THE PREPARATION AND STUDY OF THE CELLULASES AND HEMICELLULASES OF MYROTHECIUM VERRUCARIA

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THE PREPARATION AND STUDY OF THE CELLULASES AND HEMICELLULASES OF *MYCOTHECIUM VERRUCARIA*

By

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AN ABSTRACT

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The present investigation was undertaken to study (1) the possible multiplicity of cellulolytic enzymes and (2) the relationships among the cellulase, hemicellulase, and β-glucosidase activities of culture filtrates of the fungus Myrothecium verrucaria. Previously, Whitaker (1953) obtained evidence indicating that apparently a single cellulase enzyme is elaborated by the fungus, but Reese and Gilligan (1953) reported that there are two separate enzymes involved in the hydrolysis of such soluble substituted cellulose derivatives as carboxymethylcellulose. Furthermore, Jermyn (1952) obtained evidence suggesting that several of the protein components in preparations from Aspergillus oryzae possess the ability to catalyse the hydrolysis of β-glucosides as well as cellulose derivatives.

Culture filtrates of M. verrucaria grown in submerged culture were initially concentrated by slow partial freezing. Attempts to fractionate the concentrate by salt and solvent precipitation and by chromatography on cellulose columns were generally unsuccessful, but after further concentration of the proteins by precipitation with seventy-five percent acetone or saturated ammonium sulfate, electrophoresis-convection yielded preparations containing different ratios of the protein components. Fractionations by this method were controlled by estimation of enzymatic activity, using cellulose sodium sulfate as the substrate, determination of protein nitrogen content, and electrophoretic analysis of some of the fractions.

Six electrophoretic components in pH 6.9, ionic strength 0.146 phosphate buffer were observed in the unfractionated enzyme concentrates. These components were numbered starting with the slowest-migrating protein. The first and most active of the three final enzyme preparations used to study the enzymatic properties of the constituents, contained primarily components 1 and 2, along

with a small amount of component 3. The second preparation contained no component 1, but had all the others, and the third preparation was devoid of components 1 and 2, but had all the remaining components.

Cellulose sodium sulfate, wheat straw cellulose, two hemicellulose preparations from wheat straw, and salicin were used as substrates to study the enzymatic properties of the three enzyme preparations. The specific activities (defined as the milligrams of reducing sugars, calculated as glucose, split from 100 milligrams of the substrate under specified conditions, per milligram of protein nitrogen) of the three enzyme preparations acting on the five substrates were determined and compared. Specific activity-pH curves for each enzyme preparation with each substrate were constructed. The end products obtained from cellulose sodium sulfate and the hemicellulose preparations with each of the enzyme solutions were studied by paper chromatographic techniques.

The preparation containing components 1, 2, and 3 was the most active, and the one containing only components 3, 4, 5, and 6 the least active. However, the results indicated that more than one component was enzymatically active with all of the polysaccharide substrates. Component 2 appeared to be the most active enzyme, but its share of the total activity varied with the substrate. This component apparently contributed most of the hydrolytic action observed with cellulose, somewhat less with cellulose sodium sulfate, and still less with the hemicellulose preparations. Although the distribution of the remaining activity among the other components was not clearly indicated by the results, component 1, as well as one of the other proteins, probably had some action on cellulose sodium sulfate and the hemicelluloses. The results indicated clearly that nearly all of the β-glucosidase ("salicinase") activity was associated with component 1.

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INTRODUCTION

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The Importance of the Cellulases and Hemicellulases

The enzymes concerned with the degradation of celluloses and the hemicelluloses are of great importance to all life. Their action on the polysaccharides of plant residues is an indispensable link in the carbon cycle. By the process of photosynthesis, the green plants of the earth fix carbon dioxide at the rate of about three percent of the atmospheric supply annually (Rabinowitch, 1945). At this rate, atmospheric and reserve carbon dioxide available for photosynthesis would be depleted in a relatively short time if it were not constantly replenished by degradative processes.

A major portion of the biologically fixed carbon is found as cellulose and related polysaccharides. Although other reactions, such as the burning of coal, gas, petroleum, and wood, contribute measurably to the replenishment of atmospheric carbon dioxide, the greatest single factor is the respiration of living organisms, especially the soil bacteria and fungi (Meyer and Anderson, 1939). Since cellulosic materials make up so large a portion of the biological carbon, and the first step in their decomposition is caused by cellulases, the great importance of these enzymes in the carbon cycle is obvious.

Incidental to the contribution of the cellulose-destroying microorganisms toward the perpetuation of the carbon cycle, but of nearly as great significance is their role in soil fertility. Their activity on plant residues, along with that of many other soil microorganisms,

initiates a long and complex series of reactions leading to the formation of humus (Waksman, 1936). During this process, all the cellulose and most of the hemicelluloses are destroyed, serving as a source of energy and microbial cell substance. Certain nutrients from the plant residues are released or changed into forms more accessible to succeeding generations of plants. The lignin remaining after most of the other plant constituents have been removed is relatively resistant to microbial attack and serves as the nucleus for humus formation. The chemical and colloidal properties of humus account for much of the difference between ground and weathered rock and fertile soil.

The symbiotic relationship between their hosts and many species of microorganisms inhabiting the intestinal tracts of the higher animals is well known. In the case of ruminants, this relationship has been evolved to a high degree, and much of the credit for this successful symbiosis is due to the cellulolytic organisms present in the rumen. Their action not only converts part of the ingested cellulose and hemicelluloses into forms of carbohydrate, acids and proteins available to the host, but also makes other nutrients more accessible (Huffman, 1953). Although feeds other than the cellulose-containing roughages are necessary for an efficient ration, it is perhaps not an exaggeration to state that our entire dairy and beef industries depend to a great extent on the cellulases and hemicellulases.

The possibility of using cellulolytic organisms for the industrial production of acids and alcohols has been explored. Although this application shows some promise, it is apparently not economically

feasible at present because of the presence in wood and plant wastes of lignin and other encrusting materials which prevent a rapid utilization of the cellulose and an adequate accumulation of useful products (Siu, 1951). Among some of the other commercial uses of cellulolytic organisms may be mentioned the production of edible mushrooms, rubber production, and sewage and garbage disposal.

While it is difficult to overemphasize the importance of the cellulases and hemicellulases to the material and energy balance of the earth, man has perhaps concerned himself more with the harmful aspects of their action. Given the proper conditions, cellulolytic organisms will attack any suitable substrate. Textiles, lumber, paper, almost every kind of cellulosic item manufactured, even living plants, sustain a staggering amount of microbial damage. Such enormous losses have stimulated much research on the subject, but most of this work has been directed toward practical application, the mildew-proofing of textiles in particular (Siu, 1951).

In spite of their significance, the cellulases and hemicellulases have remained until very recently a relatively little studied group. Furthermore, mostly because of the heterogeneous nature of the substrates and enzyme preparations used, much of the earlier information is somewhat contradictory. Recently, Whitaker (1953) isolated from culture filtrates of the fungus Myrothecium verrucaria, a cellulase preparation which appears to be homogeneous. On the basis of his results, Whitaker concluded that, at least in the case of this organism, the process of cellulose hydrolysis is unienzymatic. However, the

results reported by Jermyn (1952, 1952a) and by Reese and Gilligan (1953) indicate that there may be more than one cellulolytic enzyme in preparations from M. verrucaria and several other fungi.

The object of the present investigation was to study the possible multiple nature of the cellulase activity of M. verrucaria preparations, using techniques somewhat different from those employed by Jermyn and by Reese and Gilligan, and further, to study the relationships among cellulase, hemicellulase, and β-glucosidase activities.

Review of Literature

The cellulases and hemicellulases appear to have a wide and similar distribution. They have been found in plants, in the digestive secretions of some invertebrates, and in many species of bacteria, fungi, actinomycetes, and in some protozoa. Their occurrence in the digestive secretions of insects such as termites and roaches is open to some question (Pigman, 1950).

In Table I a list of some of the sources of cellulase is presented. This compilation has been restricted to those sources from which extracts or filtered culture media have been tested for cellulolytic activity, thus excluding a great many species of bacteria, fungi, and actinomycetes which have been shown to be cellulolytic in live culture. This is an arbitrary restriction and is not meant to imply that the excluded organisms do not possess the enzyme. Siu (1951) has compiled tables of these organisms containing 357 species of fungi, 103 species

TABLE I

SOME SOURCES OF CELLULASES

Source	Substrate	Optimum pH	Reference
Bacteria			
Cellulobacillus myxogenes	Paper pulp	6.7	Simola (1931)
Cellulobacillus mucosus	Paper pulp	6.7	Simola (1931)
Cytophaga globulosa	Cellophane	7.0	Fahreus (1947)
Rumen bacteria, mixed population	Carboxymethylcellulose and powdered filter paper	5.5	Kitts and Underkofler (1954)
Fungi			
Aspergillus aureus	Cellodextrin		Grassman *et al.* (1933b)
Aspergillus aureus	Cellophane	5.0	Holden (1950)
Aspergillus aureus	Carboxymethylcellulose		Reese *et al.* (1950)
Aspergillus oryzae	Cellodextrin	4.5	Grassman *et al.* (1933a)
Aspergillus oryzae	Wood cellulose	4.7	Freudenberg and Ploetz (1939)
Aspergillus oryzae	Cellodextrin, carboxymethyl-cellulose	3.8-5.9*	Jermyn (1952a)
Aspergillus niger	Phosphoric acid-swollen cotton	4.5	Walseth (1948)
Aspergillus fumigatus	Carboxymethylcellulose	4.8-5.0	Reese *et al.* (1950)
Aspergillus flavus, *Aspergillus luchuensis*, *Aspergillus sydowi*	Carboxymethylcellulose		Reese *et al.* (1950)
Merulius lacrymans	Cuprammonium cellulose	4.7	Ploetz (1939)
Myrothecium verrucaria	Ball-milled cotton	5.5	Saunders *et al.* (1948)
Myrothecium verrucaria	Carboxymethylcellulose	4.9-5.0	Reese *et al.* (1950)
Myrothecium verrucaria	Cellulose sulfate		Levinson *et al.* (1951)
Myrothecium verrucaria	Swollen linters, reprecipitated cellulose		Whitaker (1953)

TABLE I concluded

Source	Substrate	Optimum pH	Reference
Protozoa			
Trichomonas termopsidis	Cuprammonium cellulose	5.3	Trager (1932)
Eudiplodinium neglectum	Filter paper		Hungate (1942)
Molluscs			
Snail (Helix pomatia)	Cuprammonium cellulose		Seilliere (1906)
Snail (Helix pomatia)	Cuprammonium cellulose	5.28	Karrer and Illing (1925)
Snail (Helix pomatia)	Viscose rayon, thiocyanate-swollen cellulose		Karrer (1930)
Snail (Helix pomatia)	Cross and Bevan cellulose wood holocellulose	5.2-5.5	Freudenberg and Ploetz (1939)
Snail (Helix pomatia)	Hydroxyethylcellulose	4.51	Ziese (1931)
Snail (Helix pomatia)	Cellophane	5.6	Holden (1950)
Shipworm (Bankea setacea)	Sawdust, filter paper		Boynton and Miller (1927)
Marine wood borer (Teredo pedicillata)		6.0-6.8	Lane and Greenfield (1953)
Insects			
Stromatium fulvum (larvae)	Filter paper	5.6	Mansour and Mansour-Bek (1937)
Higher plants			
Barley malt	Hydroxyethylcellulose	3.68	Ziese (1931)
Barley malt	Hydroxyethylcellulose	4.0	Kristiansson (1950)
Barley malt	Swollen cellulose		Pringsheim and Baur (1928)
Tobacco leaves (sap)	Carboxymethylcellulose	5.4	Tracey (1950)
Beet roots, bean leaves, asparagus butts	Carboxymethylcellulose and phosphoric acid-swollen filter paper		Tracey (1950)

of bacteria, and 32 species of actinomycetes.[1] Although the criteria for classification of microorganisms as either cellulolytic or non-cellulolytic are quite definite (either the demonstration of growth on media containing cellulose as the only source of carbon, or the demonstration of cellulose utilization, or preferably both), Siu (1951) criticized some of the methods applied to bacteria and stated that some of the species classified as cellulolytic are highly suspect. On the other hand, Norman (1937) has pointed out that the usual criteria for demonstrating cellulolytic activity may be too rigid and the methods too exclusive. Two examples are given in which certain microorganisms, although not considered cellulolytic by the usual tests using native cellulose, nevertheless are capable of decomposing the cellulose as well as some of the other polysaccharides when they are offered simultaneously in such natural substrates as straw. Further, Norman stated that instances are known in which one organism is able to utilize cellulose only in the presence of certain others.

In the same connection, it is interesting to note that although Siu (1951) lists Aspergillus niger, A. oryzae, A. flavus, and A. sydowi among the noncellulolytic fungi, all have been shown to elaborate enzymes capable of hydrolyzing the β-1,4-glucosidic linkages of various modified celluloses and cellulose derivatives, and are thus included in Table I. A possible explanation of these discrepancies has been advanced by Reese et al. (1950) and will be discussed in later paragraphs.

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[1] Among the bacteria and fungi, Siu distinguished between those already isolated from exposed cotton fabrics and those not yet recorded as isolated from cotton fabrics. All the actinomycetes species listed by Siu came from the soil.

Cell-free cellulolytic preparations are more difficult to obtain from bacteria than from the fungi. With the latter, crude active preparations are obtained simply by filtering the liquid culture medium to obtain the culture filtrate, which contains nearly all the activity, or by pressing the liquid from moist solid substrates on which the fungus has grown. Bacterial culture filtrates, on the other hand, contain very little of the activity possessed by the intact culture (Hungate, 1942; Kitts and Underkofler, 1954). Failure to demonstrate the activity in the culture filtrate could be explained in several ways. Complete adsorption of the active protein on the filter could account for it (Zaremska, 1936), as would the postulation of some workers (Meyer, 1943; Khouvine, 1923) that these enzymes are strictly intracellular. However, it is difficult to conceive of a mechanism whereby the insoluble cellulose substrate could be taken into the bacterial cell.

At present, there seems to be little doubt that bacterial as well as the fungal cellulases are extracellular (Siu, 1951). Perhaps the most widely accepted explanation of the failure to detect appreciable amounts of activity in bacterial culture filtrates is that the bacterial cell liberates the enzyme only on very close approach or actual contact with the substrate (Hungate, 1942). One could further postulate that the enzyme is tightly adsorbed on the outer surface of the cell membrane. That the bacterial cellulases are apparently adaptive enzymes (Siu, 1951) can be used as an argument in favor of the first suggestion. Direct microscopic studies of bacterial attack on natural and regenerated cellulosic fibers, showing "corrosion figures" and

"enzymic cavities" immediately surrounding the firmly attached bacterial cells, also lend support to these interpretations (Baker and Harriss, 1947).

A list of some of the demonstrated sources of hemicellulases is presented in Table II. The substrates used in these investigations are also given. It should be noted that in most instances, even when given a definite designation such as xylan, the substrates used were either impure or contained more than one type of sugar residue (Pigman, 1950).

The hemicellulases, like the cellulases, are demonstrably extracellular, at least in the case of the fungi and the snail. They are also obtained by the same procedures used for the preparation of cellulase.

Comparison of the sources of cellulases and hemicellulases (Tables I and II) brings out the provoking, if not entirely surprising observation, that in every instance in which a hemicellulase has been found, cellulase activity is also present. In other cases, hemicellulase activity has not been sought. From the ecological point of view, this situation is perhaps not at all unexpected, since the natural substrates of the cellulolytic microorganisms are nearly always intimate mixtures of cellulose and one or more hemicelluloses.

The occurrence of discrete sources of enzyme preparations which will act on several different but related substrates is of course rather common. An example appropriate for the present discussion is the digestive juice found in the crop of the snail, Helix pomatia. Holden and Tracey (1950) have compiled from the literature a list of 30 enzymes reported to have been found in this single source. Twenty of these are

TABLE II

SOME SOURCES OF HEMICELLULASES

Source	Substrate	Optimum pH	Reference
Bacteria			
Anaerobic bacteria	Ivory nut mannan		Pringsheim (1912)
Fungi			
Aspergillus niger	Crystalline xylans from paper birch and barley straw		Yundt (1949)
Aspergillus niger	Irish moss (extracts)		Saiki (1906-1907)
Aspergillus oryzae	Irish moss (extracts)		Saiki (1906-1907)
Aspergillus oryzae	Xylans and mannans from beechwood		Grassman et al. (1933a)
Aspergillus oryzae	Xylans from fruit pits and beechwood	4.65	Voss and Butter (1938b)
Aspergillus oryzae	Arabans from sugar beet and orange peel		Ehrlich (1929)
Aspergillus oryzae	Oakwood hemicelluloses		O'Dwyer (1940)

TABLE II concluded

Source	Substrate	Optimum pH	Reference
Molluscs			
Snail (Helix pomatia)	Wheat straw xylans	4.65 (citrate) 5.28 (phosphate)	Ehrenstein (1926)
Snail (Helix pomatia)	Mannans, mannogalactans		Bierry and Giaja (1912)
Snail (Helix pomatia)	Pentosans of wood	4.7	Ploetz (1939)
Snail (Helix pomatia)	Xylans from corn		Holden and Tracey (1950)
Snail (Helix pomatia)	Irisin		Holden and Tracey (1950)
Shipworm (Bankea setacea)	Sawdust		Boynton and Miller (1927)
Higher plants			
Barley malt	Barley endosperm	5.0	Luers and Volkamer (1928)
Barley malt	Xylans from fruit pits and beechwood		Voss and Butter (1938a)
Barley malt	Wheat straw xylans	5.28	Ehrenstein (1926)
Barley malt	Salep mannan		Pringsheim and Genin (1924)

carbohydrases. The list of substrates includes cellulose, lichenin, the starches, glycogen, fructosans, arabans, xylans, mannans, mannogalactans, chitin, polygalacturonic acid, several naturally occurring di- and trisaccharides, stachyose and α- and β-glycosides. The question which arises in this and in similar cases is whether the activities are possessed by a few enzymes of relatively low specificity, or by many enzymes of absolute or nearly absolute specificities.

There have been very few investigations specifically designed to establish the identity or nonidentity of the cellulases and hemicellulases. Grassman and co-workers (1932, 1933) found that crude commercial enzyme preparations from A. oryzae hydrolyzed cellulose and a xylan, and had some action on a mannan. The latter activity was lost on dialysis, and treatment with animal charcoal removed the xylanase, leaving only the cellulase. They concluded that mannanase, xylanase, and cellulase are separate specific enzymes.

In the absence of similar studies on different preparations, it is perhaps unwise to extend Grassman's conclusions to other organisms.

The possibility of the identity of cellulase with β-glucosidase has been mentioned by Veibel (1950) on the basis of experiments reported by Helferich and Thieman (1944). The latter found that glycol β-D-cellobioside anhydride was slowly hydrolyzed by the β-glucosidase of sweet almond emulsin. They admit, however, that this type of evidence is only suggestive.

According to Pigman and Goepp (1948), the β-glucosidases and the polysaccharidases should be considered separately, not necessarily on the basis of differences in specificity, but rather because of the great

quantitative differences in rates of the reactions that they catalyze. The possibility of a close relationship or even identity was not implicitly denied.

Jermyn (1952, 1952a) has reported the results of an investigation of the cellulase and β-glucosidase activities of A. oryzae preparations. Filter paper electrophoresis revealed the presence of at least eight separate protein components in the crude enzyme preparations. The distribution of activity along the paper, against various substrates, was determined by spraying each developed paper with one of the buffered substrates, incubating, drying, and spraying again with a reducing sugar reagent. The substrates used were salicin, aesculin, para-nitrophenyl-β-glucoside, and carboxymethylcellulose. At least seven of the eight components were found to be active against both salicin and carboxymethylcellulose. The distribution of "aesculinase" activity was not so well resolved, but again was spread the length of the paper. Only with para-nitrophenyl- β-glucoside was there but a single well defined area of activity.[1]

Attempts by Jermyn to fractionate the crude material by solvent and salt precipitation methods gave erratic results. In one experiment, the enzyme active against carboxymethylcellulose remained in solution when alcohol was added up to a concentration of forty percent. Addition of solid ammonium nitrate to the alcoholic solution, held at a temperature

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[1] It should be mentioned that with this substrate, a sugar reagent spray was not used, since free para-nitrophenol is itself a colored compound. If this procedure is not as sensitive as the detection of reducing sugars, it seems possible that other areas of hydrolysis might have been found with the sugar reagent spray.

of 10° C., brought about an immediate precipitation of some of the active material. Active protein continued to be precipitated with each small increment of ammonium nitrate up to about 85 g. per 100 ml. of original solution.[1] There were only slight differences between the activities of the various fractions.

These results, along with others to be mentioned later, led Jermyn to conclude "that in the A. oryzae system the enzymes breaking down monomeric β-glucosides do not differ qualitatively from those breaking down polymeric β-glucosides", and that there is no need to assume the existence of a specific cellulase to explain the hydrolysis of soluble cellulose derivatives. Jermyn instead thinks that any estimation of cellulase or β-glucosidase activity is the summation of the separate activities of a whole battery of β-glucosidases, each with its own ratio of activities against any one substrate.

Although obtaining evidence of similarly complex systems in almond emulsin and Stachybotris atra preparations (1953), Jermyn is cautious about applying the results generally.

It will be noted that nearly all the substrates listed in Table I are celluloses modified to some degree from native cellulose. Most investigators in this field have found it necessary, or at least desirable, to modify cellulose from its native form in order to obtain sufficient hydrolysis for accurate measurement.

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[1] After the addition of each increment of salt, one hour was allowed for completion of precipitation. The adequacy of this period of time might be questioned.

Seillière (1906), after confirming earlier observations that cotton and filter paper seem to be very resistant to attack by the snail enzyme, found that cotton cellulose reprecipitated from solution in Schweizer's reagent (cuprammonium hydroxide) was much more susceptible to hydrolysis. The reaction did not go to completion even after the addition of more enzyme. It was also found that swelling cotton with zinc chloride or sodium hydroxide had somewhat the same effect (Seillière, 1910).

These observations have since been repeatedly confirmed and extended. Karrer and Illing (1925) found that by repeated treatment of reprecipitated cotton cellulose with fresh snail enzyme, 96 percent hydrolysis could finally be attained. Others have not been able to attain such an extent of breakdown. There is always a resistant residue.

Karrer et al. (1925a) reported that filter paper and mercerized cotton are less resistant to enzymatic attack than native cotton, but alkali celluloses are strongly attacked (Karrer and Schubert, 1926).

Pringsheim and Baur (1928) studied the differences in rate of hydrolysis, by barley malt enzymes, of cotton cellulose swollen by different means. Cellulose treated with calcium thiocyanate was most rapidly attacked, lithium chloride was somewhat less effective, and treatment with cold dilute sulfuric acid produced the least susceptible substrate.

Syrupy phosphoric acid has also been used successfully for the preparation of cellulosic substrates susceptible to enzymatic attack. Walseth (1948) found that ground, dewaxed cotton linters were only about five percent hydrolyzed by A. niger enzymes after six days incubation. In the same time, an equal concentration of enzyme hydrolyzed a

phosphoric acid-treated sample of cotton linters to the extent of about 80 percent. This effect can be graded by control of the time of swelling treatment.

Since all of the treatments listed above result in a shortening of the cellulose chains, the simplest explanation for their effectiveness in increasing the enzymatic susceptibility of native cellulose would be that the rate of hydrolysis is a function of the degree of polymerization. This idea has been proposed by Hajo (1942). However, the extensive work of Karrer and his associates indicates that the degree of polymerization is not as important as another variable property, namely, the degree of crystallinity. Karrer and Schubert (1928) could find no constant relation between the degree of polymerization, as determined viscometrically on cuprammonium solutions of different celluloses, and the rate of enzymatic hydrolysis by Helix enzymes. Instead, a constant inverse relationship between the amount of breakdown and the degree of crystallinity was noted (Karrer and Schubert, 1926).

In further experiments, Karrer (1930) and Faust et al. (1928) compared the action of snail cellulase on two series of viscose rayons, prepared under identical conditions up to the spinning stage. One series was set with tension, the other with no tension. The samples spun without tension were hydrolyzed more rapidly and to almost twice the extent. X-ray diagrams of the samples revealed that tension applied during the spinning process increased the orientation of the cellulose micelles.

Karrer (1930) concluded that the effect of swelling and of regeneration treatments on the susceptibility of cellulose to the action of

enzymes can be ascribed to an increase in exposed surface due to a change in the physical arrangement of the micelles.

More recently, Walseth (1948) has provided further good evidence that the degree of crystallinity[1] is the more important factor. Different samples of phosphoric acid-swollen cotton linters which varied markedly in their enzymatic susceptibility (A. niger), varied only slightly in their degree of polymerization. In another series of experiments, a sample of swollen cotton linters was about 28 percent hydrolyzed by an A. niger preparation in 24 hours. When the residual cellulose was recovered, washed, and treated with fresh enzyme, very little further breakdown was obtained. However, when it was treated again with phosphoric acid, it was rapidly hydrolyzed by the enzyme (about 50 percent in 24 hours). Walseth also found a direct correlation between the extent of hydrolysis in a given time and the equilibrium moisture contents of various samples of swollen linters. The equilibrium moisture content has been found to reflect the degree of accessibility (Howsmon and Sisson, 1954).

Among the substrates listed in Table I are three soluble cellulose derivatives: carboxymethylcellulose, cellulose sulfate, and hydroxyethylcellulose. Others which have been used are methylcellulose (Reese et al., 1950; and Tracey, 1953) and ethylmethylcellulose (Tracey, 1953).

Soluble substrates have obvious advantages. Solutions of these

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[1] Howsmon and Sisson (1954) favor the term "accessibility" rather than the older term "degree of crystallinity", because the crystalline and amorphous areas of cellulose fibers cannot be strictly delineated, whereas accessibility to any single reagent can be accurately measured.

derivatives form homogeneous solutions[1]; consequently substrate concentration becomes a more meaningful term, and the uncertainties of substrate surface area and degree of crystallinity are of less concern. The initial rate of hydrolysis of these substrates is much more rapid because of the great increase in surface area in going from a solid to a colloidally dispersed substrate.

As Tracey (1953) has pointed out, however technically advantageous the use of soluble substituted celluloses may be, it should be kept in mind that these substrates are not cellulose. To be satisfactory, any substitute should faithfully reflect, by some known function, the susceptibility of the natural substrate. This is by no means true of carboxymethylcellulose, as has been shown by Reese et al. (1950) and Reese and Levinson (1952). The latter determined the activity of several fungal metabolic filtrates toward ground cotton and carboxymethylcellulose. The ratio of these activities varied considerably, the range being from about five to about forty. Tracey (1953) has supplied confirmatory evidence of the same nature.

As with native or modified cellulose, the degree of polymerization (DP) seems to have little effect on the rate or extent of enzymatic hydrolysis of substituted celluloses. Reese et al. (1950) could demonstrate no differences in the rate of hydrolysis of carboxymethylcellulose with DP values of 125, 150, and 200 by Aspergillus fumigatus

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[1] Jermyn (1952) finds that solutions of carboxymethylcellulose of more than about 0.5 percent are not homogeneous; centrifugation separates part of the material as a gel-like mass. Furthermore, the rate of hydrolysis of these solutions by A. oryzae cellulase is not appreciably influenced by changes in concentration.

culture filtrates. However, as pointed out by the authors, this range is rather narrow.

Siu (1949) and Greathouse (1950) have shown that the nature of the substituent in substituted celluloses is apparently of only secondary importance in determining the resistance to attack by live microorganisms. What is more important, they found, is the degree of substitution (DS) i.e., the average number of substituent groups per anhydroglucose residue.

Reese, Siu, and Levinson (1950), using four samples of carboxymethylcellulose of about the same DP (150), but varying in DS from 0.52 to 1.2, found that the resistance of these substrates to attack by active metabolic filtrates from cultures of several fungi is an inverse function of the DS. The sample of DS 0.52 was about eight percent hydrolyzed in two hours (as indicated by the appearance of reducing sugar); the sample of DS 1.0, about 3.5 percent; whereas the sample of DS 1.2 was not hydrolyzed to any measureable extent in this time. They concluded that no enzymatic hydrolysis is possible if each anhydroglucose unit of the polymer bears a single substituent, i.e., if the DS is unity. The small amount of hydrolysis observed with the sample of DS 1.0 was probably due to a nonhomogeneity of substitution.

Holden and Tracey (1950), using a somewhat different approach and snail instead of fungal enzymes, obtained evidence which suggests that both members of a pair of anhydroglucose residues in carboxymethylcellulose of DS 0.45 must be unsubstituted to render the linkage between them susceptible to hydrolysis. With ethylmethylcellulose (DS 1.2), only one of a pair of contiguous residues need be unsubstituted. These conclusions were based on the limited extent of hydrolysis achieved by

adding fresh enzyme to the substrate until no more increase in reducing sugars could be detected. The limiting extent of hydrolysis (40 percent for the carboxymethylcellulose and 17 percent for ethylmethylcellulose) was then compared with calculated estimates of the frequency of occurrence of two consecutive or of a single unsubstituted residue in the two substrates. Calculations were based on Spurlin's (1939) statistical treatment of the arrangement of substituents in partially substituted cellulose derivatives, which takes into account the degree of substitution and the relative reactivities of the primary and secondary hydroxyl groups of cellulose.

In considering the early and most of the recent work on cellulases and hemicellulases, the nature of the active preparations should be kept in mind. Most of the enzyme solutions used have been crude, unfractionated extracts or culture filtrates. Another possible source of confusion is the multiplicity of sources used. In the absence of a pure cellulase from more than one source, it is difficult to make comparisons between enzymes from different sources. Part of the seemingly contradictory evidence concerning the end products of cellulose breakdown by cellulase is due to this circumstance.

One of the end products of the action of crude cellulase preparations on cellulose and its derivatives has been shown, beyond a doubt, to be D-glucose. This has been shown for preparations from plant tissue (Pringsheim and Baur, 1928), snail enzyme (Seillière, 1910; and Karrer and Illing, 1925), protozoa (Trager, 1932; and Hungate, 1942), bacteria (Simola, 1931) and fungi (Saunders et al., 1948; Whitaker, 1953; and Whistler and Smart, 1953) by the isolation of D-glucose as the osazone

from the hydrolysates. With the snail enzyme, Karrer and Illing (1925) found that the conversion to glucose was almost complete. Walseth (1948), using A. niger preparations, was also able to account quantitatively for the loss in weight of swollen cotton linters by the appearance of glucose alone.

In contrast to these findings, Pringsheim (1912) was able to demonstrate the accumulation of cellobiose as well as glucose in cultures of anaerobic cellulolytic bacteria after growth was arrested by addition of such metabolic poisons as toluene, chloroform, or iodoform. He also found that by heating the arrested cultures to about 70° C., cellobiose accumulated to an even greater extent. These findings were interpreted to mean that cellobiose is the end product of the action of cellulase and that glucose is liberated from the disaccharide by a second enzyme, cellobiase.

This has been the prevailing theory until very recently, in spite of the fact that later workers (until recently) were unable to detect cellobiose in other systems. This failure was attributed to the presence of a very active cellobiase, an assumption which gained credence with the work of Grassman et al. (1932, 1933), who were able to separate, at least partially, the cellobiase from the cellulase of fungal preparations by adsorption of the former on aluminum metahydroxide at pH 3.5. The cellobiase was eluted with 0.04 molar sodium bicarbonate. The two fractions thus obtained were tested for their activity against cellodextrin and the β-oligosaccharides of two, three, four, and six residues. The cellobiase fraction readily hydrolyzed not only cellobiose but also the other oligosaccharides as well; however, it had

little effect on the cellodextrin. The cellulase fraction had no effect on cellobiose (in 24 hours), and hydrolyzed cellotriose and cellotetraose only to the slightest extent. It attacked cellohexaose to a certain extent and cellodextrin fairly rapidly. Thus, there was some overlapping of the domains of each enzyme at the cellohexaose level.

