollen cotton is brought about by treatment with hydrochloric acid, the degree of polymerization falls to about 260 and there is little loss of weight (*2*). That the striking increase in the degradation obtained by repeated treatments was not caused by the large amounts of enzyme solution employed is evident from Figure 5 where a curve for an experiment using sixteen times the usual liquor–cotton ratio is included.

Evidence for Two Enzymes from M. Verrucaria

Although extensive degradation of unswollen cotton had been obtained by repeated treatments with cell-free culture filtrate, it was apparent that the expenditure of so large a quantity of enzyme would be an extremely uneconomical operation for the organism. Enzyme solutions, apparently exhausted by their attack on unswollen cotton yarn, were still capable of weakening fresh yarn samples and had lost none of their ability to reduce the viscosity of a solution of carboxymethylcellulose. The process could be repeated several times, a series of fresh yarns each being weakened in turn by about 15% during a 24-hour exposure to exhausted culture filtrate. This loss of 15% was, of course, much less than that produced by treatment with fresh filtrates, but the fact that exhausted filtrates could produce any degradation at all suggested that some small part of the fiber was more accessible to the cellulase than the remainder. This view was supported by the fact that cotton yarn already weakened by exposure to culture filtrate was not further degraded by a filtrate that had already been twice exposed to cotton yarns for 24 hours. These observations suggested that cellulases of different molecular sizes were present in the filtrate, a concept borne out by later work (*11*).

The contrast between the complete loss of activity toward pretreated cotton and the persistence of a limited activity toward fresh cotton suggested that at least two enzymes were present, the loss of activity being due to the exhaustion of one of them. The exhaustible cellulase without which pretreated cotton could not be further weakened was called A-enzyme and the other, which accounted for the residual activity toward fresh yarns, B-enzyme. A-enzyme appeared to attack those linkages in fibrous cotton that must be broken to produce losses in strength of more than 15 to 20%. B-enzyme, on the other hand, did not attack these linkages and, in consequence, could not by itself reduce the strength of the cotton by more than about 15%. A-enzyme was rapidly exhausted by its attack on the cotton and had to be replaced to ensure extensive degradation. It was detected and assayed by using as a substrate cotton that had been pretreated by exposure to culture filtrate; B-enzyme was incapable of attacking such pretreated cotton. Although direct estimation of either enzyme against cotton yarn was difficult because of the lack of proportionality between enzymic activity and loss of strength, a reasonable estimate of B-activity in solutions free from A-enzyme was obtained by finding the dilution that would cause a fixed (8%) loss of strength of a fresh yarn after a single 24-hour incubation. B-activity in a culture filtrate was lost only slowly by repeated 24-hour incubations with fresh yarns, and carboxymethylcellulase activity was lost at about the same rate. This supported the view that B-enzyme was a distinct entity from A-enzyme and that it was responsible for most, if not all, carboxymethylcellulase activity.

The ability of B-enzyme to attack swollen cellulose could be demonstrated by comparing the action of fresh and exhausted filtrates on undried, mercerized cotton. Exposure for seven days to exhausted culture filtrate reduced the strength of the swollen cotton by 79% compared with the 15 to 20% found for

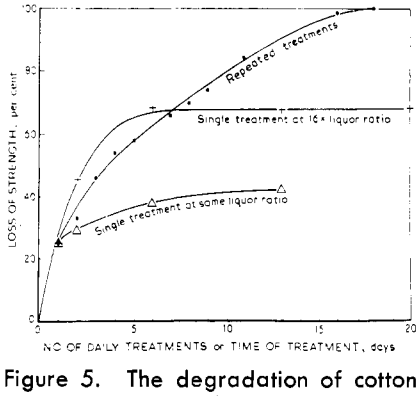

Figure 5. The degradation of cotton yarn by repeated treatments with culture filtrate from *M. verrucaria*

By courtesy of the *Biochemical Journal* **88**, 288 (1963)

its attack on unswollen cotton. The fresh culture filtrate, as might have been expected, caused more loss of strength of both substrates (100% and 30%, respectively), since A-enzyme if present attacks more extensively than B-enzyme.