More recently, Whistler and Smart (1953) provided the first unequivocal proof of the formation of cellobiose as an intermediate in the enzymatic degradation of cellulose. They pointed out that the cellobiose found by Pringsheim in arrested bacterial cultures might be a metabolic product rather than an intermediate or end product. Whistler and Smart were able to detect only D-glucose in the hydrolysates of swollen cellulose produced by (1) the crude extract of A. niger, (2) the preparations obtained from ammonium sulfate or acetone fractionations, (3) the crude extract held for various periods at elevated temperatures or in strong acid or alkali, or (4) the fractions from columns of ion-exchange resins, charcoal, wheat starch, Kaolin-Supercel, bauxite, or aluminum hydroxide. Finally, a fraction was obtained by sorption of the crude extract at pH 3 on a powdered cellulose column and elution with a pH 9 borate buffer which hydrolyzed swollen cellulose to D-glucose and cellobiose in detectable amounts. The latter was isolated both by paper and column chromatography and identified by its melting point, X-ray diffraction pattern, and by the formation of its phenylosazone. Apparently the cellobiase was not entirely eluted from the cellulose powder, thus allowing cellobiose to accumulate in the hydrolysates. A search for other oligosaccharide intermediates and phosphorylated sugars, using paper chromatographic techniques, produced negative results.

Reese, Siu, and Levinson (1950), using metabolic culture filtrates from several different fungi, were unable to detect any cellobiase activity. Although no specific search for cellobiose in the hydrolysates of ground cotton cellulose was made, these workers postulated that glucose can be liberated directly from the cellulose chains without the intermediation of a cellobiase.

In a subsequent publication, Levinson, Mandels, and Reese (1951) reversed their stand and reported, after using enzymatic techniques for the simultaneous and specific determination of both glucose and cellobiose, that cellobiose appeared in the hydrolysates before glucose could be detected. With the culture filtrates of some fungi, the amounts of cellobiose produced exceeded the amounts of glucose. The cellobiose was hydrolyzed very slowly, which indicated that there is little, if any, β-glucosidase in these filtrates. The results were qualitatively confirmed by paper chromatography. No other intermediates were detected when powdered cellulose and alkali cellulose were hydrolyzed, but at least two spots other than those representing glucose and cellobiose were noted when cellulose sulfate was used. These unidentified products were probably sulfate-substituted glucose or oligosaccharides, since they did not appear in the hydrolysates of unsubstituted cellulose. Furthermore, incubation of the hydrolysates with β-glucosidase before chromatography did not eliminate these spots.

On the basis of the results just described, Levinson *et al.* (1951) hypothesized that the principal final product of the enzyme which splits the β-1,4 linkages of cellulose is cellobiose.

As mentioned previously, it has been found by Reese et al. (1950) that all the cellulolytic fungi tested could utilize both native cellulose and carboxymethylcellulose (of low DS), and that culture filtrates from these organisms could also attack these substrates. Unexpectedly, it was also found that certain noncellulolytic fungi, while not having the ability to utilize solid cellulose, did grow when carboxymethylcellulose was used as the substrate. Their culture filtrates reflected these characteristics. These results led the authors to suggest that all cellulolytic organisms possess two enzymes. One of these, which they designated C_1, and which Siu (1951) later suggested should be called cellulase, supposedly brings about some change in native cellulose making it amenable to hydrolysis of the β-1, 4 linkages by the second enzyme, C_x. C_x, according to Reese et al., occurs in all cellulolytic and in many noncellulolytic fungi as well. The precise action of C_1 was not stated, but the authors speculated about the possibility that it splits the nonglycosidic cross-linkages postulated to be present in native cellulose. Siu (1951) even suggested that the action of C_1 may be that of a "hydrogenbondase" which would serve to separate the chains in the relatively more crystalline areas of the native fibers. In any event, the result of its action is supposedly the production of straight polyanhydroglucose chains available to the C_x enzyme.

The extreme variations in the ratio of activity on cellulose sulfate or carboxymethylcellulose and solid cellulose substrates from one enzyme source to another (Reese and Levinson, 1953; and Tracey, 1953) can be explained in terms of this hypothesis. It is only necessary to assume differences in the proportions of C_1 to C_x in different fungi.

The validity of Reese's multiple enzyme hypothesis is difficult to assess in the absence of pure enzyme preparations. Final proof or disproof of its general validity can only await the isolation of one or both of the enzymes in a high state of purity from several sources.

Whitaker (1953) has succeeded in isolating an active cellulase from culture filtrates of Myrothecium verrucaria, apparently in a high state of purity. The preparation appears to be homogeneous at pH levels above, near and below the isoelectric point, and shows only one peak in the ultracentrifuge at pH 5.03. The procedure used for the isolation involved concentrating the culture filtrates by vacuum evaporation and by slow partial freezing, followed by precipitation of the proteins by saturation with ammonium sulfate at 1° C. The specific activity (units of cellulase activity per unit of protein) at this stage was little changed from the original culture filtrate, but some undesirable impurities of unspecified nature were removed at 30 percent saturation with ammonium sulfate. The next step, fractionation with alcohol at low temperature, low ionic strength, and pH 4.95, yielded a fraction at 25 percent alcohol which contained about two-thirds of the protein and had a somewhat higher specific activity. The final step was a fractional precipitation with polymethacrylic acid at pH 4.35. The protein liberated from the complex by addition of barium chloride had a specific activity about 2.25 times as great as the starting material.

The ammonium sulfate precipitates were analyzed electrophoretically and found to contain five components, two major slow-moving peaks and three minor ones migrating at successively faster rates. These runs were made in phosphate buffer of pH 6.8 with an ionic strength of 0.2.

Protein concentrations for the electrophoretic analyses had to be kept low because of the dark color of the material.

Whitaker, Colvin, and Cook (1954) determined the diffusion constant (5.61×10^{-7} at 20° C. in water) and the sedimentation constant (3.72×10^{-13} at 20° C. in water) of the purified cellulase. In the diffusion measurements, no evidence of deviation from a Gaussian distribution was found, another indication of the homogeneity of the preparation. From these values and an assumed partial specific volume of 0.74 ml. per gram, the molecular weight was calculated to be $63,000 \pm 1600$.

During the purification procedure, the enrichment in specific activities against all substrates (phosphoric acid swollen cotton linters, reprecipitated cellulose, unswollen linters, carboxymethylcellulose, and cellobiose) was approximately the same. Although this finding, coupled with the demonstrated homogeneity of the final product, is considered by Whitaker to suggest a unienzymatic mechanism of cellulose hydrolysis, he suggested that it is not necessarily at variance with the work of Reese. According to Whitaker (1953), Reese's findings can be interpreted as an indication that the cellulase of the cellulolytic fungi has the capacity to form an effective complex with all types of cellulose, whereas the enzyme of the noncellulolytic fungi loses its ability to hydrolyze the β-1,4 linkages when it is adsorbed on a solid substrate.

The end products of cellulose hydrolysis by the purified cellulase were shown to be glucose and cellobiose in about equimolecular amounts. The enzyme has so low a cellobiase activity that the amount of glucose produced could not originate from cellobiose. Whitaker (1953) concluded that the appearance of glucose is not dependent on formation via cellobiose.

Whitaker (1952) found that the presence of any one of several proteins in the cellulase assay medium has a decided stimulatory effect on the action of partially purified cellulase preparations on insoluble substrates. The effect was not so marked when carboxymethylcellulose was used as the substrate. Evidence was presented showing that the effect is not one of neutralization of an inhibitor nor of protection of the enzyme against denaturation.

Basu and Whitaker (1953) found that at low pH levels, basic dyes and neutral salts are also stimulatory, whereas acid dyes are inhibitory. At higher pH levels, the reverse effect was found. It was suggested that the effect of proteins and dyes may be due to their effect on the surface potential of the solid cellulose substrate.

Whitaker's work seems to indicate that the cellulolytic activity of Myrothecium verrucaria filtrates is that of a single protein component. These results are in distinct contrast to those of two other investigators.

Jermyn's (1952) findings indicating the presence of perhaps seven different components in A. oryzae preparations active against carboxymethylcellulose have been mentioned previously. Reese and Gilligan (1953) also obtained evidence suggestive of a multiple nature of their C_x enzyme. Their procedure was somewhat like that of Jermyn. They used chromatographic separation of the components on zein-treated paper, rather than paper electrophoresis, and determined the C_x activity distribution along the length of the developed strip by cutting it into short sections and incubating these sections with carboxymethylcellulose in buffered solution. Plotting the activity contained in the cut

sections against their location on the original chromatograms gave curves showing at least two maxima for seven of the eight culture filtrates tested. The exception, a culture filtrate from Trichoderma viride, contained only one active component. The curve for M. verrucaria had two different maxima, a slow-moving component barely migrating from the starting point and a faster moving component migrating somewhat less than half the distance of the solvent front. With other culture filtrates, the fast-moving component almost kept pace with the solvent front. In all cases but one, every section of the developed strip had some activity, and the curves did not reach the base line (zero activity) at any point.

Further evidence that the maxima on the curves represented distinct protein species was afforded by successful attempts to separate them by other means. The single, slow-moving component of T. viride could be almost completely adsorbed on cellulose (Solka Floc). Only the slow component, representing about 30 percent of the C_x activity of M. verrucaria filtrates was adsorbed. The other component could be removed by adsorption on kaolin, leaving the slower one in solution. The two enzymes thus must have distinct physical properties. Furthermore, with M. verrucaria, the relative amount of the fast-moving component can be influenced by changes in the conditions under which the organism is cultured.

The question of whether the action of cellulase on the cellulose chain is an endwise attack or proceeds in a random manner has received some attention. The most convincing evidence in favor of the random cleavage mechanism would be the isolation of an oligosaccharide intermediate in enzymatic hydrolysates of cellulose. On the other hand,

failure to find any such intermediates, in spite of concerted efforts to do so (Whistler and Smart, 1953; Levinson, Mandels, and Reese, 1951) does not constitute a repudiation of the random cleavage hypothesis, in the light of a recent report by Whitaker (1954). Using his purified M. verrucaria cellulase, Whitaker found that cellotetraose, cellopentaose, and cellohexaose are very rapidly hydrolyzed. Cellobiose is very slowly attacked, and cellotriose occupies an intermediate level of susceptibility.[1] All possible hydrolysis products of each substrate were determined at short intervals. The results suggest that with cellotetraose the enzyme shows a preference for splitting the central rather than the terminal linkages, but with the higher members of the series, there is a tendency toward increasing randomness of chain-splitting with increasing chain length.

Whitaker suggested that the detection of the higher soluble oligosaccharide intermediates is likely to be very difficult, since their rate of hydrolysis greatly exceeds the rate at which they could be formed from an insoluble cellulose substrate.

At the same time that the above paper appeared, Nash and King (1954) published a short account of the demonstration of an oligosaccharide intermediate in the hydrolysis of cellulose by M. verrucaria cellulase. Their technique was to remove possible diffusible intermediates from contact with the enzyme by carrying out the reaction in a collodion sac

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[1] Turnover numbers (moles of substrate degraded in one minute by one mole of enzyme) were: cellobiose 5-6, cellotriose 50-200 (depending on initial concentration of substrate), cellotetraose 400, and both cellopentaose and cellohexaose, at least 450.

suspended in a large volume of buffer. After a suitable incubation period the outer solution was concentrated and chromatographed on paper. Besides spots corresponding to cellobiose and glucose, a third spot with a lower R_f value was noted. A small amount of the material isolated from this area was tentatively identified as cellotetraose by estimating its molecular weight through comparison of its reducing power with that of cellobiose and determining total glucose in the molecule by the anthrone method. That the oligosaccharide is an intermediate and not an end product was indicated by its rapid hydrolysis to cellobiose and glucose on incubation with the M. verrucaria enzyme.

Other evidence supporting the hypothesis of random attack on cellulose by this enzyme is the finding that samples of cellulose differing only in degree of polymerization are hydrolyzed at about the same rate by fungal cellulase (Walseth, 1948, 1952). If the mechanism were primarily an endwise process, the rate of hydrolysis would logically be expected to increase with a decrease in the degree of polymerization.

With the soluble cellulose derivatives, it has been found that the viscosity of the solutions being hydrolyzed by snail or plant cellulases falls appreciably before a significant increase in reducing end-groups occurs (Holden and Tracey, 1950). Similar results were obtained by Levinson and Reese (1950), using fungal cellulases. These findings can be interpreted in favor of an extension of the random cleavage mechanism to the soluble derivatives.

Other investigators, notably Clayson (1943), have expressed the belief that the process necessarily proceeds endwise. Kitts and

Underkofler (1954), the first to extract a cell-free cellulase from rumen bacteria, were unable to detect any products of cellulose degradation by this crude preparation other than glucose and cellobiose, and believed that this tends to confirm Clayson's hypothesis of endwise attack.

It seems to be generally assumed that the enzymatic breakdown of cellulose is a hydrolytic process. However, according to Siu (1951), this question is not yet settled. Simola (1931) reported that the cellulolytic activity of a cell-free bacterial preparation was increased almost three times over that of the controls by 0.033 M phosphate. Greater concentrations were inhibitory. Norkrans (1950) reported that the stimulatory effect of phosphate on Tricholoma nudum enzymes occurred only in the presence of calcium. Lane and Greenfield (1953) found that the cell-free cellulase system obtained from the gut of the mollusc Teredo pedicellata is stimulated by the presence of adenosinetriphosphate, the production of reducing sugar being increased ten times. Nevertheless, no phosphorylated sugars have been found in enzymatic hydrolysates of cellulose (Whistler and Smart, 1953).

Table I includes a column giving the pH optimum for the particular system and conditions used. It can be seen that there are considerable differences between the optima for different enzyme sources and even with the same source as determined by different investigators. In his study of the stimulating effect of inactive proteins on the action of M. verrucaria cellulase, Whitaker (1952) found that the added protein also caused a relatively great shift in the optimum pH. He also found that the shift depends on the particular substrate used. This may have

some general bearing on the lack of agreement noted in Table I. The possibility of the existence of more than one cellulase in a given source (Jermyn, and Reese and Gilligan, vide supra) might also explain some of these differences. This might also be pertinent to the observation that most of the pH response curves seen in the literature are rather broad and flat. In contrast to the latter curves, showing a single optimum (or optimal range), Freudenberg and Ploetz (1939) found three distinct pH optima (4.7, 6.1, and 6.9) for a commercial A. oryzae preparation.

The kinetics of the enzymatic degradation of insoluble celluloses have been studied in a few instances. Most investigators agree that the experimental results are difficult to interpret because of the inhomogeneity of the substrate (areas of different accessibility) and the impurity of the enzyme solutions. Karrer and Illing (1925) found that with viscose rayon and snail cellulase preparations, the reaction at first follows a monomolecular course, changing later to coincide with the Shutz rule.[1] Walseth (1948) obtained similar results with swollen cellulose and A. niger enzymes. This type of rate curve, a relatively rapid primary phase followed by a much slower secondary phase, with a rapid transition between the two phases, can be interpreted to be the result of the nonhomogeneity of the substrate, the rapid part representing the hydrolysis of the more accessible regions of the substrate.

With both carboxymethylcellulose and cellodextrin, a linear relationship between concentration of enzyme (A. oryzae) and the initial

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[1] The quantity of substrate changed is proportional to the square root of the reaction time.

rate of reaction was found by Jermyn (1952). When the enzyme concentration was held constant and the substrate concentrations varied, Lineweaver-Burk plots of the data yielded straight lines for both substrates. The Michealis constants obtained from these data were 3.2 and 4.4×10^{-3} for cellodextrin and carboxymethylcellulose, respectively.

Norman's (1937) definition of the hemicelluloses is the one employed in the present work. Since this definition includes the hexosans, pentosans and hexo-pentosans, as well as the polyuronide hemicelluloses, any enzyme hydrolyzing any linkage of these substrates might be called a hemicellulase. This is perhaps unfortunate, since the term is not descriptive of the specificity which may be involved. On the other hand, until the specificity relationships for a member of the group are thoroughly worked out, a specific name is not in order.

The specificity requirements of the hemicellulases have not been rigidly defined in any case, since none has been isolated in a condition pure enough to warrant such a project. As mentioned before, Grassman et al. (1933) presented evidence which seems to indicate that the xylanase and mannanase activities of an A. oryzae preparation are due to separate enzymes, and that both are different from cellulase.

The early observations of Bierry and Giaja (1912) afford further evidence that the hemicellulases are a group of enzymes with different specificities. These workers found that although the digestive juice of Helix pomatia hydrolyzes the mannogalactans of alfalfa seed and fenugreek and the mannans of the date palm and the ivory nut, that of certain crabs and lobsters attack only the ivory nut mannan. Action of the digestive secretion of the crayfish on the mannogalactans produced much more galactose than mannose.

Such observations as the latter are not surprising, considering the wide variety of sugars and types of linkage to be found among the hemicelluloses. In the absence of precise knowledge of the composition and structure of a hemicellulose substrate, end products of the reaction should be at least qualitatively identified in order to give significance to the results.

Pringsheim and Genin (1924), using a two-months old malt extract as the source of hemicellulase, observed an almost quantitative cleavage of salep mannan to mannose. When a six-months old malt extract was used, the reducing power of the final hydrolysate was only one-half as great. Mannobiose was identified by means of its phenylhydrazone. The authors suggested that there are two enzymes involved, a fairly stable mannanase and a less stable mannobiase. This observation is of particular interest since it is entirely analogous to the mechanism of cellulase action postulated by Pringsheim (1912), and to the action of β-amylase on starch.

Xylose has been repeatedly identified as the main end product of hydrolysis of various xylans by snail (Ehrenstein, 1926; and Voss and Butter, 1938), malt (Luers and Volkamer, 1928), and fungal (Grassman et al., 1932; and Yundt, 1949) preparations. To the author's knowledge, xylobiose or higher oligosaccharides of xylose alone have never been reported. Hydrolysis of the xylans seems never to go to completion, even with the addition of fresh enzyme solution. The limiting extent of hydrolysis varies not only with the xylan, as might be expected, but also with the enzyme (Ehrenstein, 1926) and with the method of dispersion (Yundt, 1949).

The nature of the resistant residues has been investigated in a few cases. O'Dwyer (1937, 1939, 1940) found that the portion of several oak hemicelluloses resistant to A. oryzae enzymes had a composition corresponding to six xylose residues to one of a methylhexuronic acid. The linkages involved were not determined.

Some pH optima for various hemicellulase preparations acting on various hemicelluloses are given in Table II. It can be seen that the range reported (4.65 to 5.28) is much narrower than for the cellulases. Ehrenstein (1926) found that the nature of the buffer affected the pH optimum of snail xylanase. In citrate buffer it was 4.65, in phosphate, 5.28. No further reference to this phenomenon could be found in the literature.

There have been few studies of the kinetics of hemicellulase action. Ehrenstein (1926), using a wheat straw xylan and snail enzyme, found that the reaction followed neither a monomolecular course nor Shutz's rule. Voss and Butter (1938) reported that the hydrolysis of xylans by both snail and A. oryzae enzymes did proceed according to Shutz's rule over an intermediate range.

Statement of the Problem

As mentioned previously, Veibel (1950) has discussed very briefly, and left open, the question of the identity of β-glucosidase and cellulase. Although Pigman and Goepp (1948) separate the two activities completely, at least for purposes of discussion, the question seems never to have been specifically answered one way or the other. Whitaker's purified cellulase would seem to present a good opportunity to investigate

this problem, but up to the present time, no investigations pertinent to the question, other than the demonstration of a relatively slow rate of attack on cellobiose by the purified cellulase, have appeared.

Jermyn's work, suggesting the identity of not one but several β-glucosidase and cellulase components in A. oryzae preparations, should stimulate further research along these lines. His results, along with those of Reese and Gilligan, have emphasized the complexity of the problem.

If the possibility is conceded that cellulase and β-glucosidase activities can be possessed by a single enzyme, at least in some cases, a discussion of the known specificity requirements of the β-glucosidases would be pertinent. Veibel (1950) has recently reviewed this subject, with special emphasis on the β-glucosidase of sweet almond emulsin.

The investigations of several authors, references to whose work will be found in Veibel's review, may be summarized briefly as follows. The specificity with regard to carbon atoms 1, 2, and 3 seems to be absolute. In other words, the substrate must have the β-configuration, and substitution or epimerization of carbons 2 and 3 (to a configuration opposite to that of D-glucose) gives a product which is resistant to attack by β-glucosidase.

With regard to the rest of the molecule, the specificity is more relative. For example, β-methylmaltoside (which may be regarded as a β-methylglucoside substituted in the 4 position with an α-glucopyranose unit) is readily hydrolyzed to methanol and maltose by β-glucosidase. This is of interest to the present discussion since the

constituent residues of cellulose and of many of the residues in the hemicelluloses are linked at the 4 position.

At carbon atom 5, the only change which seems to be tolerated is substitution of the entire $-CH_2OH$ group by hydrogen, yielding a β-D-xyloside. It must be pointed out, however, that this type of glycoside is hydrolyzed by β-glucosidase of almond emulsin at a much slower rate than are the β-glucosides.

Certain substitutions at carbon atom 6 may be made without complete loss of hydrolyzability. The volume and the relative electrical charge of the substituent appear to be the important factors. A positively charged group is inhibitory.

The aglycon specificity requirements of the β-glucosidase of almond emulsin have received a great deal of attention. It is sufficient for the present discussion to repeat Veibel's statement that no case of real absolute specificity caused by the aglycon has been reported, and also to point out that this generality extends to various disaccharides such as cellobiose, gentiobiose, celtrobiose, and phenyl- α-cellobioside.

None of these specificity requirements rules out cellulose and some β-glycosidic linkages of many hemicelluloses as suitable substrates for β-glucosidase. The work of Grassman et al. showing that the cellobiase (a β-glucosidase) and cellulase activities of A. oryzae preparations can be partially separated suggests that cellulase and β-glucosidase are not identical. Whitaker's demonstration of the relatively weak cellobiase activity of his purified M. verrucaria cellulase may also be cited. In partial answer, it can be pointed out that the β-glucosidases of different origin are known to differ considerably in

their spectrum of relative specificities toward various substrates (Veibel, 1950). Furthermore, Jermyn's results and those of Reese and Gilligan demonstrating the multiplicity of the β-glucosidase and cellulase activities of fungal preparations leave the question still open, in the opinion of the author.

If the specificity requirements of cellulase resemble those of β-glucosidase, cellulase would be expected to have some action on certain hemicelluloses, particularly those containing β-D-xylosidic linkages. The nature of the aglycon side of the molecule and the position and nature of the substituent would be expected to have some influence on the rate of the process. Thus, there occurs the question of whether it is necessary to invoke a special hemicellulase (or xylanase) in those cases in which both cellulase and hemicellulase (xylanase) activities are known to occur. As far as the author is aware, this question has been specifically investigated only once: Grassman et al. were able to remove the xylanase activity of a fungal preparation by treatment with animal charcoal. The cellulase activity remained in solution. On the basis of this single observation, the question cannot be considered settled.

At the beginning of the investigation to be described in the remaining pages, the object was to isolate, purify, and characterize the extracellular cellulase of Myrothecium verrucaria. However, after the appearance of Whitaker's work on the purification of the same cellulase, and of Jermyn's and Reese and Gilligan's demonstrations of the possible multiple nature of the activity, a different approach was thought to be possibly more fruitful. The final plan was to isolate from the original

multicomponent culture filtrate of M. verrucaria a series of fractions containing different ratios of the various protein components and to determine, if possible, which of the components is active against various monomeric and polymeric β-glycosidic substrates. Greatest emphasis was placed on the relationships among cellulase, hemicellulase, and β-glucosidase, with a view toward establishing their identity or nonidentity.

EXPERIMENTAL

EXPERIMENTAL

The course taken for the investigation of the problem stated previously may be outlined briefly as follows. Culture filtrates of Myrothecium verrucaria grown in submerged culture were initially concentrated by slow partial freezing. Further concentration of the proteins was accomplished by precipitation with acetone or with ammonium sulfate. Aqueous extracts of these precipitates served as the starting materials for fractionation of the protein components. Attempts to fractionate the concentrates by salt and solvent precipitation and by chromatography on cellulose columns were generally unsuccessful, but electrophoresis-convection finally yielded preparations containing different ratios of the protein components. Fractionations were controlled by estimation of enzymatic activity and determination of protein nitrogen as well as by electrophoretic analysis. Cellulose, two different impure hemicelluloses, cellulose sodium sulfate, and salicin were used as substrates to study and compare the enzymatic properties of the final preparations.

Materials and Methods

Production of Culture Filtrates

The fungus Myrothecium verrucaria[1] was chosen as the source of enzyme material because, according to Siu (1951), it is one of the strongest cellulose decomposers known. Its culture filtrates reflect

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[1] Obtained from the American Type Culture Collection, Washington, D. C.

this property (Saunders et al. 1948). Moreover it is not pathogenic, as are some of the Aspergillus species.

Filtrates were obtained from submerged cultures of the organism grown in the medium devised by Saunders et al. (1948). This culture medium contained only mineral salts and cellulose as the sole source of carbon. The mineral portion of the medium is given in Table III.

TABLE III

COMPOSITION OF THE CULTURE MEDIUM USED FOR M. VERRUCARIA

Salt*	Final concentration	Salt	Final concentration
	g./l.		µg./l.
Na_2HPO_4	1.5	$CuSO_4 \cdot 5H_2O$	2.5
$NaH_2PO_4 \cdot H_2O$	2.0	$Fe_2(SO_4)_3$	43.0
$NaNO_3$	3.75	H_3BO_3	57.0
K_2HPO_4	0.15	$ZnSO_4 \cdot 7H_2O$	50.0
KH_2PO_4	0.20	$MnSO_4$	5.5
NH_4NO_3	0.60	$(NH_4)_2MoO_4$**	120.0
$MgSO_4 \cdot 7H_2O$	0.30		

*All the chemicals used throughout this investigation were reagent (or C.P.) grade unless specifically stated to be otherwise.

**Ammonium molybdate was substituted for ammonium phosphomolybdate on the basis of its molybdenum content.

Cellulose was supplied at the rate of 20.0 g. for each liter of culture medium. Solka-Floc[1] was used in all but two cultures, in which it was replaced by Whatman ashless powdered cellulose (medium grade).

The medium was prepared by placing 240 g. of cellulose and seven liters of the mineral salts solution in the incubation vessel illustrated in Figure 1. With the top assembly in place and all openings covered with cotton caps, this vessel and five more liters of mineral salt solution distributed among four two-liter Erlenmeyer flasks were sterilized in the autoclave at fifteen pounds pressure (120° C.) for 45 minutes. The remainder of the salt solution, while still hot, was introduced into the incubation vessel by means of suction. Care was taken during these manipulations to maintain the sterility of the culture medium.

The inoculum, consisting of a suspension of ungerminated spores, was prepared by growing the fungus on filter paper disks placed on mineral salts agar[2] in Petri dishes. The filter paper disks, cut to cover about 80 percent of the area of the agar slab, were moistened with a small amount of the salt solution and sterilized by autoclaving in a Petri dish. One disk was placed on each solidified agar slab and

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[1]Solka-Floc is a wood cellulose product of The Brown Company.

[2]In accordance with instructions from the American Type Culture Collection, the composition of this medium was: dibasic potassium phosphate, 0.5 g., sodium nitrate, 2.0 g., magnesium sulfate, 0.2 g., agar, 17.0 g., and water, one liter. As a precaution against bacterial contamination, penicillin and streptomycin were added at the rate of 25 units per ml. The antibiotics were dissolved in a small amount of water, sterilized by filtration, and introduced after the agar had been autoclaved (120° C., 30 min.).

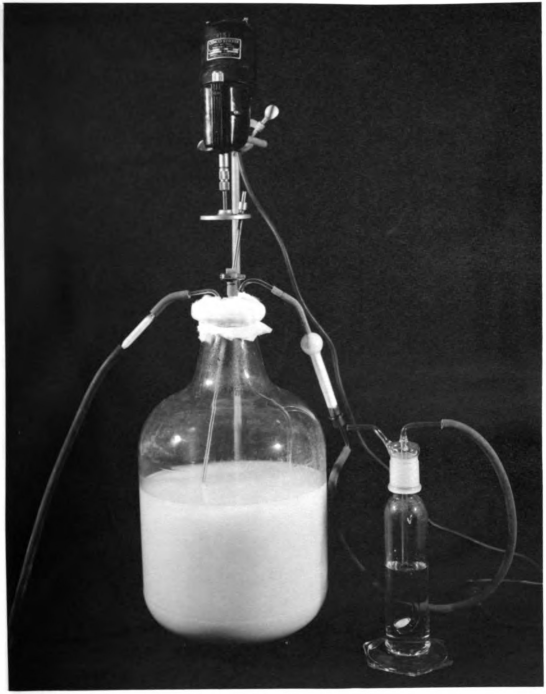

Figure 1. Incubation vessel for the submerged culture of *M. verrucaria*.

liberally streaked with spores from a previous culture. Best results were obtained at room temperatures, under which conditions noticeable vegetative growth first became visible after three or four days. Sporulation started in about a week and at the end of two weeks nearly the entire surface of the filter paper disk was covered with the greenish-black spores.

The inoculum was obtained by flooding the surfaces of two or three of the Petri dishes which contained the sporulated cultures with about five ml. of sterile salt solution (composition as in Table III) and scraping the spores into a suspension with a wire loop. Individual suspensions were transferred first to a sterile test tube and then, after mixing, to the cooled culture medium in the carboy. Enough of the spore-suspension was saved to make a spore count.

Spore counts were made on an appropriate dilution of the suspension with the aid of a Spencer bright line counting chamber and a microscope. Although the spores are small, staining was not necessary because of their density and dark color. Serious clumping of the spores was never encountered so the distribution in the counting chamber was satisfactory.

In some cases, a solution of 300,000 units of penicillin and 300,000 units of streptomycin, sterilized by filtration, was added to the large culture to prevent bacterial contamination.

Incubation of the large culture was maintained at room temperature with continuous aeration and stirring. Aeration was provided by means of a water aspirator. Air entering the system was washed with water and filtered through a tightly packed sterile cotton column and then

introduced below the surface of the culture (see Figure 1). The air flow rate was not measured, but was kept rapid at all times.

Vigorous, continuous stirring was provided by means of a stainless steel rod pivoted in the rubber stopper and connected at the top to a "roto-stir unit" driven by a heavy duty motor (See Figure 1). The stirring rod was fitted very tightly in the rubber stopper, but the entire thickness of the stopper could not be used as a pivot. Instead, wells several times the diameter of the rod were sunk from both surfaces of the stopper. A hole somewhat smaller than the rod was then cut through the remaining portion. Autoclaving tended to seal the rubber tightly to the rod.

In order to follow the increase of cellulase activity, small samples of the culture were asceptically removed at two or three-day intervals and assayed, using cellulose sodium sulfate as the substrate. The incubation was terminated when the activity level had reached a plateau, or was increasing very slowly. The time of incubation varied between two and three weeks.

Fifteen such cultures were prepared, but two were discarded because of gross bacterial contamination. Contamination was indicated by cloudiness of the filtrates and the appearance of bacterial colonies on dextrose agar streaked with some of the material. The cellulase activity of the contaminated culture filtrates could not be estimated before dialysis because of the presence of an oxidizing agent which interfered with the end point. The oxidant could be removed by dialysis, but the cultures were nevertheless discarded because of bacterial contamination.

Culture filtrates were obtained by filtration through a mat of glass wool in a large Buchner funnel. (Filter paper is rapidly weakened by the enzyme). The first portions of the filtrate were returned to the funnel until the residual cellulose and the fungal mycelium formed an efficient filter, after which the filtrates were clear.

The color of the filtrates was light amber. In the later fractionation steps it was found that this color tended, at least in part, to accompany the protein. It was only partially removed by dialysis and was nearly completely precipitated along with the proteins by ammonium sulfate, acetone, and alcohol, but not so completely by trichloroacetic acid. In some of the more concentrated fractions, the darkness of the color made it necessary to dilute in order to obtain satisfactory electrophoretic patterns. Whitaker (1953) reported the same difficulty.