Separation and Preparation of A- and B-Enzymes

Because of the strong adsorption of cellulases on cellulose an attempt was made to increase the yield of A-enzyme by extraction of the culture solids, which were normally rejected. Although the enzyme is not released at the pH of a growing culture, complexes of cellulases and carbohydrate can be split by washing them through a cellulose powder column with dilute alkali (*14*). The culture solids from *M. verrucaria* were therefore mixed with Celite 535 and eluted with sodium tetraborate at pH 9.0. Peak activities were comparable with those of culture filtrates. The material eluted first had similar activity toward cotton as had later fractions but twice the carboxymethylcellulase activity, thus indicating the possibility of achieving at least a partial separation of the A- and B-enzymes. Significantly, the maximum yield of A-activity from culture solids was obtained after only two days' growth and before any appreciable carboxymethylcellulase activity had appeared in the filtrate (Figure 6).

The elution technique was subsequently improved by using a competing soluble substrate. Using phosphate buffer (pH 5.5) to which carboxymethylcellulose had been added, an appreciable increase in A-activity was obtained, as it was with buffer plus methylcellulose.

The knowledge that separation of the two types of enzymes could be effected gave impetus to attempts to secure a more complete separation in order to study their properties individually. The method ultimately employed (*11*) was gel filtration on Sephadex G-75 (Pharmacia AB, Uppsala, Sweden), by which separation of a mixture of solutes into fractions of different molecular size can be achieved under extremely mild conditions. Temperature changes cause irregular behavior because of dimensional changes in the gel particles, but if temperature is controlled, the flow behavior is extremely reproducible.

A column of gel was calibrated by determining the elution volume of a number of materials of known molecular weights, ranging from thyroglobulin (mol. wt. 670,000) to dinitrophenylalanine (mol. wt. 255) and plotting the fraction number at which the peak of absorbancy for each material emerged against the logarithm of its molecular weight.

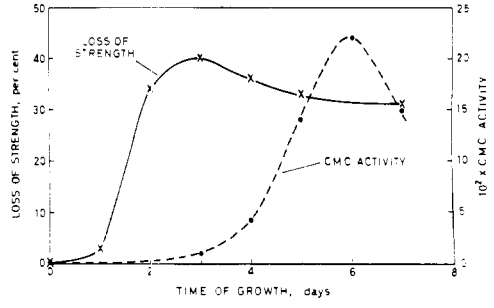

Figure 6. The rate of appearance of A-activity on the solids and of carboxymethylcellulase in the filtrates from shake cultures of *M. verrucaria*

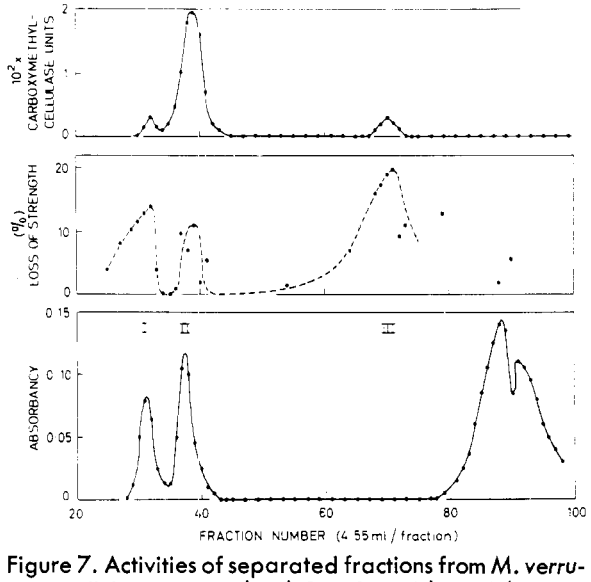

Figure 7. Activities of separated fractions from *M. verrucaria* cellulase toward (top) C.M.C. and (middle) cotton

Bottom represents absorbancy (protein content) of the fractions
By courtesy of the *Biochemical Journal* **94**, 578 (1965)

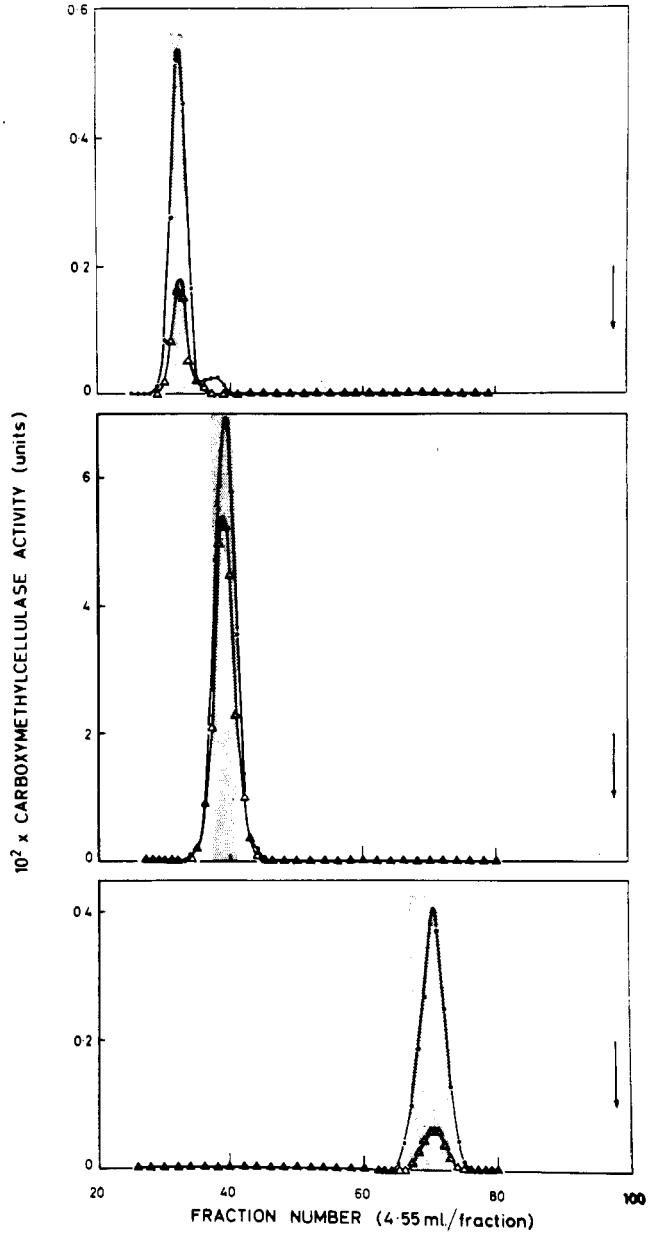

Figure 8. Effect of exposure to cotton on the components from the fractionation of *M. verrucaria*

Top. Component I rerun before (•) and after (△) exposure to cotton
Middle. Component II rerun before (•) and after (△) exposure to cotton
Bottom. Component III rerun before (•) and after (△) exposure to cotton
On each graph the shading shows which fractions were united
By courtesy of the *Biochemical Journal* **94**, 578 (1965)

A sample of filtrate from a surface culture of *M. verrucaria* was separated into four components of which three contained cellulases (Figure 7) with different molecular weights and with different activities toward soluble and insoluble cellulosic substrates. The greater part of the carboxymethylcellulase activity was in component II and the greater part of the activity toward yarn in components I and III. The approximate molecular weights of the cellulases, judged from the positions of their peaks, were 55,000, 30,000, and 5300. The small size of the cellulase in component III was somewhat surprising, but a cellulase of low molecular weight (11,000) has also been separated from *Polyporus versicolor* (*6*).

The cellulase in component III was obtained only in very small yield. In order to obtain a reasonable yield and also to obtain a better idea of the relative activities of the three enzyme components, a 10-fold concentrate of culture filtrate was similarly fractionated.