Concentration of Culture Filtrates

The concentration of protein in the filtrates was very low. Since there was some nonprotein nitrogen present and there was no assurance that all the protein could be precipitated, determination of protein nitrogen was not attempted at this point. Total nitrogen, exclusive of nitrate nitrogen was found to vary between 0.022 and 0.041 mg./ml. If this were all protein nitrogen and the usual conversion factor of 6.25 were to apply, then the protein concentration would be from 13.7 to 25.6 mg./100 ml. Inasmuch as this concentration is too low for application of the usual fractionation procedures the culture filtrates were concentrated.

The volumes of the filtrates were too large (nine to ten liters) to permit freeze-drying with the apparatus available. Instead, the

method of slow partial freezing to remove water with a minimum of enzyme loss was adopted (Whitaker, 1953). About three liters of filtrate were placed in each of two four-liter beakers wrapped with a thick layer of cotton and gauze and then placed in boxes containing about two inches of cotton. Covers with an inner lining of about two inches of cotton extending into the beaker close to the surface of the filtrate were placed on the beakers. When placed in a freezing cabinet, freezing took place slowly from the outside inward. At the end of 24 to 36 hours, the concentrated solution in the center was poured off and the ice was chipped and allowed to drain, with frequent stirring, in a large funnel. The small amount of melt from the ice was combined with the concentrate, and the process repeated until the volume had been reduced at least five times.

Protein nitrogen and cellulase activity (cellulose sodium sulfate) were determined on the concentrates. The cellulase activity of the material removed as ice was also estimated. The protein nitrogen content of the concentrates varied from about 0.08 to 0.21 mg./ml.

Fractionation of the proteins by salt or solvent precipitation was not very successful, but it was noted during these experiments that much of the activity of the concentrates could be precipitated by acetone or ammonium sulfate. Consequently some of the concentrates were further concentrated by these means.

Acetone precipitations were carried out as follows. Three hundred and fifty ml. of the concentrate was placed in a two-liter Erlenmeyer flask which was clamped in a large battery jar filled with ice and water. The entire assembly was then put on a magnetic stirring unit. When the

temperature of the concentrate reached about 2° C., 1400 ml. of chilled reagent grade acetone was added down the side of the flask over a period of three to four hours. As the acetone concentration increased, salt was added to the ice bath until the temperature of the acetone solution reached about -10° C. Stirring was continued for one-half hour after all the acetone had been added, and then the precipitate allowed to settle for about two hours in a freezing cabinet. The precipitate was heavy and granular and settled rapidly. Most of the supernatant solution was siphoned off and the precipitate obtained by centrifugation washed three times with small amounts of cold acetone and then dried in a vacuum desiccator. The dried powders were tan in color, and their weight indicated that they were mostly inorganic salts. They were stored in this form over calcium chloride in a desiccator.

In an attempt to improve the recovery of activity and proteins, the acetone precipitation was also applied to a dialyzed concentrate. Dialysis was against twenty volumes of distilled water for 12 hours at about 4° C., then against the same amount of 0.02 M phosphate buffer, pH 6.9 for 24 hours.

When the acetone powders were to be used, the proteins were extracted by slowly adding one part by weight of the powder to five parts of water with continuous mechanical stirring. The water was cooled to about 0° C. in an ice and salt bath. This procedure was found to be necessary because of the heat of solution of the salts. After the powder was added, the ice bath was removed and the mixture allowed to warm slowly to about room temperature while stirring was continued. A voluminous dark-colored precipitate was removed by centrifugation,

extracted once with a small amount of water, and saved for nitrogen analysis. An aliquot of this second extract was saved for cellulase assay and the remainder combined with the first extract. The combined extracts were then stored in a refrigerator (ca. 4° C.) overnight, during which time a rather large amount of a salt (probably $Na_2HPO_4 \cdot H_2O$) separated in the form of large crystals. These were removed by filtration through glass wool and washed with a small amount of ice-cold water. The combined filtrate and washings were then dialyzed first against about twenty volumes of distilled water and then against the pH 6.9 phosphate buffer used for electrophoresis.

When ammonium sulfate precipitation was used as a means of concentrating the proteins, the procedure was as follows. The concentrate was placed in a two-liter beaker on a magnetic stirrer in the cold room (ca 4° C.) and an eighteen-inch length of large diameter viscose cellulose tubing, tied at the bottom and packed with enough ammonium sulfate to make the solution about 50 percent saturated, was suspended in the liquid. Stirring of the outside enzyme solution was continuous, so that as ammonium sulfate diffused through the membrane it was immediately mixed and diluted. At the same time, water diffused into the cellulose tubing, bringing about a decrease in the volume of the enzyme solution. Small samples of the outside solution were removed at intervals and analyzed for ammonium sulfate by steam distillation of the ammonia released by strong alkali, as in the Kjeldahl procedure to be described later. When the solution was 30-35 percent saturated, it was centrifuged if any precipitate was present. The precipitate was dissolved in a small amount of water and analyzed for cellulase activity

(cellulose sodium sulfate as substrate) and protein nitrogen. More ammonium sulfate was then added to the concentrate in the same way as before until the solution was 90-100 percent saturated. The precipitate was collected by centrifugation (in the cold room) and dissolved in a small volume of water. The small amount of insoluble material was removed by centrifugation. After dialysis first against distilled water (about 20 volumes for 12 hours) and then phosphate buffer (pH 6.8, ionic strength 0.146) as described in the section on electrophoresis, the solutions were analyzed for protein nitrogen and cellulase activity.

Fractionation by Salt or Solvent Precipitation

Preliminary experiments to determine the feasibility of fractionation by precipitation with alcohol or acetone were done in the same manner. Several 10-ml. portions of a concentrate were measured into flasks held in an ice bath. Absolute ethanol or acetone was delivered slowly and with gentle agitation into the flasks until a predetermined amount had been added to each. Final concentrations of solvent ranged from 33 to 80 percent by volume. The flasks were stoppered and allowed to stand at 0° C. for one hour in one experiment and for 30 minutes in a second. The mixtures were then transferred to test tubes and centrifuged in the cold room. The supernatant solution was decanted and the precipitates washed once with about five ml. of an aqueous solution of the solvent at the corresponding concentration and then recentrifuged. The solvent was removed by decantation and allowed to drain for several minutes; then the precipitate was dissolved in five or ten ml. of cold phosphate buffer (pH 6.9). Any insoluble material was removed by

centrifugation. The resulting solutions were analyzed for protein nitrogen and cellulase activity.

In some of these experiments, pH and buffer concentration of the starting material were controlled by dialyzing some of the concentrate against an appropriate buffer.

Fractionation by means of ammonium sulfate precipitation was also attempted. The procedure was the same as that previously described for the concentration of culture filtrate proteins. The precipitates were recovered at several intermediate stages of ammonium sulfate saturation by centrifugation in the cold room and washed once with ammonium sulfate solution of the same or slightly higher concentration. Wash solutions were prepared by dissolving the required amount of ammonium sulfate in the pH 6.9 phosphate buffer used for electrophoresis. It was not considered necessary to add buffer to the enzyme concentrates before the addition of ammonium sulfate because all were strongly buffered at about pH 7. The washed precipitates were dissolved in a small volume of water and dialyzed against pH 6.9 phosphate buffer for about six hours to remove most of the ammonium sulfate. The dialyzed solutions were then diluted to a convenient volume and analyzed for protein nitrogen and cellulase activity.

Fractionation by Chromatography on Cellulose Columns

Whistler and Smart (1953) reported that the cellulase in a crude A. niger extract could be purified by adsorption of the proteins on cellulose columns at pH 3 followed by elution of the cellulase with borate buffer of pH 9. Since none of the methods of fractionation

described above were very successful, this chromatographic technique was tried.

A column, 150 x 10 mm. was packed with wet Whatman ashless cellulose powder (medium grade) to deliver about 0.5 ml. per minute when the receiver was attached to the aspirator. The concentrated filtrate, first dialyzed against distilled water to remove most of the salts, was adjusted to pH 3 with dilute hydrochloric acid. Twenty ml. were passed through the column. Washing with a total of 60 ml. of water adjusted to pH 3 was begun as soon as the filtrate had passed into the column. The effluent liquids were collected in several portions. The column was then eluted with 120 ml. of Clark and Lubs borate buffer at pH 9. Eluates were collected fractionally. All the collected fractions were assayed for enzymatic activity (cellulose sodium sulfate) and some for protein nitrogen.

Variations in the conditions of adsorption and elution are recorded in the section on results.

Fractionation by Electrophoresis-convection

The only fractionation method which gave any degree of success was electrophoresis-convection. Since this method has only recently been developed to the extent that it is a generally useful technique for the fractionation of proteins and is not yet as familiar a procedure as electrophoresis, its theory and application will be discussed in more detail.

Electrophoresis-convection is an adaptation and refinement of electrodecantation, a method first used for the partial separation of

amylose and amylopectin by Samec and Haerdtl (1920). It also resembles a method devised by Clusius and Dickel (1938) for the separation of gas mixtures.[1] In electrodecantation, the colloid mixture to be separated is placed in a large central compartment separated from electrode compartments at either end by semipermeable membranes. When a direct current is applied, the mobile component accumulates near one end of the center compartment and, because of the density gradient thereby created, tends to settle to the bottom. In the Clusius column, the gas mixture is placed in a long narrow vertical channel, one wall of which is heated, the opposite one cooled. Differences in thermal diffusion rates cause convection currents to carry the heavier gas to the bottom, the lighter to the top of the channel.

Kirkwood (1941) first suggested that protein mixtures might be separated in somewhat the same manner, proposing however, that density gradients across the channel be established by the application of a horizontal electric field through channel walls of semipermeable membranes, rather than by a temperature differential.

The first apparatus designed to perform fractionations in this manner was developed by Nielsen and Kirkwood (1946) and later improved by Cann, Kirkwood, Brown and Plescia (1949). A more recent modification, designed by Raymond (1952)[2], was used in the present study.

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[1] It is interesting to note that in their reports on the development of electrophoresis-convection, only the Clusius column was mentioned by Kirkwood (1941, 1946) and co-workers.

[2] Obtained from the E-C Apparatus Company, New York.

The electrophoresis-convection cell in which fractionation takes place consists of a narrow vertical channel, formed by semipermeable membranes, with reservoirs at the top and bottom. In operation, the reservoirs and channel are filled with the dialyzed protein solution and immersed in the buffer solution between two large electrodes. On application of a direct current horizontally across the channel, the mobile proteins migrate toward one of the poles and start to accumulate at the membrane barrier. The density gradient thus established induces a convection current which deposits the mobile proteins in the bottom reservoir. The concentration of any immobile component which might be present remains approximately constant in both reservoirs. In theory, the solution in the top reservoir should be almost completely cleared of the mobile components. In practice this is seldom achieved in a single step because of the very long time required. It is usually advantageous to perform the fractionation in two or more steps, using the top fraction in each case for the starting material for the succeeding step.

This fractionation procedure has certain advantages over other methods. Since it is a purely physical method, losses due to denaturation by excessive salt or solvent concentration are not encountered. Mechanical losses are small. Contamination with foreign metals, precipitants, or solvents is not a problem since the solution comes in contact only with innocuous buffer. In some salt or solvent fractionations, the fractions which do not precipitate at reasonable concentrations are either lost or, at best, are difficult to recover. While this is a matter of little concern in many cases, it is important in

those, like the present, in which several components are suspected of having enzyme activity. On the other hand, it should be pointed out that in some cases loss of material due to adsorption on the membranes may occur.

The theory of transport by electrophoresis-convection in a one-component system, or in a two-component system in which one component is immobile, has been thoroughly worked out in mathematical form by Kirkwood *et al.* (1950, 1951). Although a complete presentation of this treatment is beyond the scope of this thesis, it is perhaps desirable to present the final equations in order to point out the manner in which certain experimentally controllable variables affect transport.

In this situation (one mobile component), the useful quantity to be calculated is the time, t, required to transport a fraction, $1-y$, of the mobile protein from the top reservoir to the bottom. This time may be predicted by means of the expression:

$$t = \theta\, I(y)$$

in which θ, called the characteristic time, is the time in hours required to reduce the concentration of mobile protein in the top compartment to 0.193 of the original concentration, and $I(y)$ is an integral[1] relating θ to the time required for the transport of any other fraction.

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[1] $I(y) = (5/3)^{5/4}\int_0^{1-y}\left[(1-y)^{-3/5} - (1+y)^{-3/5}\right]^{5/4} dy$. Kirkwood *et al.* (1950) solved this expression for several values of y between 1 and 0. When $y = 0.193$, the value of $I(y)$ is 1.00. Consequently, at this point, $\theta = t$.

The characteristic time is calculated by means of the equation:

$$\theta = \frac{2(\alpha \rho\, g)^{1/4}}{(4\eta)^{1/4}} \times \frac{D^{3/4} C^{1/4} V}{b l^{5/4} \mu^2 E^2}$$

in which $\alpha\rho$ is the density increment produced by one gram of protein per 100 ml. of solution, g is the acceleration of gravity, and η is the viscosity of the solvent. In the second term, D is the diffusion constant of the mobile protein, C is its initial concentration in grams per 100 ml., and μ is its electrophoretic mobility. V is the volume (ml.) of solution in the top reservoir, b and l are the width and height (cm.), respectively, of the channel, and E is the electric field strength.

The above equation shows that the time required to obtain a given degree of transport can be varied over a wide range by controlling certain variables. For example, since the characteristic time is directly proportional to the volume, it may be advantageous to concentrate the solution before subjecting it to electrophoresis-convection. The increase in concentration tends to increase θ, but only in proportion to its fourth root.

The dimensions of the channel are of some importance, but there are practical limitations here, and they cannot be varied easily.

The two most important variables affecting the characteristic time are the electric field strength and the electrophoretic mobility, since both enter as the inverse square. Thus, an increase in one of these values by a factor of two should theoretically decrease the time necessary to effect a given degree of transport by a factor of 0.25. An increase in mobility may be attained by a change in the pH or ionic

strength of the buffer solvent. The magnitude of the field strength is limited by heat effects, but by providing adequate circulation and cooling, it can be varied over a considerable range.

The theoretical treatment of transport by electrophoresis-convection was derived for certain ideal conditions which are not realized in practice. In testing the theory under practical conditions, Brown et al. (1951) found it was necessary to employ an empirically derived correction for the field strength in order to make the experimental results coincide satisfactorily with predicted results. It appears to the author that the use of the empirical correction factor is valid; its necessity is acceptably explained on the basis of experimental deviation from the ideal conditions on which the mathematical treatment was based.

The theory has also been extended by Brown et al. (1952) to the fractionation of two-component systems in which both components are mobile. The important quantity in this case is the separation factor, f_2, the ratio of the weight fraction of the slow component (2) to the weight fraction of the fast component (1) left in the top reservoir at the time of sampling. This may be calculated from theory by means of the expression:

$$f_2 = \left[1+X_2^0 \, (f_2-1) \right]^{\beta} \Big/ \gamma^{\beta}$$

in which X_2^0 is the initial weight fraction of component 2, γ is the fraction of total protein remaining in the top reservoir, and $\beta = 1-\mu_2/\mu_1$ (μ_2 and μ_1 are the electrophoretic mobilities of components 2 and 1, respectively).

It can be seen from this equation that under certain conditions the separation factor depends to a very large extent on the ratio of the

electrophoretic mobilities of the two components. Solution of the equation by Brown et al. (1952) for several different assigned values of $\underline{X}_2^o$, γ, β showed that in most circumstances the ratio μ_2/μ_1 is the most important factor in determining the separation factor. The best separations can be attained when this ratio (hence μ_2) is as small as possible. In other words, it is advantageous to work as near the isoelectric point of the slower component as practicable. These investigators, testing the theory under practical conditions, found reasonably good agreement between theoretical and experimental values of the separation factors. In this case no correction factors were necessary.

Although the theory of electrophoresis-convection is general and applies to multicomponent as well as to simple systems, it is not practical to attempt the necessary calculations for the exact planning of the fractionation of multicomponent systems. An empirical approach, guided qualitatively by the theory, is more practical.

The construction and operation of the apparatus used in the fractionation of M. verrucaria culture filtrate concentrates can best be described with the aid of Figure 2, which shows the assembled cell and cell-frame. The cell proper is formed from a suitable length of cellulose tubing. With the Raymond apparatus, either a single or a double channel may be used. For the single channel, one end of the tubing, softened in the buffer to be used, is closed tightly by tying a knot. The tubing, still flattened, is then placed vertically over the slot in one of the two face-plates making up the cell-frame. The face-plates are so constructed that when the second one is placed over the tubing and tightened into place by means of cap screws,[1] the center

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[1] These screws do not extend all the way through the cell frame.

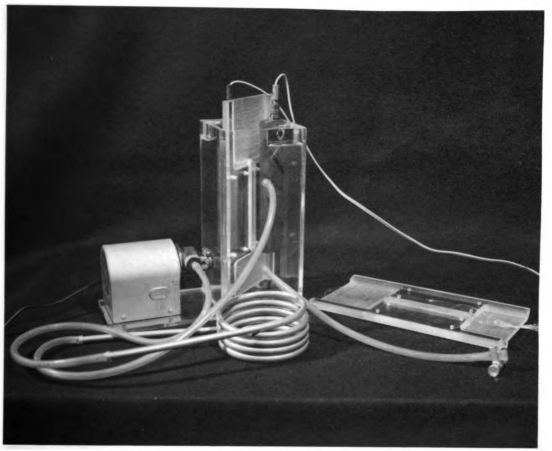

Figure 2. Electrophoresis-convection apparatus. One face-plate is shown in place between the graphite electrodes in the buffer compartment. A length of cellulose tubing has been placed over the center slot in the second face-plate.

section of the cell, partially exposed to the external buffer by opposing slots in the face-plates, will form a flat vertical channel. The upper and lower sections of the tubing are left free to expand and receive the bulk of the solution.

To use two channels simultaneously, a double length of tubing is folded in the middle to form a U-tube and placed in the cell-frame with the doubled end toward the bottom. This provides two channels and two top reservoirs with a single bottom reservoir. Double channels supposedly shorten the time required for a given degree of separation by half.

Besides providing for the use of double channels, the Raymond cell has the further advantage of volume adaptability. By adjusting the length of the tubing above and below the compressing plates and by choosing the appropriate diameter tubing (between 2.5 and 11 cm. flat width), any volume of solution between 20 and 200 ml. may be accommodated.

After assembly, the cell is filled with dialyzed protein solution and any entrapped air bubbles are removed from the bottom reservoir and channel. The assembly is then placed in the buffer compartment between the two large flat electrodes (Figure 2). The cell frame fits snugly enough to minimize current leakage around the sides of the cell, but it does stand on short pegs to allow free circulation of the buffer. Direct current, up to 35 volts or 2 amperes output, is supplied to the graphite electrodes by means of a selenium rectifier power pack. The heat evolved by the passage of the current is dissipated by means of an all-rubber circulating pump and a cooling coil of stainless steel (see Figure 2).

In the first few experiments of this investigation, the cooling coil was immersed in a mixture of salt and ice or in ethanol cooled with solid carbon dioxide. In later experiments, it was found more convenient to use the thermostat tank of the electrophoresis apparatus. In either case, the temperature of the circulating buffer was maintained between 4 and 6° C.

As in electrophoresis, it is important that the protein solution be in ionic equilibrium with the outside buffer. To accomplish this, the solutions were dialyzed in collodion sacs in the manner described in the section on electrophoresis.

Lack of success in preparing the lengths of collodion tubing required (70 cm. for a double channel cell) necessitated the use of viscose cellulose tubing. It was found that the latter could withstand the action of the enzyme for periods up to 14 hours without leakage, but extreme care in emptying the cells was necessary, since the membrane was markedly weakened and could easily be split with a gentle pull after this length of exposure. Therefore, to prevent loss of material, most runs were held to periods of less than 10 hours.

Three different pH levels were tried: 6.9, 5.9, and 5.0. The pH 6.9 phosphate buffer used for electrophoretic analysis was found most suitable, because the time required for dialysis prior to electrophoretic analysis of the bottom cut was much less with this buffer; the progressive loss of activity with increasing time of dialysis was therefore decreased. The pH 5.9 buffer was also a phosphate buffer, with an ionic strength of 0.145, and the pH 5.0 buffer was an acetate buffer of 0.15 ionic strength. The composition of these buffers is given in Appendix B.

The total protein concentration of the solutions was generally somewhat lower than ideal and varied considerably from one run to another. Higher concentrations could probably have been obtained by lyophillization and resolution, but in most cases the dark color of the bottom cut would have required dilution before the electrophoretic analysis. Then, if this fraction were to be subjected to further electrophoresis-convection, reconcentration would have been necessary. It was considered more practical to use the more dilute solutions.

The potential gradient in the channels was not calculated since there is no accurate way to measure current leakage around and under the cell, and the resistance of the cell membranes is unknown. With the semi-empirical approach necessitated by the complexity of the solutions to be fractionated, it was considered sufficient to record only the current and voltage for each run. These remained fairly constant during the experiment, but when drift was noted it was promptly corrected.

The values of the experimental variables (time, pH, current and voltage) are presented in the tables of data on electrophoresis-convection fractionations.

At first, the top and bottom fractions were analyzed for protein nitrogen and enzyme activity after a single operation with the electrophoresis-convection apparatus. Later, however, several runs were performed before these analyses were undertaken. Usually, in these cases, the bottom fraction of the first run was used as the starting material for the second run and the resulting top fraction was combined with the first top fraction. Further steps consisted of similar recombinations

and runs. These operational details are indicated in the section on results.

At the end of an experiment, small portions were taken from both of the final fractions for the determination of protein nitrogen and enzyme activities. Electrophoretic analyses were usually performed only on the original starting material and on the bottom cut. The composition of the top cut was calculated from these results. In some cases, the top cuts were concentrated by freeze-drying and analyzed electrophoretically. These control data were used to determine the manner in which fractions were combined.

During the course of fractionation it became apparent that the primary objective — the isolation of one or more pure fractions accounting for all the enzyme activity — was not attainable with the amounts of material on hand. Consequently, the secondary objective — the obtaining of several fractions containing the suspected components in widely different ratios — had to be accepted. Fractionations were continued until this objective had been attained.

Although valuable information would have been gained by determining the activity of each fraction against several different β-glycosidic substrates, time did not permit such extensive control of the fractionation procedure. Instead, reliance was placed on a few early experiments which showed that most of the activity against cellulose, two different impure hemicelluloses, and cellulose sodium sulfate, is confined to the three components having the lowest electrophoretic mobilities. These data suggested that determinations of the activity against cellulose sodium sulfate and occasional electrophoretic analyses would afford sufficient control.

Estimation of Enzymatic Activity

Although several proposals for the establishment of a standard unit of cellulase activity have been made in the past, none has become universally accepted. Each group of investigators has used a different cellulosic substrate and different experimental conditions. For example, Karrer et al. (1925) defined a unit as the amount of enzyme which will hydrolyze 200 mg. of fibrous cuprammonium cellulose in 96 hours at 36° C. in 50 ml. of a one percent suspension buffered at pH 5.28 with phosphates. The unit proposed by Grassman et al. (1933) is somewhat larger. It corresponds to the amount of enzyme required to bring about the splitting of 320 mg. of hydrocellulose in 20 ml. of solution in eight hours at 30° C. and pH 4.5 (0.04M acetate buffer).

The difference in substrates and conditions renders a quantitative comparison of results obtained in different laboratories difficult and impractical. Reese et al. (1950) suggested that carboxymethylcellulose of a low degree of substitution be used as the standard substrate. Besides being easily standardized as to degree of substitution and degree of polymerization, carboxymethylcellulose and cellulose sodium sulfate are initially more highly susceptible to hydrolysis by cellulases than are the insoluble cellulose preparations. The latter property is important because it allows the assay period to be greatly shortened. However, it was later found (Reese and Levinson, 1953; Tracey, 1953) that in spite of these advantages the soluble cellulose derivatives are not suitable as standard substrates, the reason being, as previously mentioned, that the relation between the rate of hydrolysis

of the soluble derivatives and the rate of hydrolysis of solid cellulose substrates by different cellulase preparations is not constant.

The point to be emphasized is that, because of the possibility of the existence (in crude cellulase preparations) of two or more cellulolytic enzymes, each with its own spectrum of activities toward different substrates, the establishment of a rigidly defined unit of cellulolytic activity is of doubtful value. It is perhaps better simply to report the loss in weight of the substrate or the amount of end product appearing under stated conditions.

Three different methods have been used to determine the extent of hydrolysis brought about by cellulases. In addition to the more commonly used methods of measuring substrate disappearance or end product accumulation, a viscometric method has been used with the soluble cellulose derivatives. Although Levinson and Reese (1950) stated that the viscometric method is not entirely satisfactory because of the abrupt initial decrease in intrinsic viscosity on adding the cellulase preparation, Tracey (1951) found this to be a distinct advantage in detecting very small amounts of cellulase in plant extracts.

With insoluble substrates such as cellulose and some hemicelluloses, determination of the loss of substrate weight is perhaps more convenient than determination of end products. According to Karrer and Illing (1925) and Walseth (1948), the same result is obtained by both methods when the accumulation of end products is calculated as glucose. This cannot be true in those cases in which cellobiose accumulates to an appreciable extent. With the hemicelluloses, especially those with branched chains and with more than one type of residue, the two methods might be expected to give quite different results because of the possible accumulation of soluble oligosaccharide end products whose reducing power

would be relatively small in comparison to weight. In these cases, estimation of the increase in reducing sugars would give a better estimate of the number of glycosidic bonds hydrolyzed than would the determination of the decrease in weight of the substrate.

For these reasons, since both soluble and insoluble celluloses and hemicelluloses were to be used as substrates, the estimation of end products seemed to be the method of choice for the investigation reported here.

The micro method of Somogyi (1945) was used for the analysis of reducing sugars in the hydrolysates. The use of this method, capable of giving accurate results in the range 0.01 to 0.5 mg. of glucose, allowed assays to be made at very low enzyme concentrations. (Satisfactory results were obtained with as little as two micrograms of protein nitrogen in ten ml. of assay medium, when the most active enzyme preparation acted on cellulose sodium sulfate). This is an important consideration because under these conditions the initial rate of reaction is less apt to be limited by the concentration of the substrate.

The conditions chosen for the enzymatic hydrolysis are compared in Table IV with those used by previous investigators. The conditions employed by Reese et al. (1950) served as a guide in choosing the conditions to be used in this work. Reese found that the optimum temperature for M. verrucaria enzymes acting on carboxymethylcellulose for one hour is near 50° C. However, since longer periods of reaction were to be used in the work reported here, a lower temperature was considered to be more suitable.

TABLE IV

CONDITIONS EMPLOYED BY VARIOUS INVESTIGATORS FOR THE ESTIMATION OF CELLULASE ACTIVITY

Author	pH	Temperature	Buffer type	Buffer conc.	Time	Substrate	Substrate conc.
		°C.		M	hr.		%
Karrer and Illing (1925)	5.28	36	phosphate		96	cuprammonium cellulose	1.0
Grassman et al. (1933)	4.5	30	acetate	0.04	8	hydrocellulose	
Reese et al. (1950)	5.0	50	citrate	0.075	2	carboxymethyl-cellulose	0.75
Walseth (1948)	4.5	47	acetate	0.16	—*	swollen cotton linters	2.0, 3.0
Whitaker (1953)	5.6	35	acetate	0.05	17	swollen and precipitated cellulose	1.0
Author	5.35	40	citrate	0.05	—**	—***	1.0

* Time curves were constructed.

** For routine analyses using cellulose sodium sulfate, the reaction time was two hours. For the hemicelluloses and cellulose the reaction time was 24 hours.

*** For a description of the several substrates used, see the text.

In choosing the initial concentration of substrate to be employed in the case of cellulose sodium sulfate, an important factor is the viscosity of the solution. It was found that a one percent solution flows readily enough to permit accurate sampling by pipette. Solutions of higher concentrations flow at a noticeably slower rate and the problem of accurate sampling is encountered. This problem becomes especially important when many samples of hydrolysates are to be taken at short intervals. All other substrates except phloridzin were used in one percent concentration.

Reese et al. (1950) found that the optimum pH for M. verrucaria culture filtrates acting on carboxymethylcellulose is about 5.1. This is the pH level originally chosen for this work. The stock citrate buffer solution (pH 5.1, 0.5 M) was incorporated into the assay medium at a 1:10 dilution. It was later found that the resulting solution has a pH of 5.35. Reese et al. prepared their assay solutions in the same manner, the final dilution of the pH 5.0, 0.5 M citrate buffer being 1:6.67. That the change in reaction was due to dilution and not the substrate was demonstrated by diluting the stock buffer 6.67 and ten times with distilled water. The pH's of these dilutions were 5.3 and 5.35, respectively. Thus there is reason to believe that Reese et al. (1950) were working at a somewhat higher pH than reported. They did not indicate that the reaction of the final solutions had been checked.

Substrates. The cellulose sodium sulfate used in this work has a degree of substitution of 0.4, according to information supplied by the manufacturer.[1] The water content of this material, determined by drying

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[1] We wish to thank Dr. C. J. Malm of the Eastman Kodak Company for his cooperation in supplying this substrate (cellulose sodium sulfate sample No. CAD-1045).

in the vacuum oven at 65° C., was found on one occasion to be 7.68 percent and on another occasion 7.60 percent. This moisture content was taken into account when samples were weighed. No other analytical data were obtained. Levinson, Mandels, and Reese (1951) routinely dialyzed their cellulose sulfate solutions, finding that some of the sulfate ion could be removed in this way. Since it was found in this work that dialysis had no effect on the results, this treatment was omitted.

Salicin (purified, Pfanstiehl) is orthohydroxymethylphenyl-β-D-glucoside. This material was dried in vacuo at 65° C. before use.

Arbutin (hydroquinone β-D-glucoside), amygdalin (D-mandelonitrile β-D-gentiobioside), and phloridzin (phloretin β-D-glucoside) were used as supplied, without drying or purification, since they were employed in only one experiment to determine qualitatively their susceptibility to attack by M. verrucaria enzymes.

Wheat straw polysaccharide fractions were prepared essentially by the acid chlorite delignification and alkaline extraction methods of Wise, Murphy and D'Addieco (1946). These methods were originally developed for application to wood and wood pulps but have subsequently been applied to cereal straws and grasses with satisfactory results (Ely and Moore, 1954). Three holocellulose samples, presumably with different lignin contents but otherwise the same, were prepared. From two other holocellulose preparations, two different mixed hemicelluloses and wheat straw cellulose were prepared.

A clean, dry sample of wheat straw was ground to pass a 60-mesh screen in a Wiley mill, then extracted for 16 hours with an ethanol-benzene mixture (1:2 v/v) in a Soxhlet apparatus. The air-dried

material was then submitted to the chlorite delignification procedure of Wise et al. (1946). A 10-gram sample was suspended in 320 ml. of water in an Erlenmeyer flask loosely covered with an inverted flask or beaker and heated to 70° C. in a water bath. The suspension was acidified with twenty drops of glacial acetic acid, 3 g. of sodium chlorite was added, and the mixture was heated with occasional gentle agitation for one hour.

One sample was given a single treatment as described above. A second sample was delignified in two successive steps and a third, in three steps. The second and third steps were carried out simply by adding the same amounts of acetic acid and sodium chlorite as in the first step and continuing the reaction period for another hour.

At the end of the chlorite treatments, the reaction mixture was cooled in an ice bath and filtered on a canvas filter in a large Buchner funnel. The residual material was washed repeatedly with small portions of ice-cold distilled water until the odor of chlorine dioxide was no longer detectable, and finally with generous portions of acetone. The holocellulose was then air dried.

For convenience, these three preparations are designated holocellulose-1, holocellulose-2, and holocellulose-3, the numerals signifying the number of delignification steps.

There was a distinct gradation in the color of these preparations, holocellulose-1 possessing a moderate tan color, whereas holocellulose-3 was only a shade removed from pure white.

The yields of air-dried holocelluloses-1, -2, and -3 from 10 g. of extracted wheat straw were 8.5, 8.7, and 8.4 g., respectively. When these values are corrected for moisture, ash, and protein (nitrogen x 6.25)

content they become 7.5, 7.4 and 7.4 g. of holocellulose from 8.4 g. of extracted wheat straw.

Moisture was determined by drying in vacuo at 65° C. for four hours. A second drying period under the same conditions did not further decrease the weight of the sample. The ash content was determined by first charring the weighed sample in a Coors crucible over a low Bunsen flame, then heating in a muffle furnace to 500° C. for three hours.

The nitrogen content of the holocelluloses was estimated on duplicate samples (100-200 mg.) by the semimicro Kjeldahl method to be described later.

All these analytical data are recorded in Table V. They were used to determine the weight of sample to be taken to obtain a specified amount of holocellulose for the estimation of enzyme activity.

TABLE V

MOISTURE, ASH, AND NITROGEN IN WHEAT STRAW FRACTIONS

(All values are expressed as percentages)

Fraction	Moisture	Ash	Nitrogen
Extracted wheat straw	8.04	5.84	0.30
Holocellulose-1	7.22	3.75	0.20
Holocellulose-2	7.05	4.11	0.16
Holocellulose-3	6.72	4.03	0.11
Hemicellulose X	4.70	6.22	0.05
Hemicellulose X	6.97	4.05	0.04
Hemicellulose Y	7.54	7.10	0.04
Hemicellulose Y	6.91	4.60	0.05
Cellulose	4.05	1.23	0.03

The lignin content of these holocelluloses was not determined. Fly and Moore (1954), examining the application of the same technique to cereal straws, hays, and grasses, found that a single treatment reduced the lignin content of wheat straw from 13.0 to about 2.8 percent, a second step reduced it to about 2.0 percent, and a third to about 1.8 percent. Three more successive acid chlorite treatments reduced the lignin only to about 1.1 percent and at the same time removed a considerable amount of polysaccharide material. The first three treatments resulted in very little loss of holocellulose components.

Two additional batches of holocellulose, obtained from 40 g. of extracted wheat straw by three acid chlorite treatments, were used for the preparation of two hemicellulose fractions and wheat straw cellulose.

Hemicellulose X was extracted from 30 g. of holocellulose by 750 ml. of 1.5 percent potassium hydroxide for 48 hours at room temperature. The extraction was carried out in a closed vessel with continuous stirring. At the end of the extraction period the mixture was filtered with suction and the residue washed on the filter with 250 ml. of water in several small portions. The filtrate and washings were then acidified to pH 5 with glacial acetic acid and, after cooling, poured into four volumes of absolute ethanol. The voluminous white precipitate was allowed to settle overnight. Most of the supernatant solution could then be siphoned off and the precipitate recovered by centrifugation. The precipitate was washed twice in the centrifuge bottle with 200-ml. portions of ethanol, then twice with 100-ml. portions of ethyl ether. The air-dried material was almost pure white and was easily ground into a fine powder.

The ash content of the two hemicellulose X preparations was very high (21.2 percent in the first preparation and 18.5 percent in the second.) It was found that much of this foreign inorganic material could be removed by dissolving the preparation in about 200 ml. of 0.2 percent potassium hydroxide, then dialyzing the solution, first against three changes (one liter each) of distilled water for a total of about 24 hours, then against two changes (two liters each) of one molar acetic acid for another 24 hour period. The polysaccharides were then precipitated with ethanol, centrifuged, washed and dried as previously described. These preparations were very nearly white.

Moisture and nitrogen were determined as previously described for the holocelluloses. The ash determination was modified according to the procedure recommended by Wise et al. (1946). After the weighed sample was charred and cooled, two or three drops of concentrated sulfuric acid were added and gentle heating was resumed until all fuming ceased. The crucible was then heated over a Fisher burner to a dull red glow, cooled in a desiccator and weighed. According to Wise et al. (1946), most of the inorganic material in the hemicellulose fractions is potassium acetate, although some of the potassium is probably present as the salt of uronic acid residues. The weighed ash is mostly potassium sulfate, so a correction factor of 1.13 was applied.

The results of moisture, ash, and nitrogen determinations are recorded in Table V.

The yields of hemicellulose X, corrected for moisture, ash, and protein (nitrogen x 6.25), from the two 30-g. batches of holocellulose were 5.9 and 6.1 g.

Hemicellulose Y was obtained from the residue left by the first alkaline extraction by further extraction with 500 ml. of 24 percent potassium hydroxide. The extraction was made at room temperature in a tightly sealed flask with continuous stirring for four hours. The mixture was filtered and washed on the filter with 300 ml. of water. The combined filtrate and washings were acidified and the hemicelluloses precipitated with four volumes of absolute ethanol. The precipitate was centrifuged, washed and dried as previously described.

The inorganic fraction of these preparations was initially rather large so the purification procedure used with the hemicellulose X fractions was again applied. In this case it was necessary to use 10 percent potassium hydroxide to dissolve the material. The purified, air dry preparations were pure white.

Moisture, ash, and nitrogen were determined as before, and the results recorded in Table V.

The yields of hemicellulose Y, corrected for moisture, ash, and protein were 3.4 and 4.1 g.

Wheat straw cellulose was prepared from the residue of the last alkaline extractions. This residue was washed further on the filter with copious amounts of water, then with 300 ml. of molar acetic acid, and finally with 500 ml. of water. Residual water was removed by washing with several small portions of dry acetone. Only one sample of this residual cellulose was kept, since the first contained small hard lumps of material which were very difficult to break up. The second sample was pure white, very light and fluffy.

Moisture, ash, and nitrogen were determined as before. The results are recorded in Table V. The corrected yield of wheat straw cellulose was 11.2 g.

It should be emphasized that no claim concerning the purity of the wheat straw polysaccharides is made. On the contrary, it is almost certain that both hemicellulose preparations contained more than one chemical species. Furthermore, it cannot even be stated with certainty that the different extraction procedures employed resulted in hemicellulose fractions which are chemically different from each other. According to Norman (1954):

> It is likely that all hemicellulose preparations so far obtained have been mixtures, but there is, in fact, reason for believing that the methods of separation and fractionation that have been rather generally used may have given a spuriously complex picture. ..... The major separation ordinarily employed in one form or another distinguishes only between the less-soluble and more-soluble fractions.

This quotation appears to be especially relevant to the relationship between the two hemicellulose preparations used in this investigation. Hemicellulose X was completely dispersed in concentrations up to two percent. The dispersions were cloudy to the point of opacity but were nevertheless stable. Hemicellulose Y was nearly completely insoluble in the citrate buffers used. Nevertheless, it will be shown later that there was no difference in the nature of enzymatic end products of these two preparations, and little difference in the ratios of different end products in the two hydrolysates. The same is true of the acid hydrolysates.

Wise, Murphy and D'Addieco (1946) termed the cellulose fraction "alpha-cellulose", but only for convenience. They stated that this

fraction invariably contained a small amount of furfural-yielding material, possibly xylose or uronic acid residues. This finding was confirmed in the present investigation by the demonstration of a small amount of xylose in the enzymatic hydrolysates of the wheat straw cellulose. No uronic acid was found.

A word about the nomenclature adopted for the hemicellulose fractions may be appropriate. Wise et al. (1946) used the designations hemicellulose A and B. It seems to the author that this nomenclature implies that these fractions are the same as the classical hemicelluloses A and B first described by O'Dwyer (1926). Norman (1954) has stated that the nomenclature instituted by O'Dwyer and subsequently used quite generally in reality indicates the particular method of isolation and does not uniquely define the composition of the preparations. Inasmuch as the methods used in this investigation were quite different from those of O'Dwyer, the author prefers to use a different nomenclature.

Assay procedure. Cellulose sodium sulfate was utilized more than any other substrate for following the course of fractionation procedures. The assay method described in the following paragraphs was designed for this substrate, but was easily adapted to any other substrate by the use of appropriate modifications.

Eight ml. of 1.25 percent cellulose sodium sulfate[1] was mixed with

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[1] In the author's experience, this was best prepared by adding hot distilled water to the powder. The large gummy masses which resulted were completely dispersed in 20 to 30 minutes of continuous mechanical stirring. The clear solution was cooled, diluted to volume and stored in the refrigerator. A solution was never kept more than one week.

one ml. of 0.5 M, pH 5.1, citrate buffer[1] in a 20 x 150 mm. Pyrex test tube. Since aseptic techniques were not used it was found necessary to include an antiseptic in the assay medium. 'Merthiolate'[2] was found to be entirely satisfactory when included in the buffer at a concentration of one mg./ml. The stoppered tube was placed in a water bath at 40 ± 0.2° C. for about 15 minutes prior to the addition of one ml. of appropriately diluted enzyme solution. A blank solution was prepared in the same way except that water was substituted for the enzyme solution. Duplicate aliquots of the hydrolysate were removed at exactly timed intervals and delivered directly into five ml. of the sugar reagent (Somogyi, 1945). Because of its alkaline reaction and high copper concentration, this reagent effectively stopped the action of the enzyme. The reaction time for most determinations of cellulase activity was two hours. When progress curves were to be constructed the first samples were taken at 15 or 20 minutes, and subsequent samples at increasingly longer intervals up to 24 hours in most cases.

The same procedure was used with all the monomeric β-glucoside substrates with the exception of phloridzin. This substrate is soluble at room temperature only to the extent of about 0.1 percent, so 10 mg. was dissolved in eight ml. of water and one ml. of buffer by heating the closed tube to about 60° C. The tube was cooled in a water bath to 40° C. before the enzyme was added.

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[1] The preparation of this buffer is described in the appendix.

[2] 'Merthiolate' is the trade-mark of Eli Lilly and Company for the sodium ethylmercurithiosalicylate (Thimerosal) made by it.

Certain modifications of technique were necessary when the insoluble substrates were used. Hemicellulose X, although initially soluble, was included in this group because it was found that a precipitate formed after the enzyme had acted on it for a while. A uniform suspension of the insoluble substrates was essential, so the reaction was carried out in plain Warburg flasks (without side arms or center wells) attached to the manometers of the Warburg apparatus. The flasks were shaken at the rate of about 80 four-centimeter excursions per minute. For activity determinations the time of incubation was 24 hours. When time curves were to be constructed, aliquots of the hydrolysate were taken at appropriate intervals over a 24-hour period.

Samples of the hydrolysate were obtained by removing the flask from the shaker and quickly withdrawing a volume 25-50 percent greater than the volume to be analyzed. The suspension was then centrifuged briefly and the supernatant was used for the analysis.

Early in this investigation it was found that even at the low concentrations of enzyme employed it was essential, in order that all activity determinations be reasonably comparable, to use approximately the same amount of enzyme in each assay. This was felt to be particularly important in the estimation of activity against cellulose sulfate, since this substrate was used to follow the fractionations. To meet this requirement it was necessary to define a relatively narrow range of enzyme concentrations in which to make all activity determinations. The range was defined as the amount of enzyme which would result in the production of 0.4 to 1.5 mg. of glucose (or its reduction equivalent) per milliliter of cellulose sodium sulfate medium in the two-hour

incubation period. At first, this objective was attained either by using two different dilutions of the enzyme solution or by using two different volumes of the same dilution. Usually one of the two assays was within the prescribed range. Later, when with added experience the proper dilution and amount of enzyme could be more accurately predicted, only one enzyme level was used. The determination was repeated if the first level was not correctly chosen.

Reducing sugars in the enzyme hydrolysate were determined by the copper reduction method of Somogyi (1945). In this method cupric copper is reduced to cuprous oxide by the reducing sugar on heating the mixture in a boiling water bath. The amount of copper reduced is directly (but not stoichiometrically) related to the amount and kind of reducing sugar present. Because the method is empirical, the time of heating and the concentration and composition of the reagent must be carefully controlled. The amount of cuprous oxide formed may be estimated iodometrically or colorimetrically. In this work, the iodometric method was chosen because one of the substrates, hemicellulose X, did not form a clear solution.

Reagents

Somogyi's (1945) alkaline copper sugar reagent, prepared to contain 0.005 N potassium iodate. This concentration of iodate is sufficient for the analysis of 0.01 to 0.5 mg. of glucose or its equivalent. For the details of preparation, see Appendix B.

Sodium thiosulfate, 0.005 N, prepared by dilution from a standardized, approximately 0.1 N stock solution. The inclusion of about one ml. of 2 N sodium hydroxide in 500 ml. of diluted solution helps to protect the sodium thiosulfate from atmospheric oxygen.

Potassium iodide, 2.5 percent. This solution keeps indefinitely if made slightly alkaline by the addition of a knife-tip of potassium carbonate per 100 ml. of solution.

Sulfuric acid, about 2 N.

Soluble starch, one percent.

Duplicate volumes of the test solution and reagent blank were mixed with accurately measured five-ml. portions of the sugar reagent in 25 x 200 mm. Pyrex test-tubes. The total volume was made up to 10 ml. with distilled water and the contents mixed. The tubes were covered with glass bulbs and heated in a boiling water bath for exactly 20 minutes. After cooling for about three minutes in running water, 0.5 ml. of 2.5 percent potassium iodide was carefully layered on top of the solution, then about 1.5 ml. of 2 N sulfuric acid was added rapidly with simultaneous agitation. The excess iodine was titrated with the 0.005 N sodium thiosulfate. A few drops of starch indicator were added near the end of the titration.

A procedural detail which was not explicitly mentioned in the original paper was found to be of some importance in obtaining precise and accurate results. It was found that the titrations must be done within about 15 minutes after the heating period. If the tubes were allowed to stand longer, the thiosulfate titer became noticeably greater, probably because of oxidation of the cuprous oxide. When many determinations were to be made it was found best to start four tubes every 10 minutes. With experience, four titrations can easily be completed in 10 minutes.

In the beginning, the aliquots for the blank determination were taken at the same time as the test aliquots. Later, it was found that

this was unnecessary because none of the substrates were hydrolyzed to a detectable extent in the absence of enzyme in 24 hours. Experiments proved this to be true for the whole pH range studied. With this fact established, it became the practice in later experiments involving many determinations to use a previously determined blank value for each substrate.

Before describing how the substrate blank values were established it is necessary to digress momentarily. Early in the study, a puzzling constant increase in the blank reducing titers was noted. It soon became apparent that this increase could be correlated with only one factor: the age of the Somogyi reagent. Daily titration of the unheated reagent showed that the potassium iodate was not decomposing; five ml. of the reagent always required 5.00 ± 0.05 ml. of the 0.005 N thiosulfate. On the other hand, heated reagent blanks (as opposed to reagent plus substrate blanks) always required less thiosulfate, and the difference increased steadily with the age of the reagent. The blank for a freshly prepared reagent was usually about 0.20 ml. of 0.005 N copper reduced, but after two weeks increased to about 0.40 ml.

Reducing titers, or blank values, were established for each polysaccharide substrate and for salicin by incubating the substrates without enzyme at 40° C. at a concentration of one percent in citrate buffers of pH 4.20, 5.35 and 6.70. Volumes varying in size from 0.5 to 2.0 ml. were removed at specified times from 0.5 to 24 hours. Their reducing titers, expressed as the milliliters of 0.005 N copper reduced per milligram of substrate, were determined by substracting the volume of 0.005 N thiosulfate required from that required by reagent blanks.

The results did not vary in any significant manner either with pH or with time of incubation. The average and the range of results for each substrate are given in Table VI.

TABLE VI

REDUCING TITERS OF SUBSTRATES

Substrate	Ml. of 0.005 N copper per mg. of substrate	
	Average	Range
Cellulose sodium sulfate	0.025	0.021-0.027
Wheat straw cellulose	0.013	0.011-0.017
Hemicellulose X	0.021	0.020-0.024
Hemicellulose Y	0.015	0.010-0.018
Salicin	0.010	0.008-0.011

Enzymatic activities were expressed as specific activities whenever the concentration of protein nitrogen was known. Specific activity was defined as the mg. of reducing substances (as glucose) appearing in the assay medium per milligram of protein nitrogen.[1] Unless otherwise stated, specific activity refers to a two-hour assay period when either cellulose sodium sulfate or salicin was the substrate, and to a 24-hour period when cellulose or the hemicelluloses were used.

When the protein nitrogen concentration of the enzyme solution had not been determined, activities were recorded as a concentration expression, i.e., the mg. of glucose or its reducing equivalent released from

[1]Specific activity was based on protein nitrogen rather than on protein concentration because the percentage of nitrogen in the proteins involved was unknown.

the substrate into 10 ml. of assay medium by one ml. of the original (not the diluted) enzyme solution.

In some cases, when samples were taken at incubation times short enough to permit extrapolation to zero time, the initial rate of reaction, or the mg. of reducing substances (as glucose) appearing per minute per milligram of protein nitrogen, was also calculated.

Somogyi (1945) found that 0.135 mg. of glucose reduced one ml. of 0.005 N copper when the mixture was heated in a boiling water bath for 10 minutes. He found this time to be adequate for glucose, but for some other sugars, including arabinose, a longer time was necessary. A reaction period of 20 minutes was chosen for the work reported here because of the anticipated presence of sugars other than glucose in some of the hydrolysates. Under these conditions one ml. of 0.005 N copper was the equivalent of 0.134 mg. of glucose. Each new batch of reagent was calibrated with three replicates each of three levels of glucose: 0.05, 0.10, and 0.20 mg. If the average reduction equivalent of the three levels did not fall in the range 0.133-0.135 (inclusive), the reagent was adjusted by dilution (with the alkaline-copper reagent without iodate) or by addition of potassium iodate, then recalibrated.

An example may help to make clear the method of calculation of results. A certain enzyme solution had a protein nitrogen concentration of 0.51 mg. per ml. It was diluted 25-fold and one ml. of the dilute solution was used in an assay with cellulose sodium sulfate. The final volume of the assay medium was 10 ml. Duplicate 0.2 ml. portions were taken for sugar determination after two hours of incubation at 40° C. The volume of 0.005 N sodium thiosulfate required for the reagent plus

substrate blank was 4.65 ml. and for the assay, 3.11 ml. The difference, 1.54 ml., multiplied by the glucose reduction equivalent (0.134), gave the amount of reducing substances (as glucose) in the aliquot of hydrolysate: 0.206. Therefore, in 10 ml. of the hydrolysate there were 50 x 0.206, or 10.3 mg. of glucose or its equivalent. Since the original enzyme solution had been diluted 25 times, it contained sufficient activity in one ml. to release 10.3 x 25, or 257 mg. of glucose from the substrate under the conditions of the test. The specific activity in this case was 257/0.51, or 504 mg. of glucose per milligram of protein nitrogen.

Electrophoretic Analysis

Electrophoretic analyses were performed with a Klett apparatus which follows closely the design of Longsworth (1939, 1946). The light source was a type H-4 mercury vapor lamp. A Wratten No. 22 light filter was found to be satisfactory. Visual observation of the progress of electrophoresis was made with the aid of a diagonal straight edge, cylindrical lens, and a ground glass screen. Patterns were recorded photographically by means of a schlieren scanning device.

Three different analytical cells of rectangular cross section and 11 ml. capacity were used throughout this study. In order to calculate mobilities, it is necessary to know the cross-sectional area of the center sections of the cell, and the magnification of the optical system. The former constant was calculated, for both limbs of each cell, from the volume, determined by calibration with clean, dry mercury, and the length, measured with an accurate caliper. All measurements were carried out in

duplicate and the average used. The cross-sectional areas of the three cells were: cell No. 4, 0.768 for both limbs; cell No. 5, 0.748 for the left limb, 0.756 for the right limb; and cell No. 6, 0.756 cm^2 for both limbs.

The magnification of the optical system was determined by mounting a transparent ruler vertically in the filled thermostat tank in the place usually occupied by the cell. After adjusting the components of the optical system to obtain a sharp image on the screen, a photograph of the image was taken. Calculated as length of image/length of object, the vertical magnification was found to be 1.05 at two different times.

Samples to be analyzed were in most cases dialyzed against two changes of phosphate buffer of pH 6.9 and ionic strength 0.146. The details of preparation of this and other buffers are given in Appendix B. This phosphate buffer was the only buffer used for electrophoretic analysis. Consequently, subsequent statements concerning the number of components refer only to these conditions and are to be regarded as minimum.

The duration of dialysis was not less than 12 hours for each period. The sample was placed in a collodion sac of suitable size and the sac was tied securely on the expanded end of a glass tube extending through a rubber stopper. This assembly was then placed in a one- or two-liter Erlenmeyer flask. Stirring of the outside buffer was provided by a magnetic stirrer; the agitation was vigorous enough to cause moderate swaying of the collodion sac and mixing of its contents. All dialyses were performed in a cold room at a temperature of about 4° C.

For samples up to 50 ml., one liter of buffer was used for the first period, two liters for the second.

In several of the early experiments, the conductivities of the buffer and sample were measured at the end of 12 hours of dialysis, and the dialysis allowed to continue three more hours, after which conductivities were again determined. Lack of significant change in these values showed that 12 hours of dialysis was sufficient in most cases. However, the second period of dialysis against fresh buffer was necessary in many instances because the conductivity of the outside buffer was significantly different from the original.

This procedure was changed when circumstances demanded, for example, when solutions of acetone or ammonium sulfate precipitates containing relatively large amounts of salts were used. In these cases, a preliminary dialysis against about 10 volumes of distilled water for 10 to 12 hours removed much of the salt. To avoid excessive dilution of these samples due to their high initial osmotic pressure, the outlet of the glass tube was closed tightly with a section of rubber tubing and a screw clamp. If the internal pressure appeared to be excessive at any time, it was partially relieved by loosening the screw clamp. After this preliminary treatment, the samples were transferred to two liters of phosphate buffer for the first dialysis, and two liters for the second.

Solutions from the electrophoresis-convection experiments, when the buffer was the same as that used for electrophoretic analysis, were dialyzed only once against two liters of buffer. In two instances, the samples were not dialyzed at all prior to electrophoresis. Although the conductivities of these solutions were lower than those of dialyzed solutions, the only adverse effect noted was a large boundary anomaly.

The mobilities were satisfactory, symmetry was unaffected, and no new anomalies appeared.

Collodion sacs were used for dialysis, since it was found early in the investigation that commercial viscose cellulose tubing was unsuitable. Enzyme solutions of high activity weaken cellulose tubing to the extent that it is apt to break when removed from the buffer. Moreover, loss of activity, possibly due to adsorption on the membrane or to actual leakage, was greater with viscose tubing than with collodion. The sacs were prepared on the inner surface of test tubes of appropriate size, or 200 ml. centrifuge bottles, and were stored in distilled water at least 24 hours before use to remove residual alcohol.

The specific conductances of both buffer and sample were determined after dialysis. A conductivity cell of the Shedlovsky type (Longsworth, Shedlovsky, and MacInnes, 1939) was used in conjunction with a Leeds and Northrup Wheatstone bridge. The cell was suspended in the constant-temperature bath of the electrophoresis apparatus and allowed to attain temperature equilibrium before the final measurement was taken. This procedure was always followed to make sure that the temperature of the conductivity determination and the electrophoretic analysis was the same.

The cell constant was determined, at 2° C., with a 0.1000 N potassium chloride solution prepared from the dried C.P. salt in double-distilled water. The water was freed from carbon dioxide by heating to about 80° C. and drawing carbon dioxide-free air through at a rapid rate for about 30 minutes. The resistance of water prepared in this manner could not be accurately determined with the apparatus available. The resistance of the cell filled with this water was greater than

1,000,000 ohms; when filled with ordinary distilled water it was about 100,000 ohms.

The temperature of most electrophoretic analyses was 2.0 ± 0.1° C. Certain exceptions are noted in the mobility tables.

Potential gradients varied from 6.26 to 6.93 volts/cm., depending on the current and the conductivities of buffer and sample. In the majority of cases, a potential gradient of close to 6.5 volts/cm. was used. The current, about 20 milliamperes, remained uniform, rarely varying more than 0.1 milliampere throughout the course of a run. Current measurements were performed at intervals, using a Leeds and Northrup potentiometer with a sensitive galvonometer. At the start of each experiment, the potentiometer circuit was calibrated against a standard Weston cell.

The duration of current application for each analysis was the result of a compromise between the attainment of good resolution and the maintenance of adequate height of each maximum. The protein concentration of some samples was necessarily quite low. In these cases, the peaks representing minor components tended to decrease in height and approach the base line with increasing time. This circumstance made it necessary to stop some runs short of the best resolution of components.

Base lines and initial boundaries for both the ascending and descending sides were photographed before the current was started and the final patterns were taken on the same 9 x 12 cm. Kodak Panchromatic Process or M plates by means of the schlieren scanning device. The exposed plates were developed with D-19 developer in a semi-automatic plate processor in complete darkness.

The patterns were then projected and carefully traced on graph paper at an enlargement factor of 2.5 times by means of an Omega D-II photographic enlarger. Measurements for mobility and relative concentration determinations were taken from these tracings.

Mobilities were calculated from measurements on the descending patterns by means of the following equation:

$$u = \frac{dqk_s}{mit}$$

where u is the mobility in $cm.^2$ $volt^{-1}$ $second^{-1}$, d is the distance in centimeters on the enlarged tracing between the center of the initial boundary and the particular maximum under consideration, q is the cross-section of the descending side of the cell, k_s the specific conductance of the system, m the combined magnification factors of the electrophoresis apparatus and the photographic enlarger, i is the current in amperes, and t is the time of electrolysis in seconds (Longsworth and MacInnes, 1940).

The relative concentrations of the individual components were determined by comparing the area under each peak with the total area of the complete pattern minus boundary anomalies. Areas were delineated by the minimum ordinate method (Longsworth, 1942). Area measurements were taken from the enlarged tracings with the aid of a planimeter. The average of three separate measurements for each component was used. The deviation of individual measurements from the average seldom exceeded ± 2 percent for the major components. The results from ascending and descending patterns were averaged.

Identification of End Products

Paper chromatography was the only method used to identify the end products of enzymatic hydrolysis. Identification of sugars by this means alone is admittedly not positive, but it can be quite satisfactory if the sugars which are likely to be encountered are definitely known and if the experiments are properly controlled by the use of known sugars on each chromatogram.

The hydrolysates of cellulose sulfate and of hemicellulose X contained some soluble polysaccharide residues even after exhaustive treatment with the enzyme. Although the possibility was not investigated, there also may have been some soluble polysaccharide residues in the centrifuged enzymatic hydrolysates of hemicellulose Y. Levinson et al. (1951) found that the polysaccharide residues of cellulose sulfate interfered with the migration of the sugars during chromatography, causing undesirable trailing or comet formation. These workers removed the larger molecules by precipitation with 50 percent ethanol and then evaporating the filtrate to dryness either by lyophilization or on the steam bath. The solids were then reconstituted to one-tenth of the original volume. They stated that none of these procedures significantly affected the amount of glucose or cellobiose present.

In this investigation, all hydrolysates which were to be used for the chromatographic detection of sugars were centrifuged, when necessary, to remove the insoluble residual substrate. Four volumes of absolute ethanol were then added and the mixture was allowed to stand at 4° C. for at least three days. This long period was found to be necessary to allow for the aggregation and settling of the very finely dispersed

salts. The precipitate was removed by filtration (sintered glass filters) and the filtrate was evaporated to dryness on the steam bath. The residue was extracted with several small volumes of 80 percent alcohol and the remaining solids were taken up in a small amount of water and saved. The alcoholic extract was again evaporated to dryness on the steam bath and the solids dissolved in a small volume of water. Reducing sugar analyses of both of these solutions showed that extraction of the first residue was not quantitative; only 65 to 85 percent of the reducing sugars was extracted.[1] Chromatograms of both sugar solutions later showed, however, that the extraction removed approximately the same percentage of each component.

Chromatograms obtained with these solutions were unsatisfactory because of extensive comet formation. This was probably due to the salts which persisted after the extraction of the first residue with 80 percent alcohol. Most of the remaining salts were removed by treatment with Amberlite ion-exchange resins.

The cation-exchange resin, IR-120, was prepared for use by treatment with five percent hydrochloric acid followed by washing on a filter with water until the filtrate was free of chloride. The anion-exchanger, IRA-400, was regenerated before use by washing with 0.5 percent sodium carbonate and then with water. These resins have been recommended by Phillipps and Pollard (1953).

Deionizing was done by the batch process. One-half gram of the Amberlite IR-120 was shaken for five minutes in a test tube with 2 ml. of the sugar solution. The mixture was filtered and the resin washed

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[1]Extraction of the residues with dry pyridine according to the technique described by Malpress (1949) was not satisfactory.

on the filter with 1 ml. of water. One gram of Amberlite IRA-400 was added to the combined filtrate and washings and the mixture was allowed to stand with frequent shaking until the resin no longer floated to the top with adherent bubbles of carbon dioxide. If no more carbon dioxide was evolved after the addition of a small amount of fresh anion-exchange resin, the mixture was filtered and the resin washed with a small volume of water.

The effectiveness of the deionizing treatment was tested by applying it to a 0.1 percent solution of glucose in 0.1 M sodium chloride. Glucose analyses before and after treatment showed that there was no loss of the sugar and conductivity measurements of the final solution (after boiling briefly to remove the excess carbon dioxide) indicated that the sodium and chloride ions had been almost completely removed.

The deionized sugar solutions were analyzed for total reducing sugars (as glucose). On the basis of the results, small volumes were evaporated to dryness in vacuo and reconstituted to a concentration of approximately 25 micrograms per microliter.

The chromatographic technique described below was a combination of features taken from several methods reported in the literature (Block, Durrum, and Zweig, 1955).

Appropriate volumes of the concentrated sugar solutions were transferred to a sheet of Whatman No. 1 filter paper along a pencilled line 3.5 cm. from one short edge. In most cases the paper measured 10 by 15 inches. The distance between the individual spots was not less than 2 cm. The volume of sample deposited on the paper varied between 2 and 15

microliters,[1] depending on the concentration of reducing sugars.

Known reference sugars (40 micrograms) were used on every chromatogram. For the hemicellulose hydrolysates, D-glucose, D-xylose, and L-arabinose were always used, and in some cases D-galactose, D-mannose, D-fructose, D-glucuronic acid and D-galacturonic acid were also included. Cellobiose and D-glucose were the only reference sugars used for the hydrolysates of cellulose sodium sulfate and wheat straw cellulose.

When the spots were dry, the filter paper was shaped into a cylinder by sewing the long edges together with cotton thread so that the edges did not quite touch. Since the cylinders supported their own weight even when wet with solvent, no supporting racks were necessary for ascending chromatography. The paper cylinders were simply allowed to stand upright in the solvent covering the bottom of the chromatograph chambers.

A large desiccator topped with a bell jar of the same diameter served satisfactorily as a chromatograph chamber. It provided adequate height to accommodate the cylinders, and when the ground glass surfaces of the desiccator and bell jar were greased, the joint was airtight. A second chamber which proved to be useful was a large glass jar 10 inches in diameter and 18 inches in height, with a screw cap 5.5 inches in diameter. Occasionally, three chromatograms were developed simultaneously in this chamber.

The paper cylinders were allowed to stand in the solvent-saturated atmosphere of the chromatograph chambers for 24 hours before development

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[1] To keep the area of the spots small, no more than 4 microliters were delivered at one time. The first such spot was dried under an infrared lamp before the next was superimposed. A microburette calibrated in microliters was used for this work.

was begun. They were held in 1-liter tall-form beakers standing in the solvent in the desiccator-bell jar containers, or suspended above the solvent by threads held in place by the screw cap of the other chamber.

Two different solvent mixtures were tried. The chromatograms produced by development with the alcoholic phase of n-butanol:acetic acid: water in the proportion 4:1:5 (v/v) (Bayly et al., 1951) were found to be unsatisfactory because of trailing of the spots and low R_f values. The second solvent system tried, n-butanol:pyridine:water in the proportion 6:4:3 (v/v) (Whistler and Smart, 1953), gave better results; the spots were more nearly round and the sugars migrated at a faster rate. All the chromatograms shown in the section on results were developed with this solvent.