The relative activities toward cotton and toward carboxymethylcellulose of the separated components suggested that components I and III had the properties attributed to A-enzyme, whereas component II behaved as B-enzyme. This view was supported by the differences in behavior on exposure to cotton (Figure 8). Approximately one third of each of the components was rerun to establish its homogeneity and reproducibility of behavior. An equal volume of each was also rerun after exposure at 30° to three fresh samples of cotton yarn, each for a period of 24 hours. Clearly, components I and III were greatly diminished in carboxymethylcellulase activity, whereas component II, with the bulk of the carboxymethylcellulase activity, was little affected. The eluted fractions were suitably pooled and measurements of carboxymethylcellulase activity and activity toward pretreated cotton were made. Loss of activity by components I and III, but not by component II, was apparent.

The fractionation by gel filtration of culture filtrates from *M. verrucaria* into several active components has, therefore, confirmed the heterogeneity of this cellulase system. As had been predicted from a study of the whole culture filtrate, the separated components differed in molecular size, relative activities toward different cellulosic substrates, and behavior on exposure to cotton yarn.

Behavior of Other Microorganisms

Although *M. verrucaria* has been used for the majority of this work, other microorganisms (Table I) have also been examined briefly. The strength losses with *Trichoderma viride* were higher than those found with *M. verrucaria*, particularly after 7 or more days' growth. Marked variations occurred in the pH of the filtrates, and these may account for the variation in activity. Filtrates from *Stachybotrys atra* were comparable in activity with those from *M. verrucaria*.

Hitherto, there has been some doubt whether *Chaetomium globosum* produces an extracellular enzyme, or whether its activity is due to physical penetration of the fiber. Early experiments using shake-cultures produced filtrates which were quite inactive, but surface cultures on cotton yarn produced active filtrates, the best weakening yarn by 84% after 10 treatments.

Of these microorganisms, only the filtrate from *T. viride* has been subjected to a preliminary fractionation by elution on a column of Sephadex G-75 as was done for the filtrates from *M. verrucaria*. Two clearly defined peaks of cellulase activity were found, both of them differing from those of *M. verrucaria*; their estimated molecular weights were 38,000 and 12,000. The higher molecular weight fraction was the more active toward pretreated yarn, but possessed only about one third of the carboxymethylcellulase activity found in the lower molecular weight fraction.

In contrast to *M. verrucaria*, *T. viride* gave cell-free filtrates that were not deactivated by exposure to cotton. Clearly, a further investigation of the enzyme fractions from different culture filtrates would be profitable.

Possible New Method for Prevention of Microbial Attack

Since we now believe that microbial attack is highly localized and is caused by bulky molecules capable of penetrating only a few accessible sites, it should be possible to effect substantial protection by blocking these by similarly bulky substituents applied in a solvent chosen to minimize the swelling of the cotton. The chief advantages of such selective substitution would be in economy of reagent and the probable small changes produced in the physical properties of the textile.

The reagent chosen to test this view was phenyl isocyanate in the presence of a small amount of pyridine, sometimes with an inert diluent such as petroleum ether. The degree of substitution of the resulting cellulose carbanilates was estimated by hydrolysis and colorimetric determination of the aniline produced, with *p*-dimethylaminobenzaldehyde. Samples with a range of degrees of substitution from 0.0 to 0.2 were prepared and their resistance to degradation when buried in soil is shown in Table II.

The addition of a α-methyl-D-glucoside tetracarbanilate to the cloth produced no protection, so that the inhibition of degradation must be due to the selective blocking action rather than to any antibiotic activity of the carbanilate group itself.

Ellzey, Wade, and Mack (*3*) have recently published a survey of the reactions between cellulose and aryl isocyanates in which the data on rot-resistance of carbanilated cotton at different levels of substitution are in substantial agreement with the results presented in this paper.

Since, for a very large variety of substituents, adequate protection is not obtained below a degree of substitution of about 1.0, these findings appear to be a significant step forward in the understanding of the action of the enzymes and in their implications for the better protection of cotton from attack.