The components of the hemicellulose hydrolysates were not resolved sufficiently by a single ascent of the solvent. Consequently, the multiple development technique used by Jeanes (1951) and by Pazur and French (1952) was adopted. After the solvent had made one ascent nearly to the top of the chromatogram, the cylinder was removed, dried, and then returned to the chamber for a second passage of the solvent. The time required for each ascent varied from 10 to 12 hours. In this way the effective distance of the solvent front was doubled and the efficiency of the resolution was proportionately increased.

The chromatograms were developed at room temperature, but were protected against extreme variations in temperature.

After the developed paper cylinders were dried in a gentle current of air in a hood, they were unrolled and sprayed with an alcoholic

p-anisidine phosphate color reagent[1] (Mukherjee and Srivastava, 1952). The spots containing the reducing sugars became visible after heating at 95-100° C. for five minutes.

The spots were identified by their R_g values, i.e., the ratio of the distance traveled by the unknown spot to the distance traveled by glucose (on the same chromatogram). The use of R_g rather than R_f values obviates the necessity for strictly controlled conditions; variations in the rate of migration due to temperature or other variables are automatically taken into account (Hirst and Jones, 1949). Distances were measured from the starting line to the center of the spot. The color of the spots also helped to identify the sugars. Xylose and arabinose gave dark brown spots, the aldohexoses and cellobiose light brown, and the uronic acids reddish-brown areas. Fructose was easily identified by the bright yellow color it produced with the reagent.

Some of the end products of cellulose sulfate and hemicellulose hydrolysis could not be identified by their R_g values. The unidentified products migrated on the chromatograms at a slower rate than any of the known sugars, except the uronic acids. To test the possibility that the unidentified components of the hemicellulose hydrolysates were disaccharides or higher oligosaccharides and also to determine their composition, a small amount of each component was isolated and studied in the following manner. Streak chromatograms were prepared by applying 0.07 to 0.12 ml. of the concentrated and deionized hydrolysates in a

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[1] The preparation of this reagent is described in Appendix B.

streak along the starting line of a paper cylinder and developing twice as described before. Narrow, vertical control strips were cut from both sides, treated with the color reagent, and heated as before. Horizontal sections corresponding to the spots on the control strips were cut from the rest of the chromatogram and eluted with water. The elution technique was essentially the same as that described by Dent (1947). One end of a strip was placed between two glass plates resting on the bottom and the edge of a Petri dish filled with water. The other end, cut to a point, was suspended over a small beaker. Water reached the paper by capillary attraction, irrigated the strip and dropped into the beaker. Five or six elution assemblies were placed under a large bell jar to prevent evaporation. A period of 8 to 12 hours was required to obtain 1 to 3 ml. of eluate.

A small portion of the eluate was analyzed for reducing sugar before hydrolysis, and a second portion was analyzed after hydrolysis with 0.2 N hydrochloric acid at 120° C. (autoclave) for 45 minutes. (Before the addition of Somogyi's reagent, the hydrolysate was neutralized with sodium hydroxide solution and diluted to 5 ml. with water.) A third volume of the eluate was concentrated in vacuo and transferred to a chromatogram. A fourth sample was hydrolyzed with 0.2 N hydrochloric acid in the autoclave, concentrated, and chromatographed with some of the unhydrolyzed sample.

The unidentified components in the cellulose sulfate hydrolysates were isolated in the same manner, but in this case the eluates were analyzed for reducing sugars before and after hydrolysis and tested qualitatively for sulfate ion (barium chloride) before and after hydrolysis. No chromatograms of the eluates were made.

Two-dimensional chromatography was employed in two cases to separate compounds which were not adequately resolved by the one-dimensional treatment. The solutions, concentrated eluates obtained from streak chromatograms, were spotted in a corner of a 10-inch square of filter paper. The paper was fixed in the shape of a cylinder and developed twice with the n-butanol:pyridine:water solvent system as previously described. The cylinder was then unrolled and made into a cylinder whose long axis was perpendicular to that of the first cylinder. It was then developed twice with water-saturated phenol as the second solvent.

The sugars present in the acid hydrolysates of the two hemicellulose preparations were also identified by paper chromatography. The preparations were hydrolyzed with 1.0 N hydrochloric acid in the autoclave (120° C. for 2 hours). The hydrolysates, perfectly clear but slightly colored, were concentrated *in vacuo* and then chromatographed as before.

DETERMINATION OF PROTEIN NITROGEN

Protein nitrogen was estimated by a semimicro Kjeldahl analysis of the precipitate formed when the enzyme solution was treated with trichloroacetic acid. Direct determination of the protein nitrogen in this manner gave more precise results than did the indirect method of determining total nitrogen and non-protein nitrogen. Duplicate analyses of total nitrogen usually gave results in good agreement, but duplicate analyses of the non-protein nitrogen in the trichloroacetic acid filtrates yielded results in such poor agreement that they were considered unreliable.

The final concentration of trichloroacetic acid was 2.5 percent. According to Hiller and Van Slyke (1922), trichloroacetic acid in this concentration is superior to all the other reagents they tested in separating protein from non-protein nitrogen. On the other hand, Kirk (1947) stated that in all probability, all protein precipitants precipitate some non-protein nitrogenous substances along with the protein. Strictly speaking, then, the results should be reported as trichloroacetic acid-precipitable nitrogen rather than protein nitrogen. However, for the sake of convenience, the latter term is retained, with the understanding that the results may include a small amount of non-protein nitrogen.

Duplicate volumes of the enzyme solution containing 0.2 - 2.0 mg. of protein nitrogen were delivered into test tubes and 20 percent trichloroacetic acid was added to a final concentration of 2.5 percent. Heating the tubes in a hot water bath (ca. 90° C.) for 5-10 minutes to coagulate the proteins became routine practice. In many cases in which the protein concentration was low, the solution merely became cloudy when trichloroacetic acid was added; only by heating could the protein be made to coagulate. The tubes were centrifuged for 20 minutes, the supernatant solution carefully decanted, and the tube inverted and drained for at least 5 minutes. The precipitates were triturated with 5 ml. of 2.5 percent trichloroacetic acid, centrifuged, drained, and washed a second time in the same way. After the final washing the precipitates were dissolved in 2 ml. of 0.1 N sodium hydroxide and the solutions transferred by pipette into semimicro Kjeldahl flasks. The tubes were rinsed three times with 2 ml. of water and the washings

were added to the Kjeldahl flasks.

The samples were digested for 2 hours (after clearing) with 2.5 ml. of sulfuric acid, one gram of potassium sulfate and 1.0 ml. of 10 percent copper sulfate (pentahydrate). The ammonia liberated by making the digest alkaline was distilled with steam into 10.00 ml. of 0.02 N sulfuric acid. The excess sulfuric acid was titrated with approximately 0.01 N sodium hydroxide[1]. A mixed indicator prepared by combining 5 parts of 0.02 percent methyl red (sodium salt) and 2 parts of 0.025 percent methylene blue was used[2]. Blank analyses were done with 2 ml. of 0.1 N sodium hydroxide replacing the protein solution.

Results

Before describing the results of this investigation, it may be well to review the definitions of activity and specific activity. Activity is a concentration term and is expressed as the milligrams of reducing substances (calculated as glucose) which would be produced by one milliliter of enzyme solution acting at 40° C. for a specified period on 100 mg. of substrate in 10.0 ml. of reaction mixture buffered at pH 5.35 with 0.05 M citrate buffer. The reaction time for cellulose sodium sulfate and salicin was 2 hours, for the hemicelluloses and cellulose, 24 hours.

The more meaningful term, specific activity, is defined as the milligrams of reducing substances (as glucose) released under the conditions specified above per milligram of protein nitrogen.

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[1]The sodium hydroxide was standardized by titration against the sulfuric acid each time it was used.

[2]The range of the color change from purple (in acid) to green was found to be from pH 4.6 to pH 5.8.

Production and Concentration of Culture Filtrates

Fifteen 12-liter cultures of M. verrucaria were started. Two of the cultures were discarded because of bacterial contamination. The final activities (CSS)[1] of the filtrates, the size of the inocula, and the times of incubation of the thirteen successful cultures are given in Table VII. It will be noted that the final activities cover a three-fold range: 7.26 to 21.8. The controlled variables which might be expected to influence the final activity are the size of the inoculum and the nature of the cellulose substrate.[2]

The results in Table VII show that within the limits used, there was apparently no direct relation between the size of the inoculum and the final activity. The smallest inoculum was 1.7 billion spores. In one case this number of spores produced a culture filtrate having the highest activity and in a second culture an average activity. One of the largest inocula (14.0 billion spores) produced the lowest activity. This inverse relationship was not general.

Although the activities of cultures 14 and 15, in which Whatman ashless powdered cellulose replaced Solka-Floc as the substrate, are

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[1]For convenience, the substrates used to determine activity will be indicated parenthetically, using the following abbreviations for cellulose sodium sulfate, hemicellulose x, hemicellulose Y, wheat straw cellulose, and salicin: CSS, x, Y, C, S.

[2]The time of incubation is more of an affect than a cause since each culture was continued until the activity was no longer increasing rapidly or was actually decreasing.

TABLE VII

THE INOCULUM SPORE COUNT, TIME OF INCUBATION AND FINAL ACTIVITY OF CULTURES OF MYROTHECIUM VERRUCARIA

Culture no.	Inoculum spore count	Time of incubation	Final activity (CSS)*
	billions	days	
1	1.7	14	21.8
2	1.7	19	12.3
5	14.0	26	7.26
6	9.1	16	14.7
7	10.2	14	13.6
8	7.6	18	12.7
9	7.9	21	12.5
10	5.7	17	13.4
11	7.3	17	10.4
12	11.4	14	11.1
13	14.6	15	10.1
14**	4.8	17	20.8
15**	5.6	16	18.2

*Activity (CSS) is expressed as the milligrams of reducing substances (calculated as glucose) released in 2 hours at 40° C. from 100 mg. of cellulose sodium sulfate in 10.0 ml. of reaction mixture buffered at pH 5.35 with 0.05 M citrate by one ml. of the enzyme solution.

**Whatman powdered cellulose for chromatography was the substrate for cultures 14 and 15; Solka-Floc was used for all others.

both considerably higher than the average activity (12.7) of the first eleven culture filtrates, it cannot be stated with certainty that the nature of the substrate influences the production of the enzyme. The data are too few and the occurrence among the Solka-Floc cultures of one filtrate having an activity greater than either culture 14 or 15 makes any conclusion highly tentative.

Variations in other conditions, such as the supply of oxygen and the rate of stirring cannot be invoked because, although they were not rigidly controlled, they were kept reasonably constant from culture to culture.

The rate at which the activity accumulated in four of the cultures is illustrated in Figure 3; complete data for all the cultures are presented in Table VIII. Three of the four curves in Figure 3 are diphasic; a more or less pronounced increase in the rate of accumulation of activity occurred at 10 to 12 days. The fourth curve is smooth. The inflection also occurs (at the same time interval) in the curves (not shown) for cultures 1, 2, 10, and 11, but not in those for cultures 5, 7, and 12. The possible significance of this phenomenon will be discussed later in connection with other observations to be described.

The culture filtrates, ranging in volume from 9 to 11 liters[1], were concentrated by slow partial freezing to 0.1-0.2 of their original volumes. The activity (CSS) and protein nitrogen content of the concentrates were determined; only the activity of the solution removed as ice was estimated. These results, as well as the specific activities

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[1]Part of culture filtrate 5 was lost.

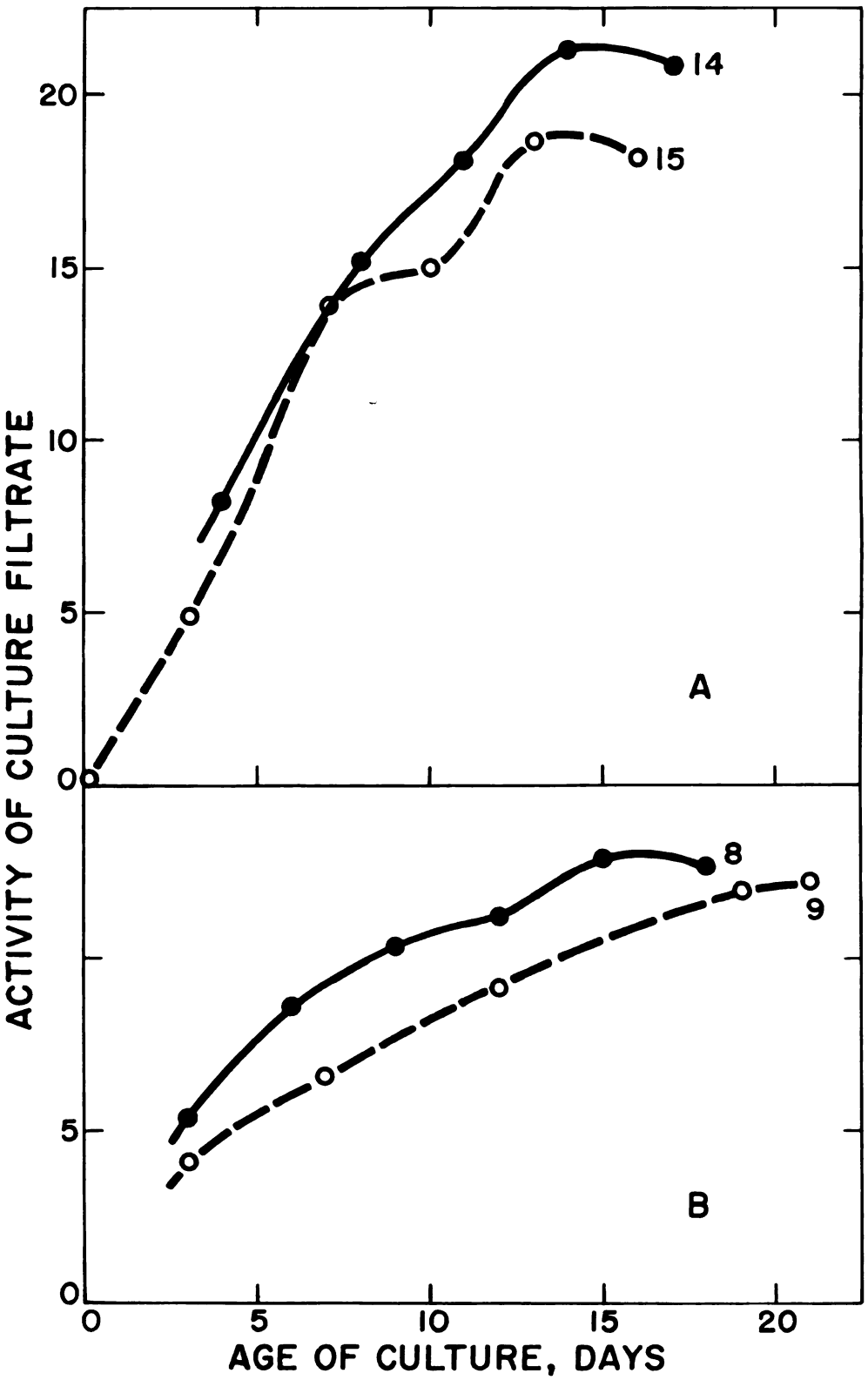

Figure 3. Accumulation of cellulase activity in cultures of M. verrucaria. The substrate for determination of enzyme activity was cellulose sodium sulfate. A. Substrate for cultures 14 and 15: powdered filter paper (Whatman ashless). B. Substrate for cultures 8 and 9: Solka-Floc.

TABLE VIII

THE ACCUMULATION OF CELLULASE ACTIVITY IN CULTURES OF M. VERRUCARIA

(Substrate: cellulose sodium sulfate)

Days	Activity
Culture 1	
3	7.80
6	12.1
10	16.6
11	17.6
13	21.6
14	21.8
Culture 2	
3	8.72
6	10.5
9	10.9
12	12.8
15	12.4
19	12.3
Culture 5	
5	3.61
9	5.95
13	7.20
19	8.20
22	8.47
26	7.26
Culture 6	
3	3.78
6	5.68
16	14.7
Culture 7	
3	3.14
7	8.10
10	11.4
13	13.5
14	13.6
Culture 8	
3	5.38
6	8.68
9	10.3
12	11.2
15	12.9
18	12.7
Culture 9	
3	4.04
7	6.56
12	9.20
19	12.3
21	12.5
Culture 10	
4	5.72
7	8.86
12	11.6
15	13.5
17	13.4
Culture 11	
3	2.24
7	6.93
11	8.08
15	10.8
17	10.4
Culture 12	
4	6.49
8	9.65
12	10.9
14	11.1
Culture 13	
12	10.4
15	10.1
Culture 14	
4	8.25
8	15.2
11	18.1
14	21.3
17	20.8
Culture 15	
3	4.93
7	13.8
10	15.0
13	18.7
16	18.2

of the concentrates and the fraction of the original activity recovered in the concentrates and in the ice, are presented in Table IX.

The specific activity (CSS) of the concentrates varied from 453 to 647. There was no apparent correlation between the activity of the original culture filtrate and the specific activity of the concentrate, although the specific activities of concentrates 14 and 15 were both somewhat higher than any of the others, indicating that the relative proportions of the proteins synthesized and excreted by M. verrucaria may be influenced by the nature of the substrate.

An interesting and puzzling feature of the results in Table IX is the recovery of activity. This was calculated from the total activity of the original filtrate (the product of the volume, in ml., and the activity). The total recovery of activity ranged from 79.9 to an apparent total recovery of 199 percent. In only one case did the total recovery amount to less than 100 percent. The calculated recovery of activity in the concentrates varied from 62.9 to 143 percent of the original. The ice contained 17.0-55.7 percent of the original activity. The distribution of activity appears in a more favorable light if the percentage recovery of activity in the two fractions is based on the total activity recovered rather than on the activity in the original filtrate. Calculated in this manner, the recovery in the concentrates was 65.8 to 78.7 percent. In general, the greatest percentage loss of activity in the ice occurred with filtrates which were concentrated to the greatest extent.

Using the same method of concentration, Whitaker (1953) reported a recovery of about 95 percent of the activity, but did not state whether

TABLE IX

CONCENTRATION OF CULTURE FILTRATES BY
SLOW PARTIAL FREEZING

Culture no.	Fraction	Vol.	Activity (CSS)	Recovery of activity	Prot. N.	Specific activity (CSS)*
		l.		%	mg./ml.	
1	Original filtrate	9.10	21.8			
	Ice	7.15	9.62	34.7		
	Concentrate	1.90	112	107	0.210	533
2	Original filtrate	9.09	12.3			
	Ice	7.63	5.40	36.8		
	Concentrate	1.45	52.5	67.9	0.116	453
5	Original filtrate	7.70	7.26			
	Ice					
	Concentrate	0.825	47.1	69.6	0.083	567
6	Original filtrate	9.16	14.7			
	Ice	7.34	3.14	17.0		
	Concentrate	1.75	48.5	62.9	0.095	511
7	Original filtrate	9.04	13.6			
	Ice	7.21	6.21	36.4		
	Concentrate	1.66	65.3	87.8	0.131	500
8	Original filtrate	10.00	12.7			
	Ice	8.22	8.60	55.7		
	Concentrate	1.65	110	143	0.206	534
9	Original filtrate	9.65	12.5			
	Ice	8.12	5.00	33.6		
	Concentrate	1.45	95.0	114	0.189	503
10	Original filtrate	9.98	13.4			
	Ice	8.76	7.04	46.0		
	Concentrate	1.18	101	88.8	0.193	523

TABLE IX concluded

Culture no.	Fraction	Vol.	Activity (CSS)	Recovery of activity	Prot. N.	Specific activity (CSS)*
		l.		%	mg./ml.	
11	Original filtrate	10.63	10.4			
	Ice	9.05	5.43	44.2		
	Concentrate	1.47	85.5	114	0.147	582
12	Original filtrate	10.36	11.1			
	Ice	8.72	4.26	32.3		
	Concentrate	1.61	54.8	76.7	0.089	616
13	Original filtrate	9.62	10.1			
	Ice	8.29	6.35	54.1		
	Concentrate	1.30	89.5	119	0.162	552
14	Original filtrate	9.93	20.8			
	Ice	8.20	11.2	44.3		
	Concentrate	1.70	123	101	0.190	647
15	Original filtrate	10.95	18.2			
	Ice	9.15	10.3	47.3		
	Concentrate	1.70	120	103	0.187	641

*Specific activity (CSS) is the mg. of reducing substances (calculated as glucose) released from cellulose sodium sulfate per milligram of protein nitrogen under the standard conditions.

this was the total recovery. The impression is left that this amount was recovered in the concentrates, and that the ice was not analyzed.

The increase in total activity accompanying the concentration process cannot be explained on the basis of a stimulatory effect of increased salt content. The dilutions necessary to bring the activity of the concentrates into the range specified for all activity determinations reduced the salt content to about the same level as that used in assaying the original filtrates. Furthermore, neither sodium chloride nor a sodium phosphate buffer (pH 5.3) had an appreciable effect on the activity of a filtrate when they were included in the assay medium in concentrations of 0.02 to 0.1 M. The activity of the filtrate without added salt was 12.4, and 0.02, 0.05, and 0.1 M sodium chloride added it was 12.6, 12.3, and 12.4, respectively. The corresponding values with phosphate buffer in the same concentrations were 12.4, 12.3, and 12.3. These variations are within experimental error[1].

Basu and Whitaker (1953) demonstrated a slight (8 percent) stimulation of the cellulase of M. verrucaria by 0.02 M sodium chloride at pH 5.0. The effect was only 3 percent at 0.05 M and disappeared entirely at 0.1 M sodium chloride.

In a second experiment, a small amount of the original filtrate of culture 9 was saved until the remainder had been concentrated. Its activity (CSS) was then determined with and without the addition of some of the concentrate which was diluted to the same extent required

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[1]In a series of ten separate activity (CSS) determinations done in duplicate on the same enzyme solution and with the same conditions, five were within ±2 percent of the mean, and nine within ±4 percent. The greatest variation from the mean was 5.7 percent.

for estimation of its activity and inactivated by autoclaving for 15 minutes at 120° C. The activity of the original filtrate was 12.3, that of the filtrate plus inactivated concentrate 12.4. The increase is not significant. Thus no explanation based on experimental evidence can be advanced for the effect of concentration by freezing.

Fractionation by Solvent and Salt Precipitation

The effect of the ethanol and acetone concentration on the precipitation of the activity of one of the concentrates is presented graphically in Figure 4. Each point represents the fraction of the original activity (CSS) which was precipitated from a separate portion of the concentrate by the slow addition of a predetermined volume of the solvent. The pH of this undialyzed concentrate was 6.95, the protein nitrogen content 0.21 mg./ml., and the specific activity 533.

Only about one-fourth of the activity was precipitated by 71 percent (v/v) ethanol. The amount was less below and above this concentration. With acetone, the results were better, 64 percent of the activity being precipitated at an acetone concentration of 75 percent.

The protein nitrogen content of the precipitates was determined when it was present in sufficient amount. The data, calculated as percentage of total protein nitrogen, are presented in Table X. It will be noted that with both acetone and ethanol, the fraction of total protein nitrogen precipitated was greater in every case than the fraction of total activity. Consequently, the specific activity of the precipitated proteins was always less than that of the starting material, and generally decreased progressively with increasing concentration of solvent. More important, the

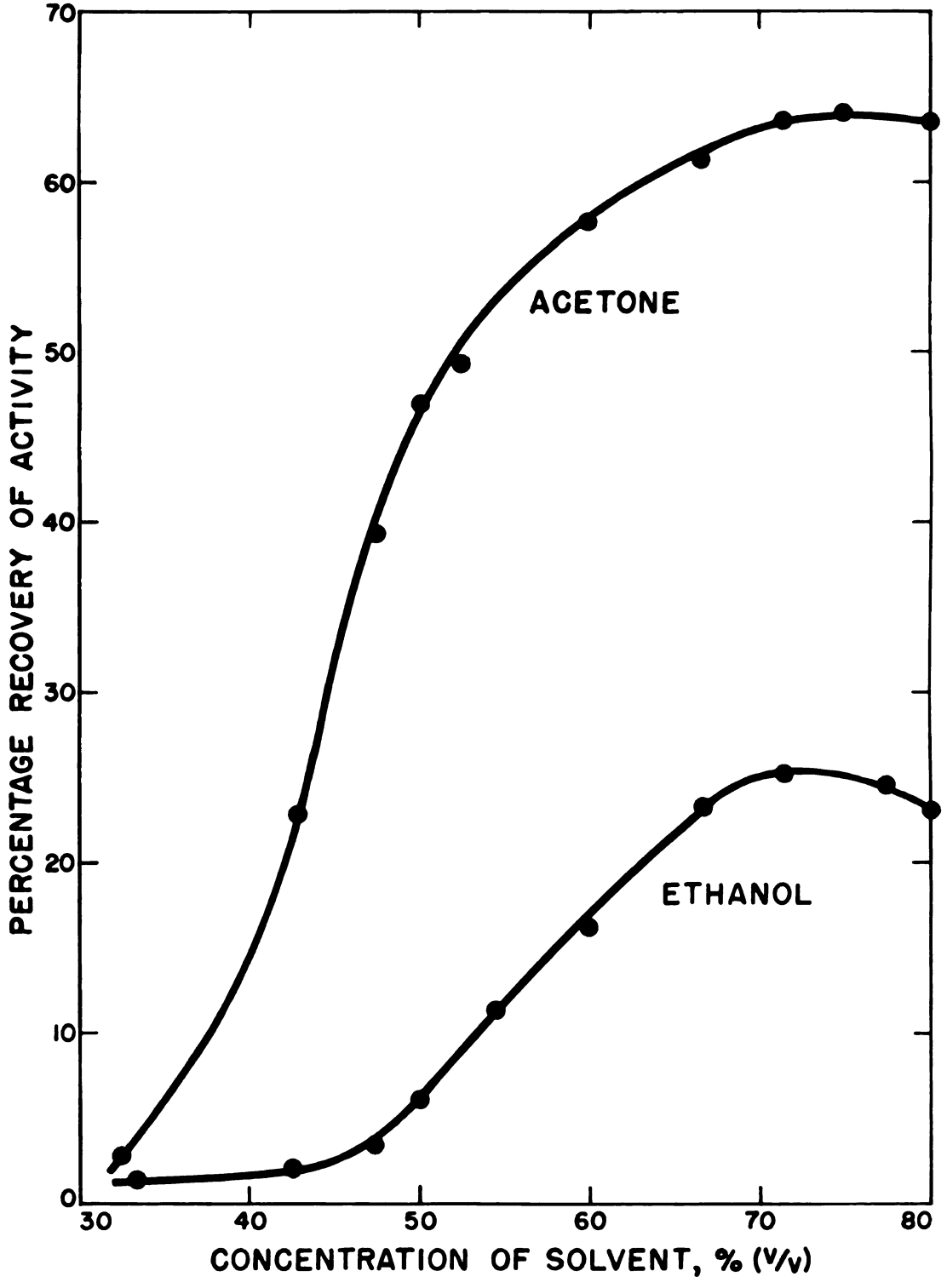

Figure 4. Effect of concentration of solvent in the precipitation of the cellulase activity of concentrated culture filtrates with acetone and alcohol.

TABLE X

EFFECT OF SOLVENT CONCENTRATION ON THE PRECIPITATION OF THE PROTEINS AND ACTIVITY (CSS) OF CULTURE FILTRATES OF M. VERRUCARIA

Solvent Concentration	Ethanol precipitate			Acetone precipitate		
	Protein nitrogen	Activity	Specific activity	Protein nitrogen	Activity	Specific activity
%(v/v)	% of original			% of original		
33.3		1.5			2.9	
42.9		2.0		24.2	22.8	502
47.4		3.4		41.2	39.3	508
50.0		6.1		53.3	47.0	471
52.4				55.7	49.3	471
54.5	14.3	11.3	424			
60.0	22.5	16.1	381	66.7	57.7	463
66.7	36.4	23.2	340	74.3	61.4	442
71.4	41.0	25.1	326	76.2	63.6	446
75.0				77.1	64.0	442
77.7	46.6	24.6	282			
80.0	47.0	23.0	261	79.0	63.6	429

calculated specific activities of the fractions precipitated between successive levels of ethanol were less than 533 in each case. With acetone as the precipitant, the calculated specific activity of the additional proteins precipitated by increasing the acetone concentration from 66.7 to 71.4 percent was 615. However, since the amount of protein was only 0.9 percent of the total, isolation of this fraction would be unprofitable. The calculated specific activity of the proteins precipitated

between all other successive acetone levels was less than 533.

The influence of salt concentration on the precipitation of the active proteins was determined by dialyzing a portion of the concentrate against 20 volumes of distilled water for 12 hours, then against the same amount of 0.02 M phosphate buffer (pH 6.9) for 24 hours before the addition of 3 volumes of acetone. Twenty-eight percent of the activity was recovered in the precipitate. Protein nitrogen was not determined. In a second experiment, the concentrate was dialyzed against 5.5 volumes of distilled water for 24 hours prior to precipitation with 4 volumes of acetone. In this case only 8.1 percent of the original activity was recovered. It appears that a high concentration of salts favors a greater recovery of activity.

In an attempt to improve the total recovery of activity, the effect of pH on solvent precipitation was studied. Portions of the concentrate were adjusted to various pH levels by dialysis against 0.5 M acetate (pH 4.0) or phosphate buffers until the pH of the concentrate was close to that of the buffer. The final concentration of ethanol and acetone was 75 percent (v/v). The results, shown in Figure 5, indicate that the best recoveries with both solvents were obtained at pH 6.7 to 7.0. At more alkaline reactions, the amount of activity precipitated fell off rapidly. The rate of decrease with change of pH toward more acid conditions was less rapid.

The results of an attempted fractionation by means of ammonium sulfate precipitation are given in Table XI. The starting material was 250 ml. of a concentrate having a protein nitrogen content of 0.116 mg./ml, a specific activity of 453, and an initial pH of 6.8. The precipitates

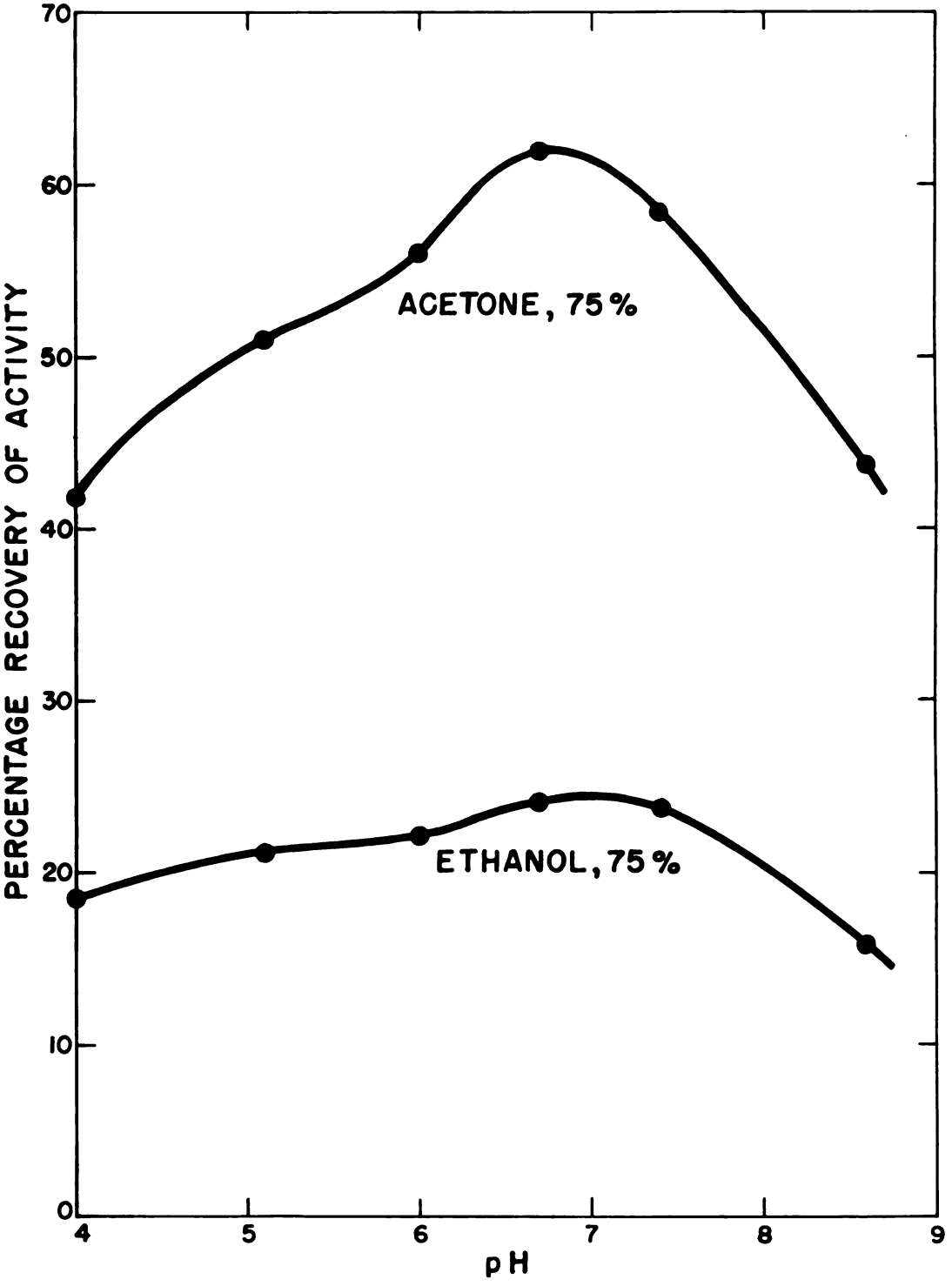

Figure 5. Effect of initial pH on the precipitation of the cellulase activity of concentrated culture filtrate with 75 percent acetone and ethanol.

TABLE XI

ATTEMPTED AMMONIUM SULFATE FRACTIONATION OF M. VERRUCARIA CULTURE FILTRATE

Ammonium sulfate	Protein nitrogen	Activity	Specific Activity
% saturation	% of original		
30		1.1	
52	30.5	26.0	387
69	13.2	11.9	408
90	32.0	20.1	286
100	4.1	2.2	243

obtained at several levels of ammonium sulfate saturation were analyzed for activity (CSS) and protein nitrogen. None of the fractions had a specific activity as great as the starting material, although the precipitate removed at 69 percent saturation approached this value. Ninety-three percent of the activity recovered, and 97 percent of the protein nitrogen recovered, were precipitated by the time the ammonium sulfate saturation reached 0.9.

Whitaker (1953), also working with M. verrucaria filtrates, found that some enrichment of enzyme activity could be achieved by fractionation with ammonium sulfate. Best results were obtained with a 1 percent protein solution and an initial pH of 5.6. The precipitate obtained between 50 percent and complete saturation had a higher specific activity than the original material (no data given), but refractionation of this material at intermediate saturation levels was not successful. The same author

also reported some success with ethanol fractionation. The starting material contained 3 percent protein in ca. 0.01 M phosphate, and had a pH of 4.95. The precipitate removed at 5 percent (v/v) ethanol, constituting a little less than one-fourth of the total protein, had a specific activity about one-seventh that of the starting solution. The protein which precipitated between 5 percent and 25 percent ethanol constituted 63 percent of the total protein and had a specific activity 25 percent greater than the original. Jermyn (1952b) reported erratic results in attempts to concentrate or fractionate the enzymes of A. oryzae preparations by salt or solvent precipitation.

Attempts to increase specific activity by salt and solvent precipitation were unsuccessful. It is possible that the use of greater protein concentrations or different conditions would have given more favorable results. It is also possible that the explanation offered by Jermyn, namely, the presence of more than one enzyme with the same type of activity, applies to these studies.

Concentration by Precipitation with Acetone and Ammonium Sulfate

The failure of fractionation by salt or solvent precipitation may have been due in part to the low concentration of protein. Further concentration was achieved by precipitation with 80 percent acetone and 90-100 percent saturated ammonium sulfate.

The results with acetone precipitation, presented in Table XII, were erratic. The recovery of protein nitrogen varied from 45.5 to 90.2, and the activity from 37.2 to 96.3 percent. The specific activity of the extract was significantly decreased in two cases and substantially increased in three others.

TABLE XII

ACTIVITY OF THE PROTEINS PRECIPITATED FROM CONCENTRATED CULTURE FILTRATES BY 80 PERCENT ACETONE

Concentrate			Extract of precipitate							
No.	Volume	Specific activity	Volume	Protein nitrogen			Activity (CSS)			
				Conc.	Total	Recov.	Conc.	Total	Recov.	Specific
	ml.		ml.	mg./ml.	mg.	%			%	
1	1580	533	75.0	1.03	77.3	48.0	429	32,100	37.2	417
2	500	453	35.0	1.08	37.8	65.1	440	15,400	58.8	407
2d*	635	453	5.0				538	2,690	8.1	
5	800	567	100.0	0.599	59.9	90.2	346	34,600	91.8	578
6	1740	511	760	0.192	146	88.5	111	77,700	92.1	578
7	1640	500	800	0.239	191	88.8	129	103,000	96.3	540
8	1635	534								
9	1420	503	1650	0.227	375	45.5	143	236,000	54.8	630
10	1145	523								
11	1455	582	290	0.634	184	86.1	355	103,000	83.1	560

*This portion of the concentrate was dialyzed 24 hours against 3.5 liters of distilled water prior to precipitation with acetone.

Further precipitation of protein could not be induced by addition of more acetone to the supernatant solution after removal of the precipitate obtained at 80 percent acetone. Some of the protein loss was probably caused by denaturation, for in each case an insoluble residue remained after two extractions of the acetone precipitate with water. The nitrogen remaining in three of the residues after two extractions with 2.5 percent trichloroacetic acid was determined. Twenty-five percent of the protein nitrogen of concentrate 1, 16 percent of concentrate 2, and 9 percent of concentrate 11 were accounted for in this way.

With ammonium sulfate precipitation (Table XIII), the recovery of protein nitrogen (50-80 percent) was generally less than with the acetone method. The percentage recovery of activity (32-77) was less than that of protein nitrogen in every case, resulting in a reduction of the specific activity. The precipitates obtained at 30-35 percent saturation with ammonium sulfate in four cases contained 2-3 percent of the total protein nitrogen and 0.5-2 percent of the total activity. The very small amount of insoluble material in the precipitates obtained by saturation with ammonium sulfate was not analyzed. Further precipitation of proteins from the final supernatant solutions could not be obtained either by complete saturation with ammonium sulfate or by addition of sulfuric acid or sodium hydroxide.

Using the same method of concentration and practically the same conditions, Whitaker (1953) recovered 86 percent of the protein and 91 percent of the activity in the saturated ammonium sulfate precipitate, and accounted for an additional 12 percent of the protein and 2 percent of the activity in the precipitate obtained at 30 percent saturation and

TABLE XIII

ACTIVITY OF THE PROTEINS PRECIPITATED FROM CONCENTRATED CULTURE FILTRATES BY AMMONIUM SULFATE

Concentrate			Extract of precipitate							
				Protein nitrogen			Activity (CSS)			
No.	Volume	Spec. act.	Volume	Conc.	Total	Recov.	Conc.	Total	Recov.	Specific
	ml.		ml.	mg./ml.	mg.	%			%	
12, 13	2880	581	170	1.03	175	50.1	538	91,500	45.1	522
14	1685	647	89.0	1.92	171	53.4	750	66,800	32.3	391
15	1685	641	140	1.79	251	79.7	805	113,000	55.9	450
6A*	690	578	240	0.442	106	80.4	247	59,400	77.4	559
7A*	780	500	105	0.887	93.1	50.0	386	40,500	40.1	435
8, 9, 10A*	1630	630	170	1.24	211	57.0	710	121,000	51.9	573
5, 11A*	375	579	90.0	1.87	168	73.0	585	52,700	39.6	313

*These concentrates were the extracts of the acetone precipitates.

the insoluble residue from the saturated ammonium sulfate precipitate. The protein nitrogen content of the enzyme solution before precipitation was 0.34 mg./ml.

The protein nitrogen concentration of six of the starting solutions in this investigation was less than 0.24 mg./ml. In the seventh (5,11A) it was 0.6 mg./ml.

Fractionation by Chromatography on Cellulose Columns

Three experiments were performed to determine the feasibility of fractionation of the cellulase solutions by chromatography on cellulose columns. The procedure, described in the section on methods followed closely the one outlined by Whistler and Smart (1953) for the purification of A. oryzae preparations.

The solution used for experiment A was 20 ml. of a dialyzed concentrate adjusted to pH 3 with dilute hydrochloric acid. The protein nitrogen content of this solution was 0.95 mg./ml., and the specific activity was 407. The wash solution was distilled water adjusted to pH 3 with hydrochloric acid, and the eluant was a Clark and Lubs borate buffer, pH 9.0.

Experiment B was the same as the first with the exception that the concentrate was not dialyzed. The protein nitrogen concentration was 1.03 mg./ml. and the specific activity was 417.

The enzyme solution used for experiment C was a dialyzed concentrate adjusted to ionic strength ca. 0.1 with sodium chloride and to pH 3 with hydrochloric acid. Twenty ml. of this solution, having a protein nitrogen content of 0.90 mg./ml. and a specific activity of 400, was used. The wash solution was 0.1 M sodium chloride adjusted to pH 3 as before. The eluant was a 0.05 M Clark and Lubs phosphate buffer, pH 8.0.

The solutions percolating through the column were collected fractionally, adjusted to pH 5.3 and analyzed for cellulase activity (CSS) and, when possible, for protein nitrogen. The results are presented in Table XIV.

In the first experiment, only 35.2 percent of the protein nitrogen and 28.6 percent of the activity was adsorbed on the column. A larger proportion of the inactive (or less active) components appears to have been adsorbed, since the specific activity of the unadsorbed material was greater than the original specific activity. Such a small degree of purification was considered unprofitable in view of the losses of both protein nitrogen and activity. Slightly more than half of the adsorbed activity was recovered in the eluates. One of the fractions of the eluate had a specific activity a little greater than the original. Eighty-one percent of the protein nitrogen and 87 percent of the activity were accounted for.

The greater ionic strength of the enzyme solution used in the second experiment (B) apparently favored increased adsorption of the more active proteins. The specific activity of the unadsorbed fraction was reduced to about one-half of the original. However, the increased adsorption of activity was of no advantage, because only 15.1 percent of the total, or about one-fifth of the adsorbed activity was eluted. The pattern of elution was similar to that of the first experiment. In this run, 55.6 percent of the protein nitrogen and 37.4 percent of the activity were recovered.

Part of the loss of activity was probably due to inactivation of the enzymes at pH 9. A loss of 14 percent of the activity was observed when

TABLE XIV

FRACTIONATION OF CELLULASE SOLUTIONS BY CHROMATOGRAPHY ON CELLULOSE COLUMNS

(See text for description of starting materials, wash and eluting solutions)

Solution from column: source and volume*	Experiment A			Experiment B			Experiment C		
	Protein nitrogen	Activity	Specific activity	Protein nitrogen	Activity	Specific activity	Protein nitrogen	Activity	Specific activity
ml.	% of start			% of start			% of start		
Starting solution - 20	58.2	62.5	435	17.5	9.4	224	50.6	46.1	364
Wash									
20	8.6	8.9	420	20.2	11.9	245	12.5	13.0	416
40		0.0			0.0			0.5**	
Eluant									
10	4.5	3.9	352	3.6	2.6	301	5.1	4.5	353
20	5.9	6.3	435	9.1	9.2	422	5.1	4.9	385
20	5.9	3.0	207	5.2	2.8	225		0.9	
20		1.5			1.1			0.4	
50		1.0***			0.4			0.0	

*The volumes recorded are approximate. The exact volume was recorded after adjustment of the pH.

**The volume of this solution was 20 ml.

***Two fractions, the first 20 ml. and the second 30 ml., were analyzed separately. The results were combined.

a small portion of dialyzed concentrate was mixed with an equal volume of the pH 9 borate buffer and allowed to stand one hour at room temperature. Similar treatment with a neutralized (pH 7) borate buffer caused no loss of activity, and the effect of a reaction of pH 3 for 30 minutes was also negligible.

The third experiment (C) was designed to test the effect of an intermediate ionic strength on the adsorption of the proteins, and to determine whether a different buffer would elute a greater fraction of the adsorbed activity.

The amount of protein nitrogen adsorbed was practically the same as in the first experiment, but the activity retained by the column was substantially greater. The pH 8 phosphate buffer was not as efficient as the borate buffer, only 10.2 percent of the protein and 10.7 percent of the activity being recovered in the eluates. There was no indication of any purification.

The degree of purification of A. niger cellulase preparations achieved by Whistler and Smart (1953) cannot be assessed quantitatively for comparison with the results obtained in this investigation because they reported no protein data. Qualitative evidence of some purification was afforded by detection of cellobiose in cellulose hydrolysates produced by the purified material. Cellobiose was not found in hydrolysates produced by the crude preparations.

Fractionation by Electrophoresis-Convection

Whereas the previous fractionation methods failed to yield the desired results with consistency, electrophoresis-convection almost invariably resulted in a substantial separation of the active from the inactive (or

less active) components, nearly every run yielding one fraction with a significantly higher specific activity and a second fraction with a lower specific activity than that of the starting material. Large losses of material were encountered with the other methods, but with electrophoresis-convection total recoveries of protein nitrogen and activity were good, both being more than 90 percent in most cases. In some of the cases in which recoveries were less, a precipitate was noted in the bottom fraction.

The procedure has been described in the section on methods; the results are presented in Table XV, which gives the volumes, protein nitrogen concentrations, activities and specific activities of the top and bottom fractions after electrophoresis convection, and of the starting solution before electrophoresis convection. Also recorded in this table are the percentage recoveries of protein nitrogen and activity in the final fractions, and in the last column, the ratios of their specific activities to that of the original solution.

It will be observed that the increase in specific activity always occurred in the top fraction, indicating that the mobility of the active component (or components) was lower than the mobilities of the inactive components. It will also be observed that the activity as well as the protein nitrogen of the bottom cut increased in most cases[1], whereas these values decreased in the top fraction. However, because the rate of change of protein concentration was greater than the rate of change of activity

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[1]In some instances, the increased volume of the bottom cut caused by rinsing the bottom reservoir with buffer obscured the results in this respect.

TABLE XV

THE FRACTIONATION OF CRUDE CELLULASE PREPARATIONS BY ELECTROPHORESIS-CONVECTION

(The conditions and starting materials used for each run appear in Table XVI.)

Run no.	Fraction*	Volume	Protein nitrogen		Activity (CSS)			Sp. A.$_F$ / Sp. A.$_S$**
			Conc.	Recov.	Conc.	Recov.	Specific	
		ml.	mg./ml.	%		%		
1***	S	35.0			620			
	T	21.7			89.3	8.9		
	B	32.5			363	54.4		
2	S	60.0			710			
	T	38.0			536	47.9		
	B	22.5			900	47.4		
3	S	125.0	0.501		328		655	
	T	80.0	0.284	36.3	250	48.7	880	1.34
	B	49.0	0.841	65.8	380	45.4	452	0.69
4	S	65.0	0.498		303		608	
	T	32.0	0.336	33.3	256	41.6	758	1.25
	B	37.0	0.507	58.0	285	53.3	562	0.92
5	S	85.0	0.470		300		638	
	T	59.0	0.342	50.5	237	54.8	693	1.09
	B	33.0	0.412	34.0	236	31.3	573	0.90
6	S	100.0	1.03		538		522	
	T	71.0	0.554	38.2	453	59.9	819	1.57
	B	36.5	1.54	51.4	560	37.9	386	0.74
7	S	87.0	1.92		750		391	
	T	46.5	1.09	30.4	713	50.8	655	1.66
	B	45.0	2.46	66.5	783	53.9	317	0.81
8	S	100.0	1.79		805		450	
	T	49.5	0.930	25.1	708	43.5	778	1.73
	B	56.5	2.12	67.0	763	53.5	359	0.80
9	S	106.5	2.00		768		384	
	T	56.0	0.946	24.9	625	42.4	660	1.72
	B	56.0	2.68	70.4	768	52.6	287	0.75

TABLE XV continued

Run no.	Fraction*	Volume	Protein nitrogen		Activity (CSS)			Sp. A.p** / Sp. A.s
			Conc.	Recov.	Conc.	Recov.	Specific	
		ml.	mg./ml.	%		%		
10	S	116.0	0.900		690		767	
	T	65.5	0.889	56.0	685	56.1	770	1.00
	B	49.5	0.871	41.4	677	41.9	777	1.01
11	S	194.0	0.760		331		437	
	T	130.5	0.330	29.3	271	55.2	821	1.88
	B	80.0	1.22	66.4	379	47.2	311	0.71
12	S	75.0	1.22		379		311	
	T	52.0	0.51	29.0	262	48.0	514	1.65
	B	35.0	1.79	68.5	366	44.9	204	0.66
13	S	395.0	0.301		203		674	
	T	271.0	0.185	42.1	179	60.6	968	1.44
	B	123.0	0.515	53.2	276	42.3	536	0.80
14	S	120.0	0.515		276		536	
	T	75.0	0.330	40.1	259	58.6	782	1.46
	B	45.0	0.750	54.7	305	41.4	405	0.76
15	S	42.0	0.750		305		405	
	T	22.0	0.418	29.2	254	43.6	608	1.50
	B	21.0	0.976	65.1	313	51.3	321	0.79
17	S	92.5						
	T	58.5	1.28		317		247	
	B	34.0	3.58		621		173	
18	S	54.0	1.28		317		247	
	T	35.0	0.626	31.7	246	50.3	393	1.59
	B	25.0	1.69	61.2	350	50.9	206	0.83
19	S	38.5	1.61		930		578	
	T	17.0	0.885	24.2	817	38.8	923	1.60
	B	22.0	2.17	76.9	950	58.4	438	0.76
20	S	62.0	1.28		1040		813	
	T	44.5	0.770	43.2	945	65.3	1230	1.51
	B	20.0	2.23	56.2	1100	34.1	493	0.61
21	S	40.0	1.10		531		483	
	T	26.3	0.534	31.8	363	45.0	679	1.41
	B	16.0	1.88	68.4	703	52.8	374	0.77

TABLE XV concluded

Run no.	Fraction*	Volume	Protein nitrogen		Activity (CSS)			Sp. A.$_F$** / Sp. A.$_S$
			Conc.	Recov.	Conc.	Recov.	Specific	
		ml.	mg./ml.	%		%		
22	S	117.5	1.37		501		366	
	T	102.0	0.863	54.7	464	80.3	538	1.47
	B	23.8	2.90	42.9	586	23.6	202	0.55
23	S	101.0	0.863		464		538	
	T	81.5	0.485	45.3	440	76.5	903	1.68
	B	22.0	1.96	49.4	622	29.2	317	0.59
24	S	41.3	2.00		2080		1040	
	T	31.5	0.940	35.6	1500	55.1	1600	1.54
	B	26.5	1.99	63.3	1750	54.0	879	0.85
25	S	118	1.00		453		453	
	T	139	0.412	48.6	245	63.7	595	1.31
	B	30.5	0.846	21.9	305	17.4	361	0.80
26	S	88.0	1.87		585		313	
	T	120.0	0.685	49.8	294	68.5	439	1.40
	B	15.0	4.99	45.4	867	25.3	173	0.55
27	S	60.0	0.399		140		351	
	T	55.0	0.182	42.6	76.7	50.2	422	1.20
	B	30.0	0.336	42.9	53.4	19.1	159	0.45
28	S	75.0	0.761		242		320	
	T	61.0	0.384	41.1	189	63.2	492	1.54
	B	16.0	1.07	30.0	216	19.0	202	0.63
29	S	138.0	1.13		510		451	
	T	110.0	0.636	51.3	342	53.4	538	1.19
	B	31.0	0.947	18.8	312	13.7	329	0.73

*The letters S, T, and B indicate the starting material, top, and bottom fractions, respectively.

**Ratio of the specific activity of the final fraction to the specific activity of the starting material.

***A collodion membrane was used for the first experiment; viscose cellulose dialysis tubing was used in all subsequent runs.

concentration, changes of specific activity occurred. In other words, although a portion of the active components was carried into the bottom reservoir, their rate of transport was less than that of the inactive components. The specific activity of the top fraction increased 40 percent or more in 18 of the 25 runs for which complete data were obtained. The entire range of increase was from 9 to 88 percent.

Factors which, according to theory, influence the degree of separation achieved in the top reservoir are pH and ionic strength (conditions which determine the ratio of the mobility of one component to that of a second; hence their differential rate of electrophoretic transport), the potential gradient across the channel and the length of time it is applied, the initial concentration of total protein and of the various components, and the volume of solution in the top reservoir. The conditions used for each run are listed in Table XVI.

The first two runs were pilot experiments to determine whether fractionation by electrophoresis convection was feasible. A collodion membrane was used for the first trial. When the cell was emptied, a relatively large amount of precipitate was found in the bottom reservoir and on the anode wall of the channel. The activity of the solution in the top reservoir decreased to about one-seventh of the original and only part of the loss was accounted for by an increased total activity in the bottom reservoir. It appeared that the protein was inactivated and precipitated in some way as it passed through the channel. Whether this was a specific effect of the collodion membrane or was due to some other cause was not established since the second experiment, in which Visking cellulose tubing was used, indicated that the use of collodion membranes was not necessary.

TABLE XVI

CONDITIONS AND STARTING MATERIALS USED IN THE ELECTROPHORESIS-CONVECTION FRACTIONATIONS

Run no.	Starting material*	pH	Time	Current
			hr.	amp.
1**	$C_{8,9,10}AcAs$	6.9	11.0	0.50
2	$C_{8,9,10}AcAs$	6.9	6.5	0.75
3	$C_{8,7,8,9,10}AcAs$	6.9	14.0	0.50
4	$C_{8,7,8,9,10}AcAs$	5.0	7.0	0.50
5	$C_{8,7,8,9,10}AcAs$	5.9	7.0	0.50
6	$C_{12,13}As$	6.9	7.5	0.50
7	$C_{14}As$	6.9	7.0	0.50
8	$C_{16}As$	6.9	7.0	0.50
9	$EC_{7,8}B$	6.9	7.0	0.50
10	$EC_{7,8,9}T$	5.0	7.0	0.50
11	$EC_{3,4,5,6}B$	6.9	7.0	0.50
12	$EC_{11}B$	6.9	7.0	0.75
13	$EC_{3,4,5,6,11,12}T$	6.9	7.0	0.75
14	$EC_{13}B$	6.9	7.0	0.75
15	$EC_{14}B$	6.9	7.0	0.75
16	$EC_{12,15}B$	6.9	7.0	0.75
17	$EC_{9,16}B$	6.9	9.5	0.60
18	$EC_{17}T$	6.9	10.5	0.80
19	$EC_{10}T,B$***	6.9	7.0	0.75
20	$EC_{13,14,15,16,18}TL$	6.9	7.0	0.80
21	$EC_{20}B$	6.9	7.5	0.75
22	$EC_{17,19,21}B$	6.9	7.0	0.75
23	$EC_{22}T$	6.9	7.0	0.75

TABLE XVI continued

Run no.	Starting material*	pH	Time	Current
			hr.	amp.
24a	$EC_{19,20,21,22}$ T-L	6.9	6.0	0.75
b	EC_{24}aB	6.9	6.5	0.75
c	EC_{24}a,bT	6.9	6.0	0.75
25a	$EC_{22,23,24}$B	6.9	4.0	0.65
b	EC_{25}aT	6.9	3.0	0.65
c	EC_{25}a,bB	6.9	4.0	0.65
d	EC_{25}cB	6.9	4.0	0.65
26a	$C_{8,11}$AcAs	6.9	7.0	0.75
b	EC_{26}aB	6.9	7.0	0.75
c	EC_{26}bB	6.9	7.0	0.75
27a	EC_{26}B	5.9	7.0	0.60
b	EC_{27}aB	5.9	4.0	0.50
c	EC_{27}bB	5.9	4.0	0.50
28a	—#	6.9	7.0	0.75
b	EC_{28}aB	6.9	7.0	0.75
29a	$EC_{25,28,28}$T	6.9	4.0	0.75
b	EC_{29}aT	6.9	5.0	0.75
c	EC_{29}a,bB	6.9	6.0	0.75

*The designations used to identify the starting materials have the following meanings: C indicates that the material is a concentrate, the numerals following specify the number of the concentrate(s), Ac and As indicate previous precipitation of the proteins with acetone and ammonium sulfate, respectively, and EC followed by subscripts and either T or B shows that the material is the top (T) or bottom (B) fraction of the previous electrophoresis-convection (EC) fractionation(s) specified by the numbers. L indicates that the solution has been lyophillized and reconstituted.

TABLE XVI concluded

**A collodion membrane was used for the first experiment; viscose cellulose dialysis tubing was used in all subsequent runs.

***The top and bottom fractions of run No. 10 were combined and concentrated by ultrafiltration.

#The starting material for run No. 28 was a mixture of several residual fractions, some of which had been stored in the refrigerator for several months.

The results of the third, fourth and fifth runs, all done with portions of the same solution[1], showed that apparently a high pH (6.9, run 3) would give better results than a low (5.0, run 4) or an intermediate pH (5.9, run 5). Conditions other than pH were not strictly comparable, but the greater volume of the third run was partially compensated by the longer time used (14 instead of 7 hours as in the other runs). A later run at pH 5.0 (run 10) ended in failure to achieve any separation whatever. According to the theory experimentally confirmed by Brown et al. (1952), the best separations should be obtained when the ratio of the lower to the higher mobility is small, i.e., when the pH is near the isoelectric point of one component. Whitaker (1953) found that the isoelectric point of his purified M. verrucaria cellulase was near pH 5.0. Thus, the results obtained in this investigation were contrary to what was expected. However, Brown et al. worked with a two-component system of plasma albumin and gamma globulin, proteins with widely different mobilities. In the present investigation there were at least six components with fairly close mobilities at pH 6.9. Furthermore, the manner in which the mobilities changed with pH was not known.

The effect of ionic strength on the degree of separation was not studied. All three buffers had nearly the same ionic strength (0.15).

Among runs reasonably comparable with respect to other conditions, increasing the current from 0.5 to 0.75 ampere (and the voltage from

---

[1]The small but significant differences in activity and protein nitrogen were caused by differences in the time of dialysis. The solutions used for the fourth and fifth runs were dialyzed 24 additional hours against the new buffers. Some loss of activity was always found on prolonged dialysis at all pH levels used.

22-23 to 30-33) seemed to have little or no effect on the results. Variations in the time of electrophoresis were not used often enough in comparable situations to allow any conclusions.

Comparing runs 3, 6-9, and 11, in which the pH and current were the same, it appears that there was a reciprocal relation between the initial specific activity and the degree of purification. Usually, when the specific activity of the starting solution was high, the separation of inactive from active components was low; the converse was also true. It also appears that the initial protein concentration influenced the results, a higher protein nitrogen concentration apparently favoring a better separation. The same tendency is not so apparent in runs 12-23, in which the current was usually 0.75. It is possible that the explanation for this difference is that with the stronger current the same percentage clearance of the faster, inactive components was reached in a shorter time, and during the remainder of the period, because of their increased relative concentration in the top reservoir, a constantly increasing proportion of the slower, more active components was transported to the bottom reservoir.

The highest specific activity (CSS) attained was 1600 in the top cut of the twenty-fourth run. This represented an increase of more than three times the average specific activity of the unfractionated starting solutions. Because of the decreased volume of the top cuts and the modest separations achieved in each run, it was necessary at several points to pool the top fractions of previous runs and use the combined solutions for further fractionation. In some cases it was also necessary to lyophilize the combined top cuts and reconstitute at a smaller volume

because of the decreased protein nitrogen concentrations. Thus, the starting solution for run No. 24 represented portions of the top cuts of all previous runs. Some of these had been previously refractionated three times.

Electrophoretic Analysis of Fractions Obtained by Electrophoresis-Convection

Fractionation by electrophoresis convection was controlled both by activity determinations and electrophoretic analysis. Twenty-five of the enzyme solutions were analyzed electrophoretically. The mobilities of the components were determined to help in their identification, and relative concentrations were calculated for use in conjunction with activity data for the purpose of determining which component was most active with a given substrate. All analyses were carried out with a pH 6.9 ionic strength 0.146 phosphate buffer. Other conditions are given with the mobility data in Table XVII. Protein distribution data are given in Table XVIII, and several electrophoretic patterns are reproduced by direct tracing in Figures 6 through 9.

The mobilities shown in Table XVII were all anodic. Only the values for the four slowest components are shown since these seemed to possess nearly all the activity. The mobilities of components 5 and 6 were calculated in several cases. The average mobility of component 5 was 5.7 and of component 6, 7.2. There was considerable variation in the mobility of each component. This might be expected on the basis of the wide variation in the concentration of a component in the different solutions. Differences in the duration of current application may also have influenced the results. In spite of the variations encountered, the mobility results

TABLE XVII

THE ELECTROPHORETIC MOBILITIES OF FOUR OF THE PROTEIN COMPONENTS OF M. VERRUCARIA CULTURE FILTRATES IN pH 6.9 PHOSPHATE BUFFER, 0.146 IONIC STRENGTH

Enzyme solution	Conditions			Mobilities			
	Time	Temperature	Potential gradient	Components 1	2	3	4
	min.	° C	volts/cm.	$cm^2/volt/sec. \times 10^5$			
EC6S	180	1.9	5.99	0.63	1.3	2.5	
EC7S	220	1.8	5.70	0.98	1.5	2.8	3.4
EC8S	220	2.0	6.56	0.86	1.4	2.5	3.8
EC7B	270	2.0	6.26	0.69	1.2	2.1	3.2
EC8B	270	2.0	6.32	0.70	1.2	2.1	3.4
EC7,8,9T	180	2.0	6.62	0.85	1.5	2.5	3.7
EC12B	130	1.8	6.75		1.4	2.8	4.5
EC20S	180	2.0	6.79	0.70	1.4	2.9	3.9
EC20B	180	2.0	6.53	0.87	1.6	3.1	4.2
EC21B	130	2.0	6.57		1.5		
EC22S	150	2.0	6.53	0.77	1.5	3.0	4.0
EC22B	150	2.0	6.53		1.3	2.7	3.7
EC23B	150	2.0	6.53	0.52	1.4	2.9	4.0
EC24S	200	2.0	6.54	0.68	1.4	2.6	3.9
EC24T	200	2.0	6.91	0.79	1.5	3.0	
EC24B	180	2.0	6.54	0.72	1.6	3.0	4.1
EC25S	130	2.0	6.62	0.65	1.8	3.1	4.5
EC25T	160	1.9	6.64	0.59	1.3	2.7	4.2

TABLE XVII concluded

Enzyme solution	Conditions			Mobilities			
	Time	Temperature	Potential gradient	Components 1	2	3	4
	min.	°C	volts/cm.	$cm^2/volt/sec. \times 10^5$			
EC$_{28}$S	150	2.0	6.69	0.85	1.1	2.5	3.8
EC$_{28}$T	180	2.0	6.64	0.84	1.3	2.6	4.1
EC$_{28}$B	130	2.0	6.69		1.3	2.7	3.9
EC$_{27}$B	130	1.9	6.53			3.0	
EC$_{28}$T	170	2.0	6.89	0.59	1.3	2.5	3.6
EC$_{28}$B	120	1.9	6.62		1.6	3.1	
EC$_{29}$B	120	1.9	6.91		1.6	2.8	3.9
			Mean	0.73	1.4	2.7	3.9
			Standard Deviation	0.122	0.17	0.29	0.31

TABLE XVIII

RELATIVE CONCENTRATIONS OF THE ELECTROPHORETIC COMPONENTS OF SEVERAL FRACTIONS PREPARED FROM M. VERRUCARIA CULTURE FILTRATES

Enzyme solution	Specific activity (CSS)	Relative concentration (%) Component no.					Figure no.
		1	2	3	4	5 & 6	
EC6S	522	13.6	25.5	25.1	— 35.8 —		
EC7S	391	24.6	12.7	21.8	28.8	12.3	6a
EC8S	450	20.6	14.0	28.4	23.3	13.8	
EC7B	317	14.0	14.4	21.6	26.5	23.5	6b
EC8B	359	14.8	14.6	22.4	34.0	14.1	
EC7,8,9T	770	23.9	25.0	17.0	32.4	1.5	6c
EC12B	204	0.0	7.3	16.9	— 75.8 —		
EC20S	813	16.0	43.7	20.4	12.2	6.7	
EC20B	493	8.1	30.5	30.1	17.1	13.8	
EC21B	374	0.0	21.8	— 59.6 —		18.1	
EC22S	366	9.9	13.1	34.1	21.0	21.9	7a
EC22B	202	0.0	5.7	29.0	23.2	41.9	7b
EC23B	317	3.2	10.1	42.0	22.9	21.6	
EC24S	1040	19.2	44.8	27.3	8.0	0.0	8a
EC24T	1600	30.7	63.3	6.1	0.0	0.0	8b
EC24B	879	8.8	39.9	40.4	10.3	0.0	8c
EC25S	453	4.2	17.5	33.2	21.5	23.3	
EC25T	595	7.8	25.4	44.4	13.8	8.6	
EC26S	313	5.5	10.9	23.1	11.7	48.7	
EC26T	439	11.6	15.9	33.3	17.6	21.5	
EC26B	173	0.0	6.0	12.3	7.1	74.5	
EC27B	159	0.0	0.0	3.8	— 96.2 —		9b
EC28T	492	9.1	37.0	26.9	10.2	17.0	
EC28B	202	0.0	6.4	24.1	— 69.5 —		
EC29B	329	0.0	19.8	38.3	25.4	16.6	9a

were valuable for identification purposes, for there was no overlapping of the ranges covered by each component.