Table I. Strength-Loss of Yarn Samples Incubated with Filtrates from Surface Cultures of Various Organisms

Organism	Age of Culture, Days	pH of Filtrate	No. of Successive Applications of Filtrate 1	3	5	10
Trichoderma viride	4	6.1	29	..	44	62
	7	3.9	74	..	100	..
	7	5.8	42	55	63	70
	7	5.9	42	49	62	70
	7	5.8	43	55	64	76
	11	3.1	62	74	85	..
Stachybotrys atra	4	5.9	28.5	..	43	50
	7	5.9	...	..	...	72
Chaetomium globosum	4	6.3	40	..	52	69
	7	6.3	45	..	68	80
	4	6.3	19	..	30	41
	7	5.6	35	..	66	84

Table II. Resistance of Cellulose Carbanilates to Soil Burial at Very Low Degrees of Substitution

D.S.	Loss of Strength (%) after Burial for (Weeks) 2	3	4	6	22	50
0.0	75	96	100	100	..	..
0.06	48	97	91	100	..	..
0.12	−1	33	...	75	..	..
0.21	−10	−6	...	15	18	24

Acknowledgment

The author thanks his colleagues who carried out this work, and especially K. Selby, who was responsible for it.

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Received for review October 20, 1964. Accepted July 12, 1965. Presented before the Division of Agricultural and Food Chemistry, Symposium on World-Wide Research Program in Agricultural Chemistry, 148th Meeting, ACS, Chicago, Ill., September 1964. This research was conducted under a project authorized by U. S. Public Law No. 480, 83rd Congress.

WORLD-WIDE RESEARCH

Role of the Universities in Agricultural Research

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The universities' essential role in agriculture, as in all fields, is the generation of new knowledge. The training of research workers is an integral part of the universities' work, since the student learns to make new knowledge by practicing doing so in apprenticeship to those who already possess the skill. The work of the universities has resulted in a knowledge explosion in biology, and we are now rapidly approaching both understanding and mastery of the processes of cell and organismal life. This new knowledge has as yet had no impact on agriculture. It is time for those of us interested in agriculture to try to foresee ways in which this impact may make itself felt.

THE ROLE of the university is to make new knowledge. This has been its traditional role since the founding of universities some 700 years ago. In those far-off years, to be sure, the universities made new knowledge only in the field of theology, but gradually they have accepted their responsibilities, spread their interests, and today their knowledge-making role extends through all fields of human interest and activity, from the arts to the exploration of the moon, from the wiring diagram of the brain to basic agriculture. We are here concerned only with agriculture, which is one facet of biology. For the agriculture-related aspects of biology, as for all biology, it is in the universities that our present knowledge explosion not only commenced, but is at present being carried forward. Let us not review past accomplishments in detail—let us merely consider a few, and then go on to the present state of biology. We will then determine what we can forecast for the future of biology and of agriculture.

From the universities have come in steady succession such new insights as our knowledge of the plant hormones, and hence of herbicides and the chemical control of senescence, the unfolding of the path of carbon in photosynthesis and our present close approach to an understanding of how light energy is converted to chemical energy in chloroplasts. This latter knowledge will one day make possible not only synthetic photosynthesis but also perhaps plant photosynthesis of improved efficiency.

The New Biology

From the university has come, too, the spectacular development of our modern insight into the nature of life. This development has all taken place within the past ten years. Its impact has not yet been felt in agriculture, nor do we know exactly the ways in which this impact will make itself felt. That its effects will be vast, we can have no doubt. Our new knowledge of life is truly revolutionary, for we know in detail what it is that makes the cell be alive. We know that all cells contain the directions for cell life written in the DNA of their chromosomes, and that these directions include specifications of how to make the many kinds of protein enzyme molecules, by means of which the cell converts available building blocks into substances suitable for making more cells. We know that to make enzyme molecules the DNA prints off RNA copies of itself, messenger RNA molecules, and that these messenger RNA molecules are decoded by ribosomes, also made by the DNA, and that the ribosome as it decodes the messenger RNA molecule uses the information to assemble a specific kind of enzyme molecule. We know that the information contained in DNA, and hence in messenger RNA, is encoded in these molecules in a sequence in which the four different kinds of monomers of which nucleic acids are constituted, succeed one another down the long linear polymeric chains of the nucleic acids, and that the sequence in which the nucleotides of the nucleic acids succeed one another in turn determines the sequence in which the 20 different kinds of amino acids are stapled together to make enzyme molecules. We