The electrophoretic pattern of an unfractionated preparation obtained by ammonium sulfate precipitation is shown in Figure 6a. Six components, four major and two minor, are evident. In this respect, the pattern presented is typical of the original solutions before fractionation. That the distribution of protein differed somewhat in the various solutions may be seen by comparison of the relative concentrations of components in the starting solutions for electrophoresis-convection runs 6, 7, 8, and 26 in Table XVIII. Whitaker (1953), using similar conditions for his analyses, found only two major and two minor components in his preparations. He used a pH 6.8 ionic strength 0.2 phosphate buffer and a time of 120 minutes. His patterns, as reproduced for publication, had no boundary anomalies.

After the solution whose pattern is shown in Figure 6a was subjected to electrophoresis-convection (run No. 7), the bottom fraction had the pattern shown in Figure 6b. It can be seen by visual inspection and by the results in Table XVIII that components 5 and 6 were increased and component 1 decreased in this fraction. The large boundary anomalies can be attributed to the fact that the solution was not redialyzed before analysis. Figure 6c is the electrophoretic pattern of the combined top fractions of runs 7, 8, and 9. Components 5 and 6 almost disappeared, and the relative concentrations of the first two components increased significantly. From the results in Tables XVII and XVIII it is evident that most of the activity (CSS) was associated with the slower moving components, since an increase or decrease in these components was accompanied by a similar change in the specific activity.

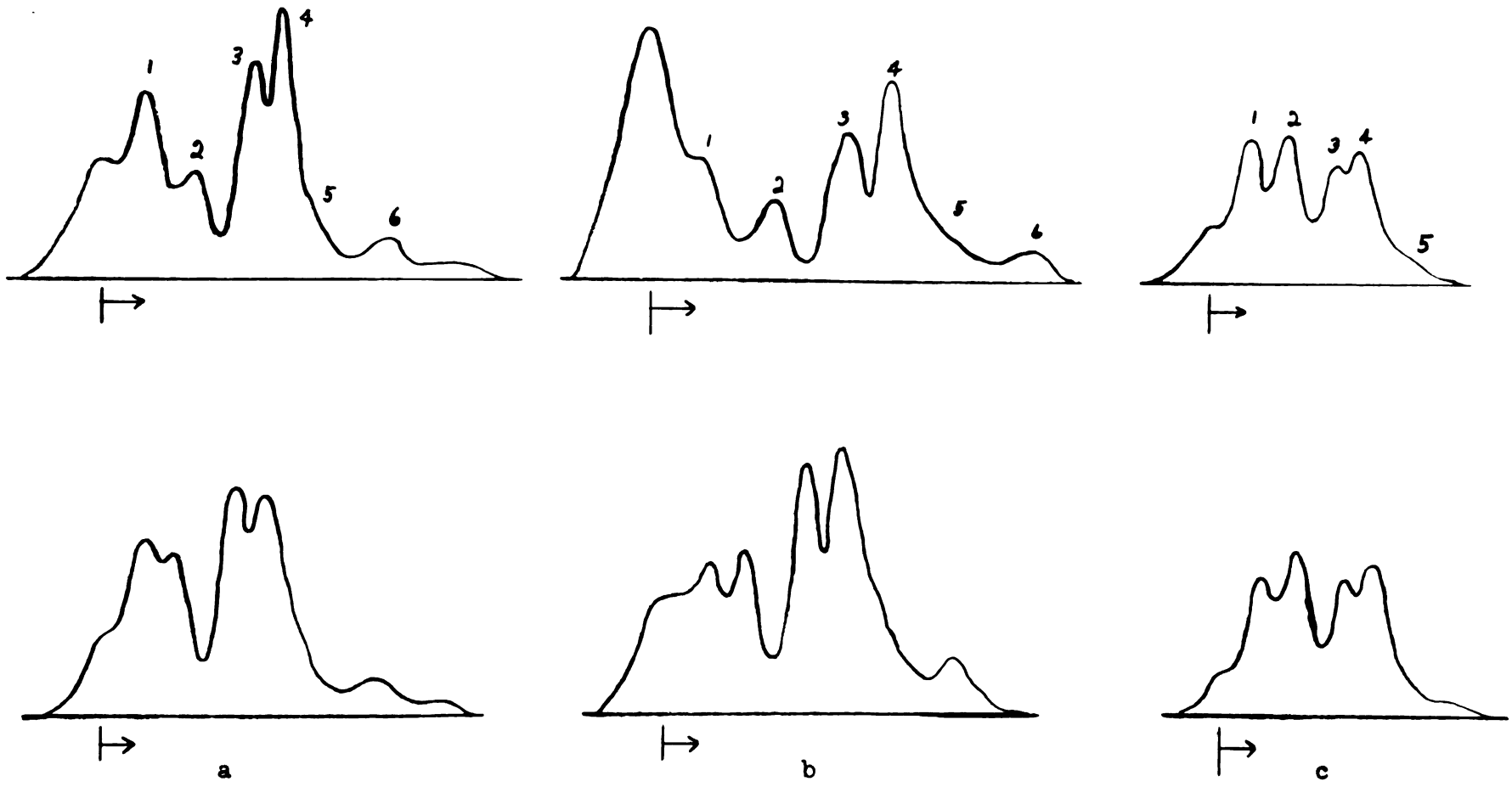

Figure 6. Electrophoretic components of (a) an unfractionated culture filtrate concentrate, (b) the bottom fraction obtained from (a) by electrophoresis-convection for 7 hours at pH 6.9, and (c) a composite of top fractions obtained under the same conditions. Upper patterns, ascending; lower patterns, descending. Migration anodic.

The electrophoretic patterns in Figure 7 are shown in order to illustrate the nonhomogeneity of two of the components. In Figure 7a, component 4 apparently comprises two distinct parts. After electrophoresis convection, the split was even more apparent in the bottom fraction (Figure 7b), and component 6 also showed two distinct peaks. These were the only components which showed this tendency; it was noted in five other instances.

Both the top and bottom fractions as well as the starting material of two runs (24 and 26) were analyzed electrophoretically, affording an opportunity to calculate the percentage recoveries of individual components and at the same time to examine the accuracy of the electrophoretic analyses. The recoveries, calculated as the sum of the percentages recovered in the two final fractions, are presented in Table XIX.

TABLE XIX

PERCENTAGE RECOVERY OF INDIVIDUAL COMPONENTS IN ELECTROPHORESIS CONVECTION FRACTIONATIONS

Run No.	Component				
	1	2	3	4	5 and 6
24	86.1	107.3	102.7	81.8	
26	106.5	98.3	96.3	103.1	91.8

The results are of the order to be expected, taking into account the overlapping of adjacent components in the electrophoretic patterns. A small error in assigning areas to the minor components for calculation of relative concentration would be magnified in the calculation of recoveries in Table XIX.

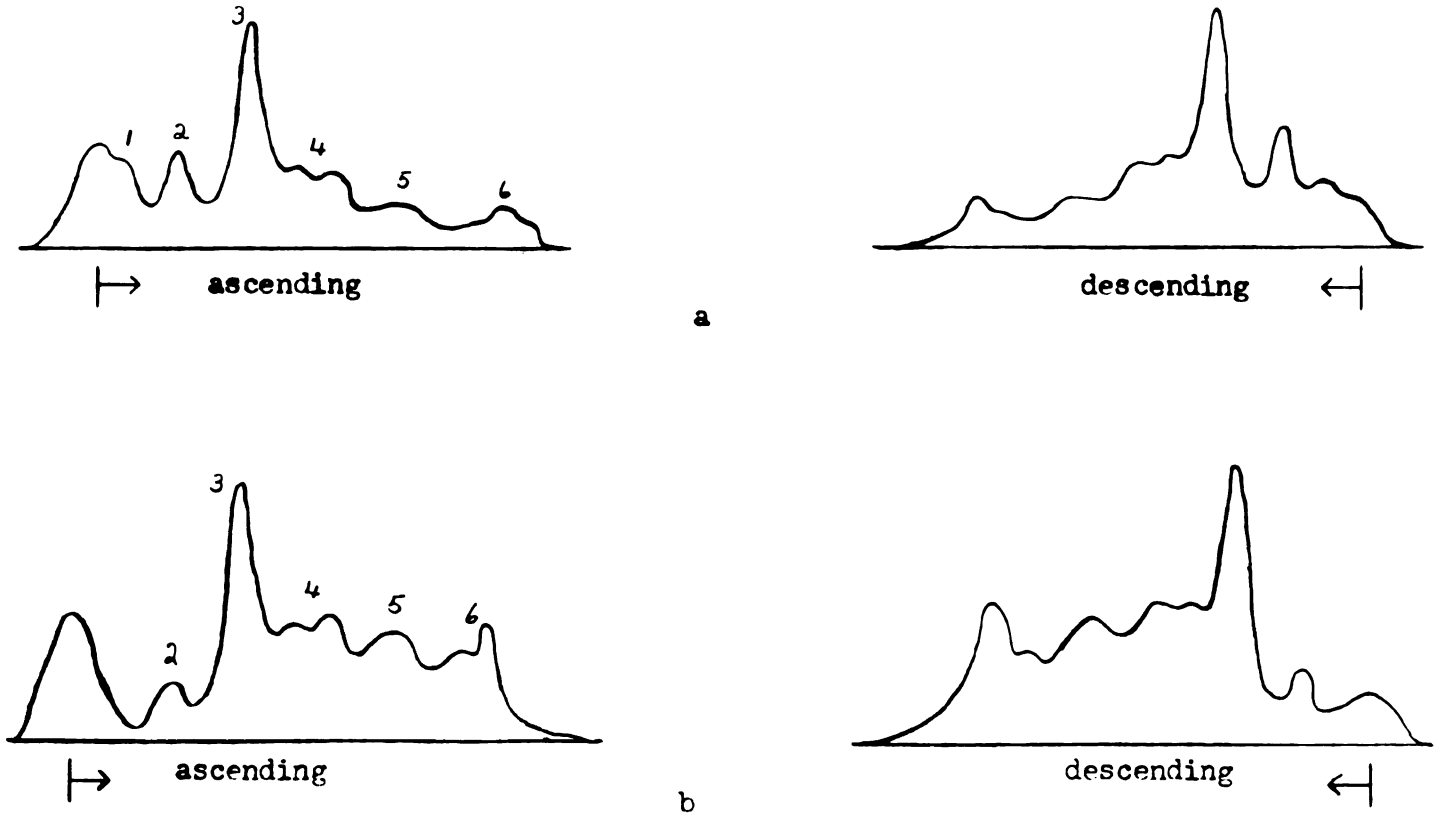

Figure 7. Electrophoretic patterns of (a) the starting solution and (b) the bottom fraction of electrophoresis run No. 22, illustrating the occasionally observed splitting of components 4 and 6 into two parts.

The objective of the fractionation originally was to obtain several of the components in highly purified condition in order to determine which ones were active against several substrates. When it became apparent that this was impractical with the amount of material available, three solutions containing the suspected components in distinctly different proportions were prepared. The electrophoretic patterns of these preparations are shown in Figures 8b, 9a, and 9b. Their sources were, respectively, the top fraction of run No. 24, and the bottom fractions of runs 29 and 27. The three solutions are subsequently designated as Preparations I, II, and III, respectively. The proteins of Preparation I were made up mostly of components 1 and 2, with only a small amount of component 3. Preparation II contained all the components except component 1, and Preparation III contained components 3-6, but no component 1 or 2. The resolution of components 4-6 in Preparation III was not satisfactory, but component 3 was well isolated and its mobility definitely distinguished it from components 2 and 4. Although the presence of a given component can usually be definitely established, the same confidence cannot be attached to statements concerning its absence. In this investigation, this was especially true of component 1, because very small amounts of this slow-moving component might be obscured by the boundary anomaly. With other components, for example 2 and 4 in Preparations III and I, respectively, amounts much less than 1 percent of the total protein probably would not be detected by the optical system of the apparatus, especially if the total protein concentration is low.

Inspection of the data presented in Table XVIII shows that most of the activity (CSS) was associated with the three proteins possessing the

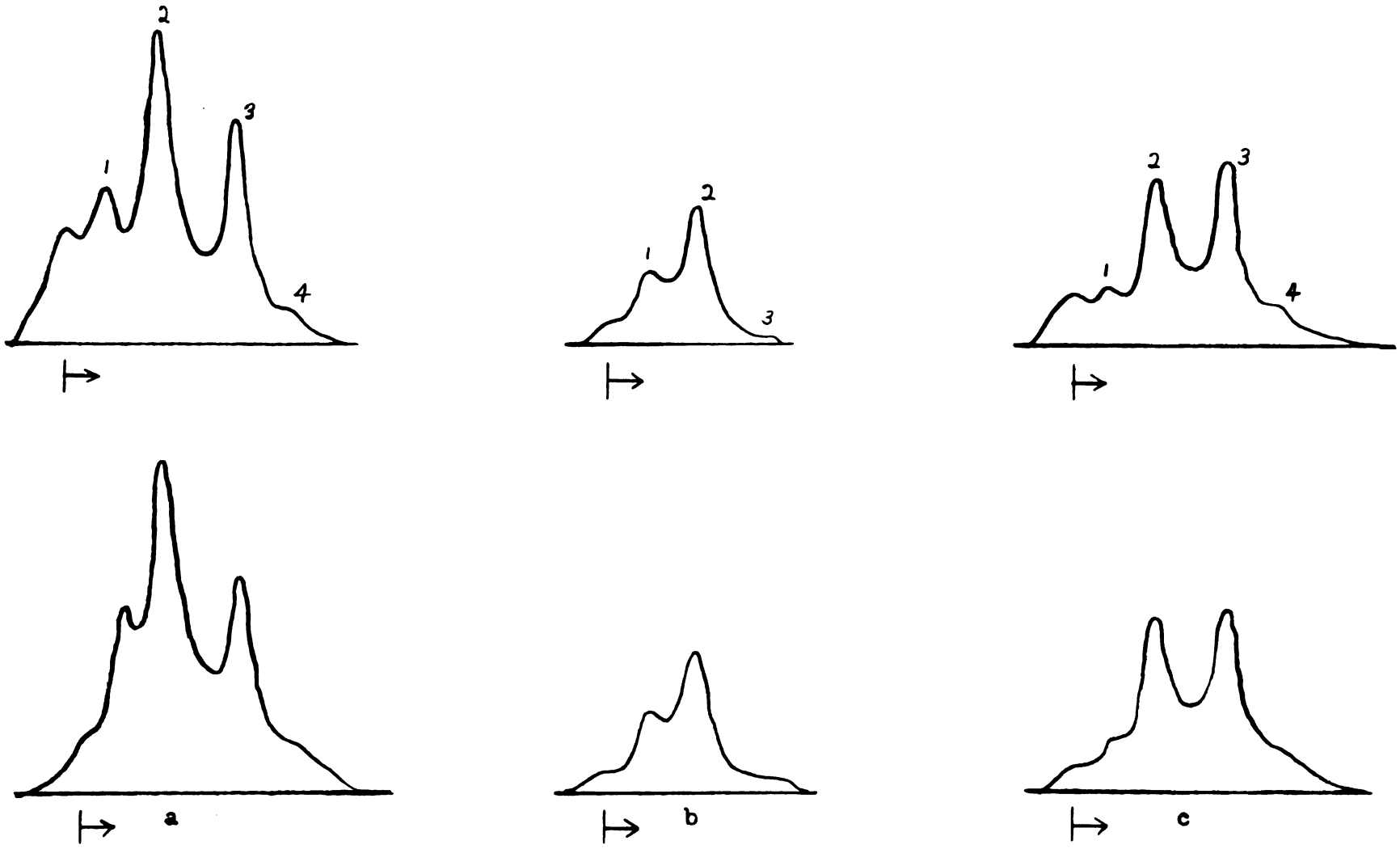

Figure 8. Electrophoretic patterns of (a) the starting solution, (b) top fraction, and (c) bottom fraction of electrophoresis-convection run No. 24. Upper patterns, ascending; lower patterns, descending. The top fraction (b) is the electrophoretic pattern of Preparation I.

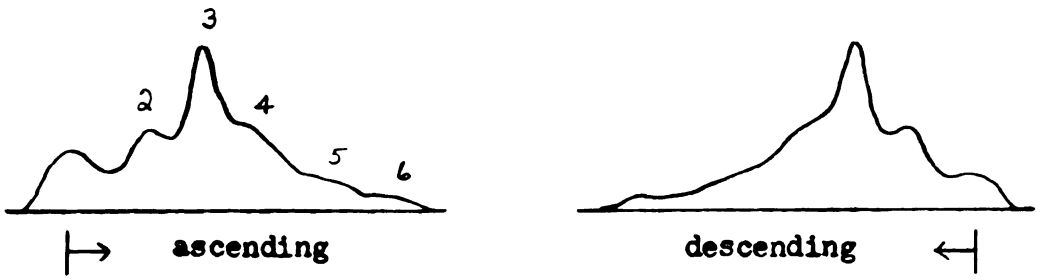

Figure 9a. Electrophoretic pattern of Preparation II.

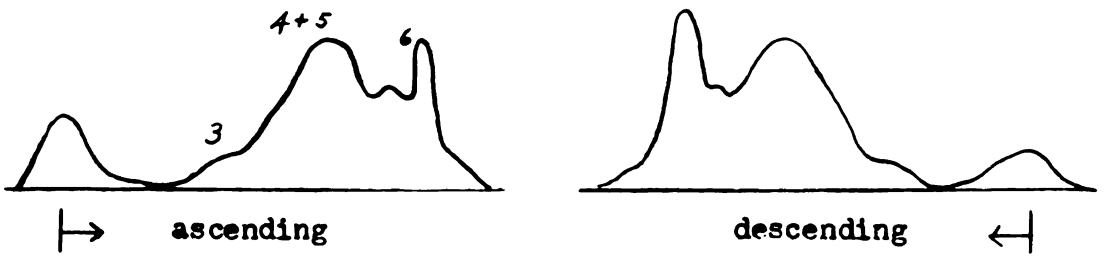

Figure 9b. Electrophoretic pattern of Preparation III.

lowest mobilities, and it appears that, of these, component 2 was the most powerful. The direct relationship between the relative concentration of component 2 and the specific activity is obvious. It also appears that components 4, 5, and 6 had very little, if any action on cellulose sodium sulfate. Although the results with Preparation III indicate that component 3 may be active, it is difficult to decide by casual inspection of the results whether component 1 had any hydrolytic action on the substrate.

The coefficients of correlation between the relative concentration of the components and the specific activity of the mixtures were calculated (Snedecor, 1946). The values are presented in Table XX. The

TABLE XX

COEFFICIENTS OF CORRELATION BETWEEN SPECIFIC ACTIVITY (CSS) AND RELATIVE CONCENTRATION OF COMPONENTS

Component No.	Coefficient of Correlation	Signficance at 5% level	Signficance at 1% level
1	0.71	+	+
2	0.92	+	+
1 + 2	0.94	+	+
3	0.43	+	-
1 + 3	0.45	+	-
2 + 3	0.72	+	+
1 + 2 + 3	0.82	+	+
4	-0.54	+ -	+
5 + 6	-0.67	+	+

conclusions stated previously concerning components 2, 4, 5, and 6 are confirmed by the coefficients. The high positive correlation between the relative concentration of component 2 and specific activity indicates that this protein was more active than any of the others. The negative coefficients for components 4, 5, and 6 indicate that they had little, if any, activity.

A coefficient of 0.71, such as the one for component 1, would ordinarily be interpreted to mean that there is a direct relationship between the two variables, especially since it satisfied the test for significance at the 1 percent level (Snedecor, 1946). However, interpretations in this case must be made with caution. It must be remembered that because of the similarity of the mobilities of components 1 and 2, any change in the relative concentration of the first component during electrophoresis convection was reflected by a qualitatively similar change in the relative concentration of the second. Thus, the relationship between relative concentration of component 1 and specific activity may be more apparent than real. On the other hand, when components 1 and 2 are considered together, the coefficient of correlation, 0.94, is higher than for either one alone. If component 1 were completely inactive, the coefficient of the combined proteins would be expected to be somewhat lower than that of component 2.

For the same reasons stated before, component 3 cannot be said to be active on the basis of the coefficient (0.43) even though the value is significant at the 5 percent level. In this case, treating components 2 and 3 together resulted in an intermediate coefficient, and adding components 1 and 3 gave a coefficient of 0.45, not significantly different

from that of component 3 alone. However, the results with Preparation III, which contained neither of the two slowest components, indicate that component 3 probably exerted weak hydrolytic action on cellulose sodium sulfate.

Action of *M. verrucaria* Enzymes on β-Glycosides, Extracted Wheat Straw, and Wheat Straw Holocellulose Preparations

The action of one enzyme preparation on arbutin, amygdalin, and phloridzin was studied. The experiment was undertaken to compare the susceptibility of these β-glycosides to the hydrolytic action of the enzymes with that of cellulose sodium sulfate. The preparation chosen, $EC_{26}T$, contained all the original protein components (see electrophoretic data, Table XVIII). The specific activity with cellulose sodium sulfate was 439. The results with the β-glycosides are given in Table XXI. The

TABLE XXI

HYDROLYSIS OF β-GLYCOSIDES WITH *M. VERRUCARIA* ENZYMES

(Enzyme preparation: $EC_{26}T$)

Substrate	Specific activity	
	2 hours	7 hours
Arbutin	8.8	20.0
Amygdalin	2.0	7.7
Phloridzin	0.6	1.6
Cellulose sodium sulfate	349.	

results with arbutin were corrected for the presence of hydroquinone, 0.935 mg. of which was found to be equivalent in reducing power in Somogyi's reagent to 1.0 mg. of glucose. In the case of amygdalin, no attempt was made to determine whether the reducing sugar end product was glucose or gentiobiose, or whether benzaldehyde contributed to the reduction of copper. The concentration of phloridzin was 0.1 percent; that of the other substrates was one percent. With this exception, the conditions were the same as usual.

The results show that, under the conditions employed, the β-glycosidic activity of the preparation was very low in comparison with its action on cellulose sodium sulfate. The highest specific activity at two hours incubation time was only 2 percent of the specific activity with cellulose sodium sulfate. Because of the low results, differences of the specific activities obtained with the three β-glycosides are probably of little significance.

The results of an experiment comparing the action of two enzyme preparations on extracted wheat straw and three wheat straw holocellulose preparations are presented in Table XXII. The purpose was to determine the effect of delignification on the availability of the substrates. The enzyme preparations employed were the top and bottom fractions of electrophoresis convection fractionation 19. The substrates have been described previously. All conditions were standard. For comparison, the results with cellulose sodium sulfate are included in the results.

Although the three holocelluloses supposedly contain different percentages of lignin (the designations 1, 2, 3 refer to the number of acid chlorite delignification steps each underwent), no consistent or

TABLE XXII

ACTION OF M. VERRUCARIA ENZYMES ON EXTRACTED WHEAT STRAW AND WHEAT STRAW HOLOCELLULOSE PREPARATIONS

Substrate	Specific activity	
	$FC_{1e}T$	$FC_{1e}B$
Extracted wheat straw	6.4	3.6
Holocellulose 1	13.9	5.8
Holocellulose 2	14.8	5.9
Holocellulose 3	12.9	5.9
Cellulose sodium sulfate	923.	438.

significant differences in their enzymatic hydrolysis could be detected. However, the extracted (ethanol-benzene) wheat straw was noticeably more resistant than the holocelluloses. Although other changes may have been involved, the removal of most of the lignin probably was an important factor in making the polysaccharides more available to the enzymes.

Comparison of Cellulase, Hemicellulase, and β-Glucosidase Activities of Three Enzyme Preparations

Comparative activities with standard conditions. The specific activities of Preparations I, II, and III with five substrates were estimated and compared to determine whether one, or more than one, enzyme was involved in each case. Standard conditions were used throughout; it will be recalled that the standard incubation time for cellulose sodium sulfate and salicin was 2 hours, and for cellulose and the hemicelluloses, 24 hours. The results are shown in Table XXIII.

TABLE XXIII

COMPARISON OF SPECIFIC ACTIVITIES OF PREPARATIONS I, II, AND III ACTING ON FIVE SUBSTRATES

Substrate	Specific activity			Relative specific activity		
	I	II	III	I	II	III
Cellulose sodium sulfate	1600.	329.	159.	1.00	0.21	0.099
Hemicellulose X	912.	177.	106.	1.00	0.19	0.12
Hemicellulose Y	484.	163.	109.	1.00	0.34	0.23
Wheat straw cellulose	93.0	58.0	15.8	1.00	0.62	0.17
Salicin	231.	20.1	13.2	1.00	0.087	0.057

For easier comparison of the results with the three enzyme preparations acting on the same substrate, relative values have been calculated by arbitrarily assigning Preparation I a specific activity of 1.00 for each substrate and calculating the relative values of the other two. These ratios are also presented in Table XXIII.

The electrophoretic composition of the three enzyme preparations has been taken from the table presented earlier and brought together in Table XXIV for greater convenience in correlating the electrophoretic and activity data.

The activity data with respect to cellulose sodium sulfate were taken from Table XVIII. Standing alone, the results agree substantially with the main body of data presented in Table XVIII. Component 2 was the most active in hydrolyzing cellulose sodium sulfate, but components 1 and 3 also probably had some hydrolytic action on the substrate.

TABLE XXIV

ELECTROPHORETIC COMPOSITION OF PREPARATIONS I, II, AND III

Preparation No.	Relative concentration of component				
	1	2	3	4	5 and 6
	percent of total protein nitrogen				
I	30.7	63.3	6.1	0.0	0.0
II	0.0	19.8	38.3	25.4	16.6
III	0.0	0.0	3.8	96.2	

The specific activities of the three preparations acting on hemicellulose X were in nearly the same ratio as with cellulose sodium sulfate, indicating that the same enzymes hydrolyzed the two substrates. In the case of hemicellulose Y, the ratio was somewhat changed, making it appear that components 1 and 3 were not as active with this substrate. The relatively high specific activity of Preparation III is difficult to interpret without assuming that components 4-6 possessed some activity.

With wheat straw cellulose as the substrate, the results were again quite different from those with cellulose sodium sulfate. In this case, component 1 probably had little activity. The distribution of activity among the remaining components is not clear from the results. Preparation II had a specific activity of 64 percent of that of Preparation I, whereas its content of component 2 was only about one-third as great. The activity of component 3 alone could not have made up the difference because Preparation III had a specific activity nearly one-half as large as that of II, but contained only about one-tenth as much component 3. Assuming that component 2 was probably the most active, it must be tentatively concluded that components 4-6 contributed a substantial share.

The results with salicin were more clear-cut. Preparation I had a high specific activity, whereas those of Preparations II and III were both very low and differed from each other only slightly. It appears that component 1 was the only really effective "salicinase". The activity of the other components was very low with this substrate.

In summary, the results indicate that component 2 was the most active enzyme with the four polysaccharide substrates. It was relatively more active with cellulose and hemicellulose Y than with cellulose sodium sulfate and hemicellulose X. Components 1 and 3 evidently had some action on the latter two substrates, but relatively less action on cellulose and hemicellulose Y. Evidence that component 4, 5, or 6 had some hydrolytic effect on all the polysaccharide substrates, especially cellulose and hemicellulose Y, is fairly convincing. The results with salicin were unique in that they indicated that a single component, 1, possessed almost all the activity.

It is interesting to note the similarity in behavior of the three enzyme preparations toward the two soluble polysaccharide substrates (cellulose sodium sulfate and hemicellulose X). The results with the insoluble substrates (cellulose and hemicellulose Y) were similar to each other but different from those with the soluble substrates.

Although it is not possible to assign specific activities to each enzyme with the data available, the evidence is good that more than one enzyme was involved with each substrate. It is also obvious that at least one enzyme acted on both cellulose and hemicellulose. In other words, at least one cellulase was identical with a hemicellulase.

Effect of pH on the rate of enzymatic hydrolysis. The effect of pH on the rate of hydrolysis of the five substrates by the three enzyme preparations was studied. In most cases, seven or eight pH levels, ranging from pH 4 to pH 7, were used. Samples of the hydrolysates were analyzed for reducing sugars at intervals over a 24-hour period. The original data, presented in Tables XXXVIII - XLII in Appendix A, were used to construct progress curves. The curves for three of the pH levels used for each combination of enzyme preparation and substrate are presented in Figures 10-24. For the sake of comparison, one of the curves representing the hydrolysis of each substrate by Preparations II and III was reproduced on the corresponding graph for Preparation I. The curves chosen for presentation were usually the ones for the lowest and highest pH's and for the pH which gave maximum activity. Because of the heterogeneous nature of some of the substrates and the possible multiple nature of the enzyme preparations, reaction rate constants were not calculated.

The curves representing the hydrolysis of a single substrate by the three enzyme preparations are very much alike, but those illustrating the action of a single enzyme preparation on different substrates are quite different. Whereas the curves for the hydrolysis of cellulose sodium sulfate (Figures 10-12) showed a steadily decreasing rate of hydrolysis, those for the hydrolysis of cellulose (Figures 13-15), hemicellulose X (Figures 16-18), and hemicellulose Y (Figures 19-21) were biphasic. In all three cases, the relatively high initial rate of hydrolysis was followed by a rapid change to a long-continued, slower rate of reaction. The effect was most pronounced with hemicellulose Y.

Walseth (1948) obtained the same type of results for the hydrolysis

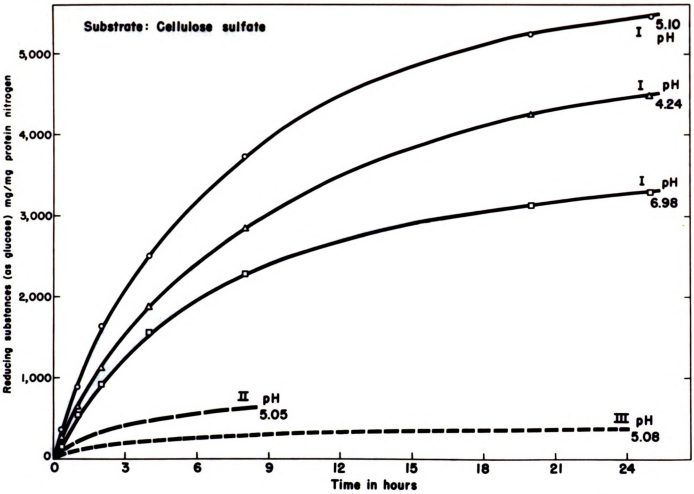

Figure 10. Progress curves for the enzymatic hydrolysis of cellulose sodium sulfate at three pH levels. Enzyme preparation I, 4.2 micrograms of protein nitrogen per 10 ml. of reaction mixture. Curves for enzyme preparations II and III are included for comparison.

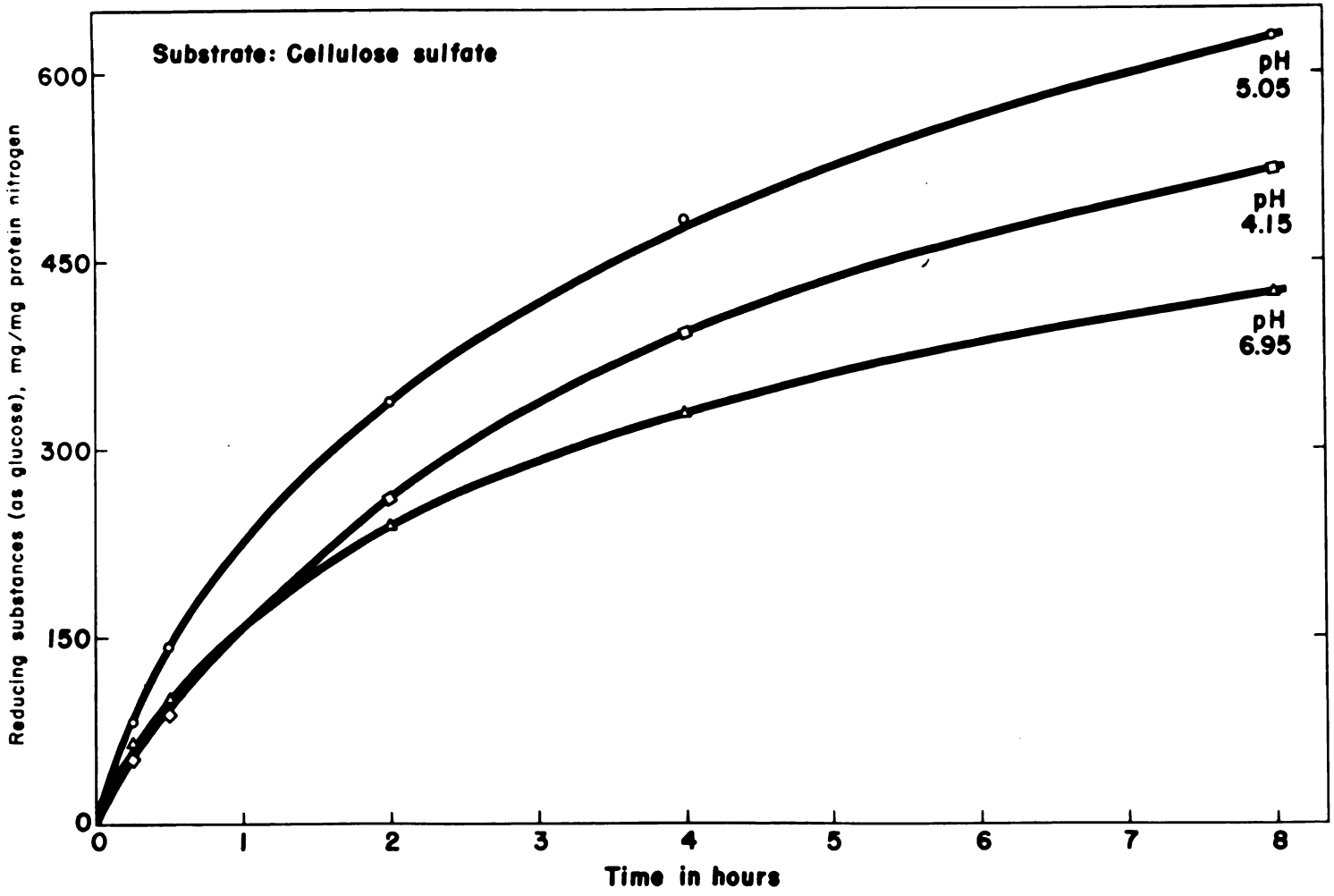

Figure 11. Progress curves for the enzymatic hydrolysis of cellulose sodium sulfate at three pH levels. Enzyme preparation II, 18.9 micrograms of protein nitrogen per 10 ml. of reaction mixture.

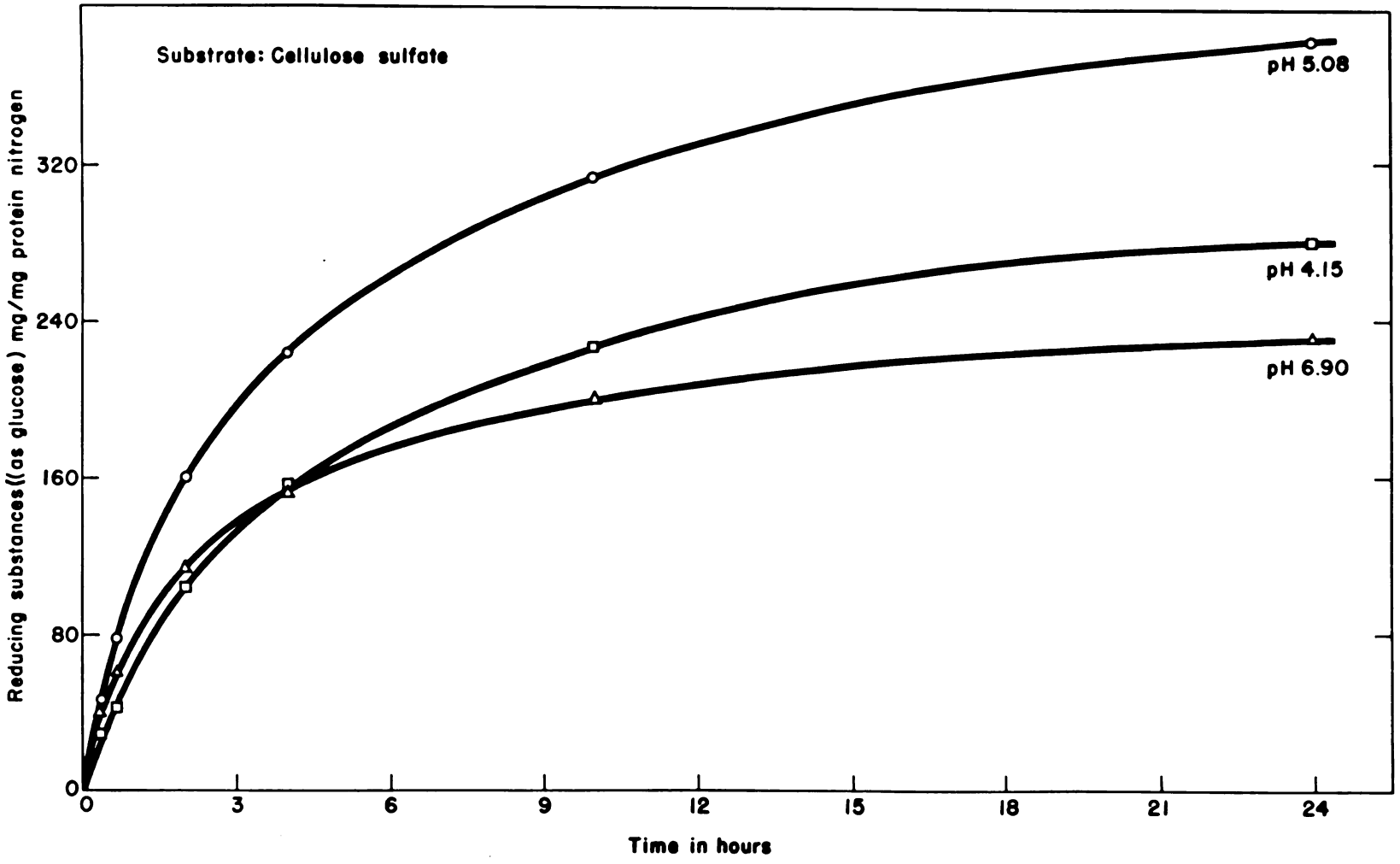

Figure 12. Progress curves for the enzymatic hydrolysis of cellulose sodium sulfate at three pH levels. Fnzyme preparation III, 33.6 micrograms of protein nitrogen per 10 ml. of reaction mixture.

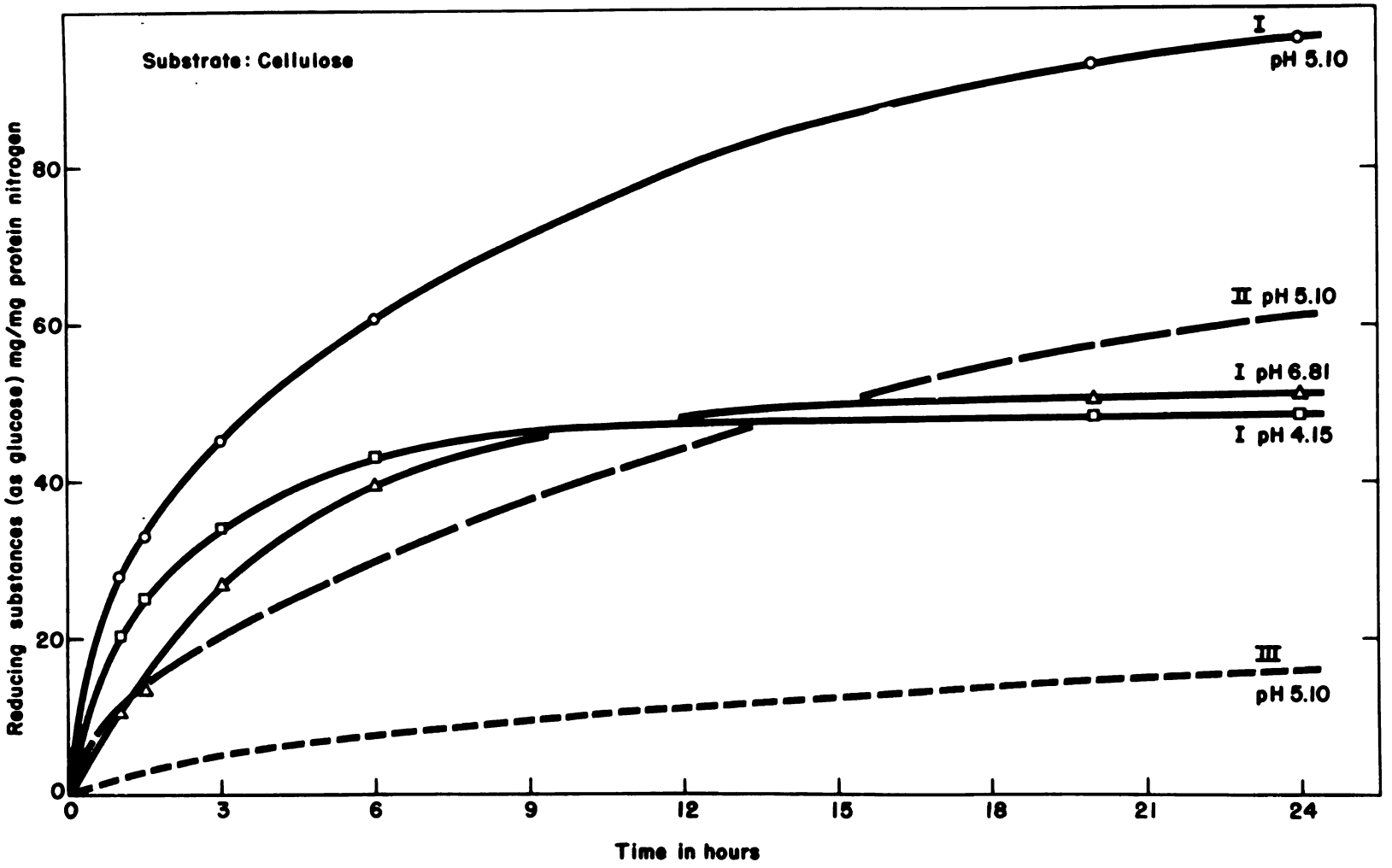

Figure 13. Progress curves for the enzymatic hydrolysis of wheat straw cellulose at three pH levels. Enzyme preparation I, 83.4 micrograms of protein nitrogen per 10 ml. of reaction mixture. Curves for enzyme preparations II and III are included for comparison.

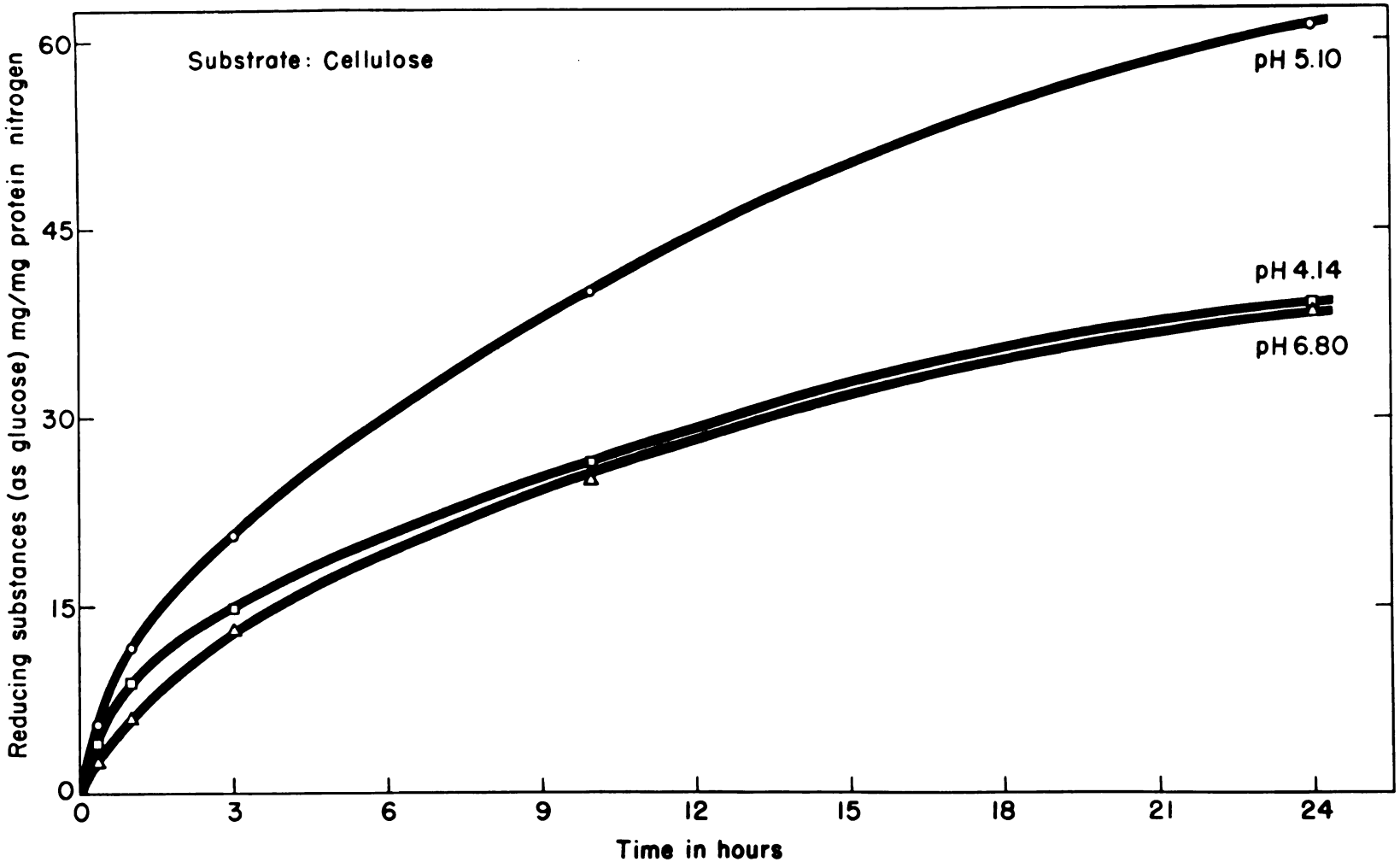

Figure 14. Progress curves for the enzymatic hydrolysis of wheat straw cellulose at three pH levels. Enzyme preparation II, 94.7 micrograms of protein nitrogen per 10 ml. of reaction mixture.

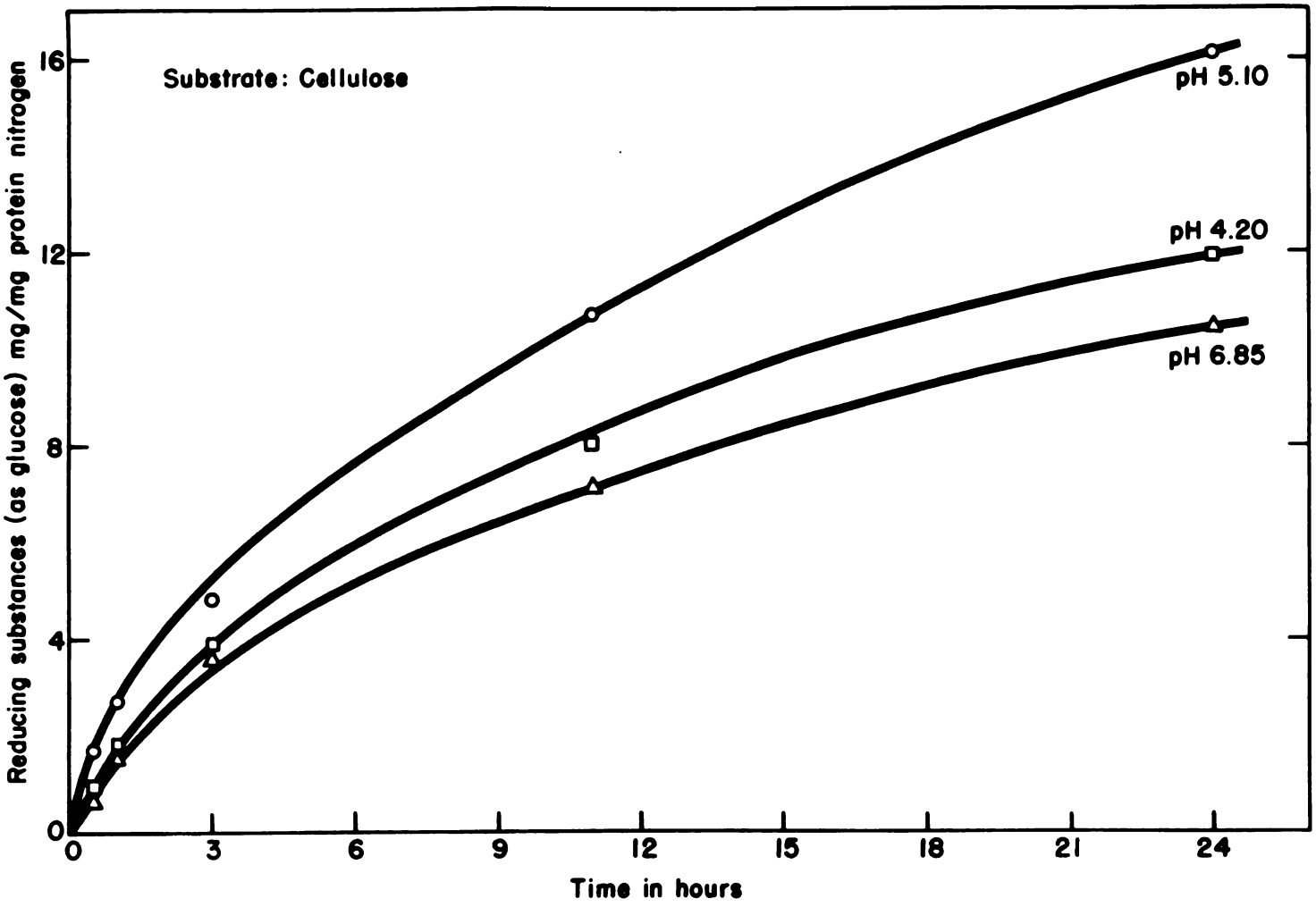

Figure 15. Progress curves for the enzymatic hydrolysis of wheat straw cellulose at three pH levels. Enzyme preparation III, 336 micrograms of protein nitrogen per 10 ml. of reaction mixture.

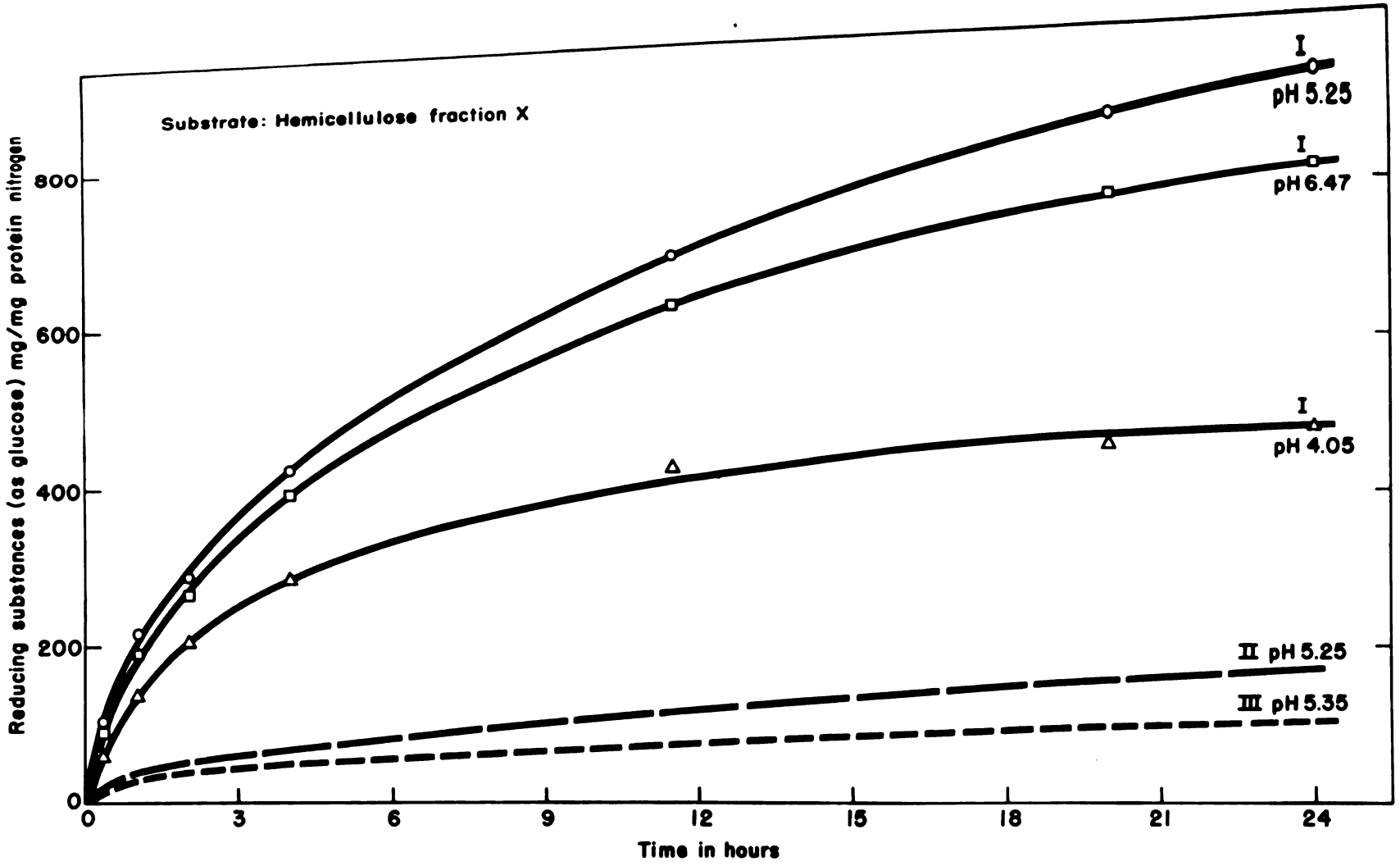

Figure 16. Progress curves for the enzymatic hydrolysis of hemicellulose X at three pH levels. Enzyme preparation I, 8.3 micrograms of protein nitrogen per 10 ml. of reaction mixture. Curves for enzyme preparations II and III are included for comparison.

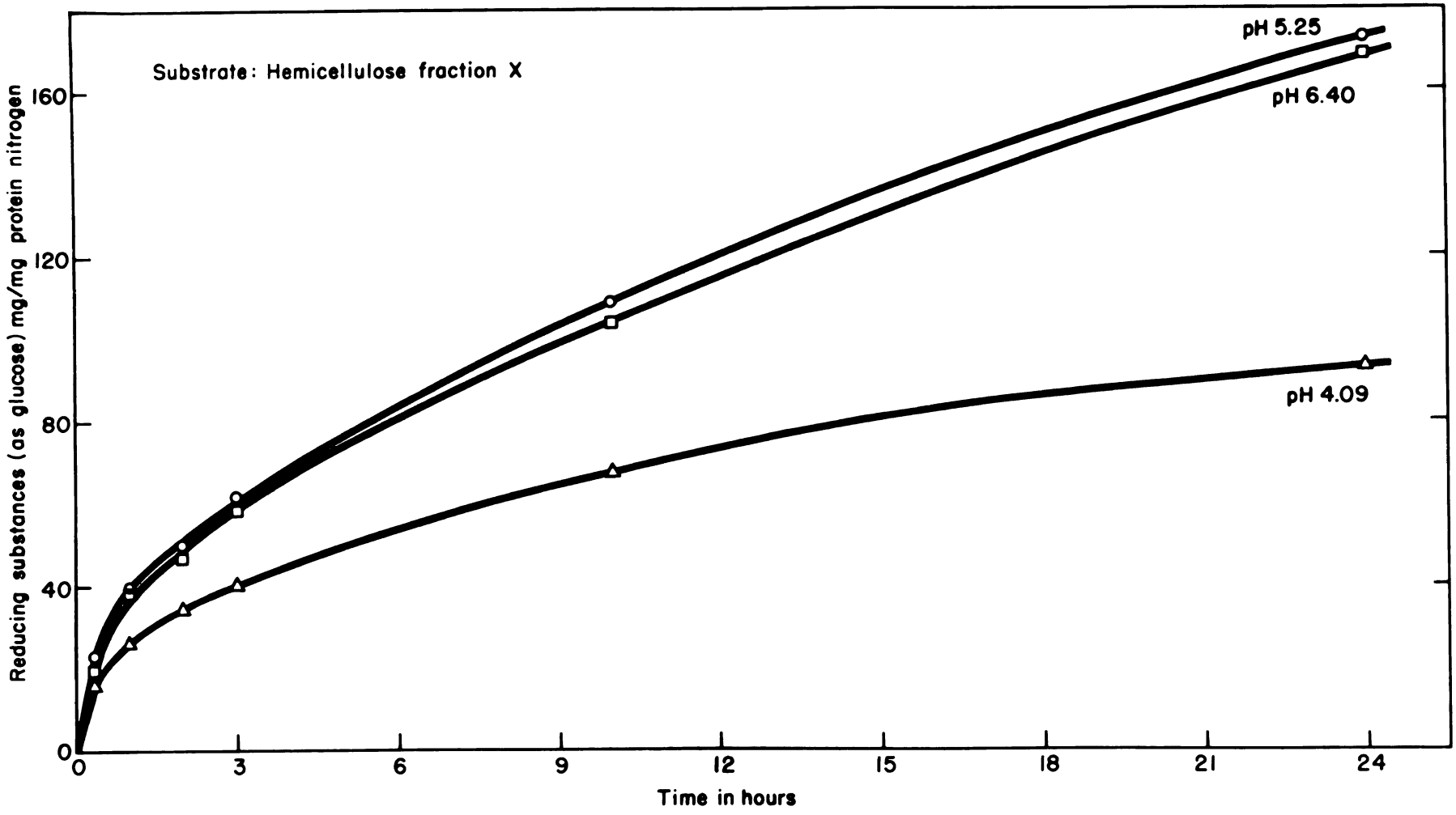

Figure 17. Progress curves for the enzymatic hydrolysis of hemicellulose X at three pH levels. Enzyme preparation II, 37.8 micrograms of protein nitrogen per 10 ml. of reaction mixture.

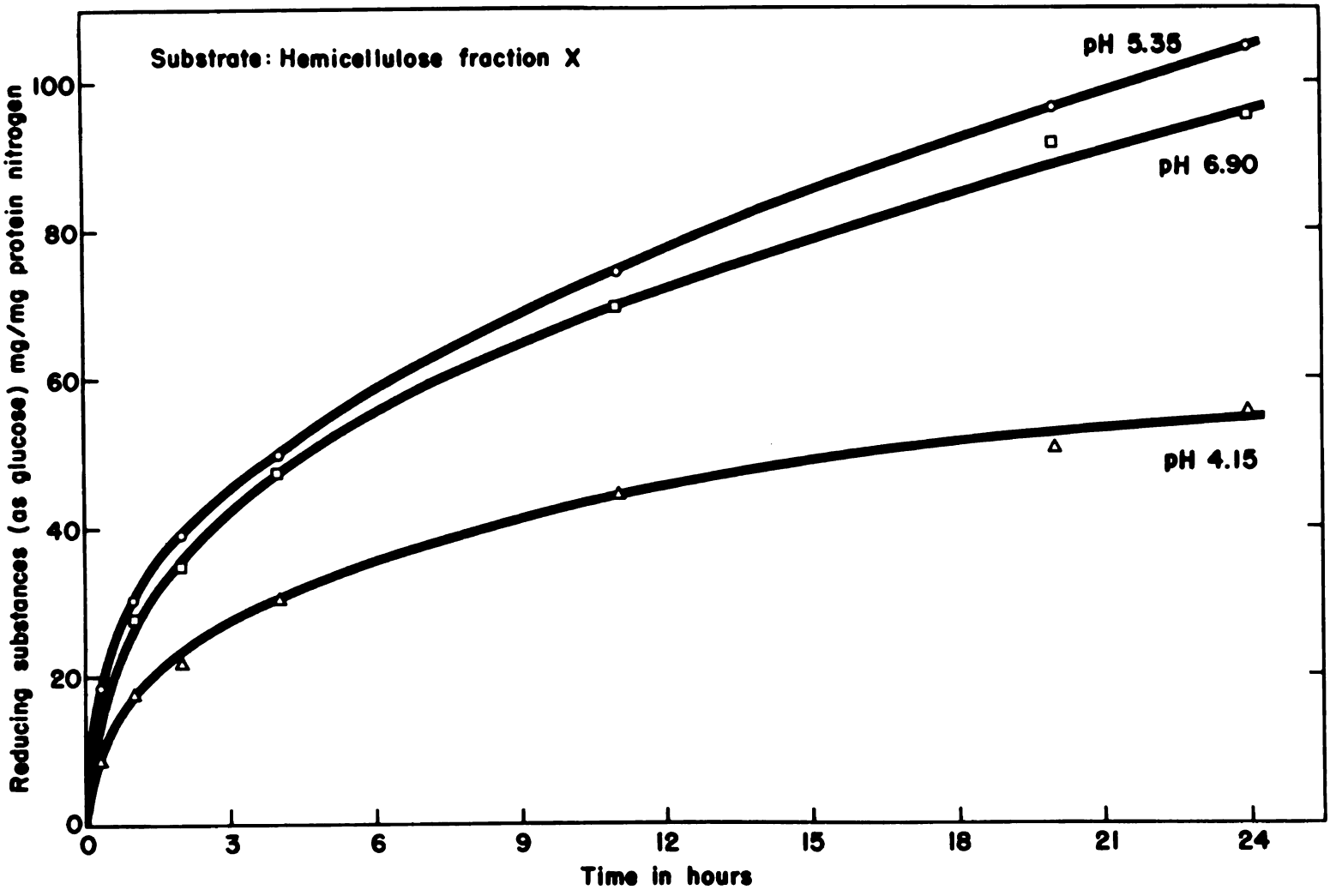

Figure 18. Progress curves for the enzymatic hydrolysis of hemicellulose X at three pH levels. Enzyme preparation III, 67.2 micrograms of protein nitrogen per 10 ml. of reaction mixture.

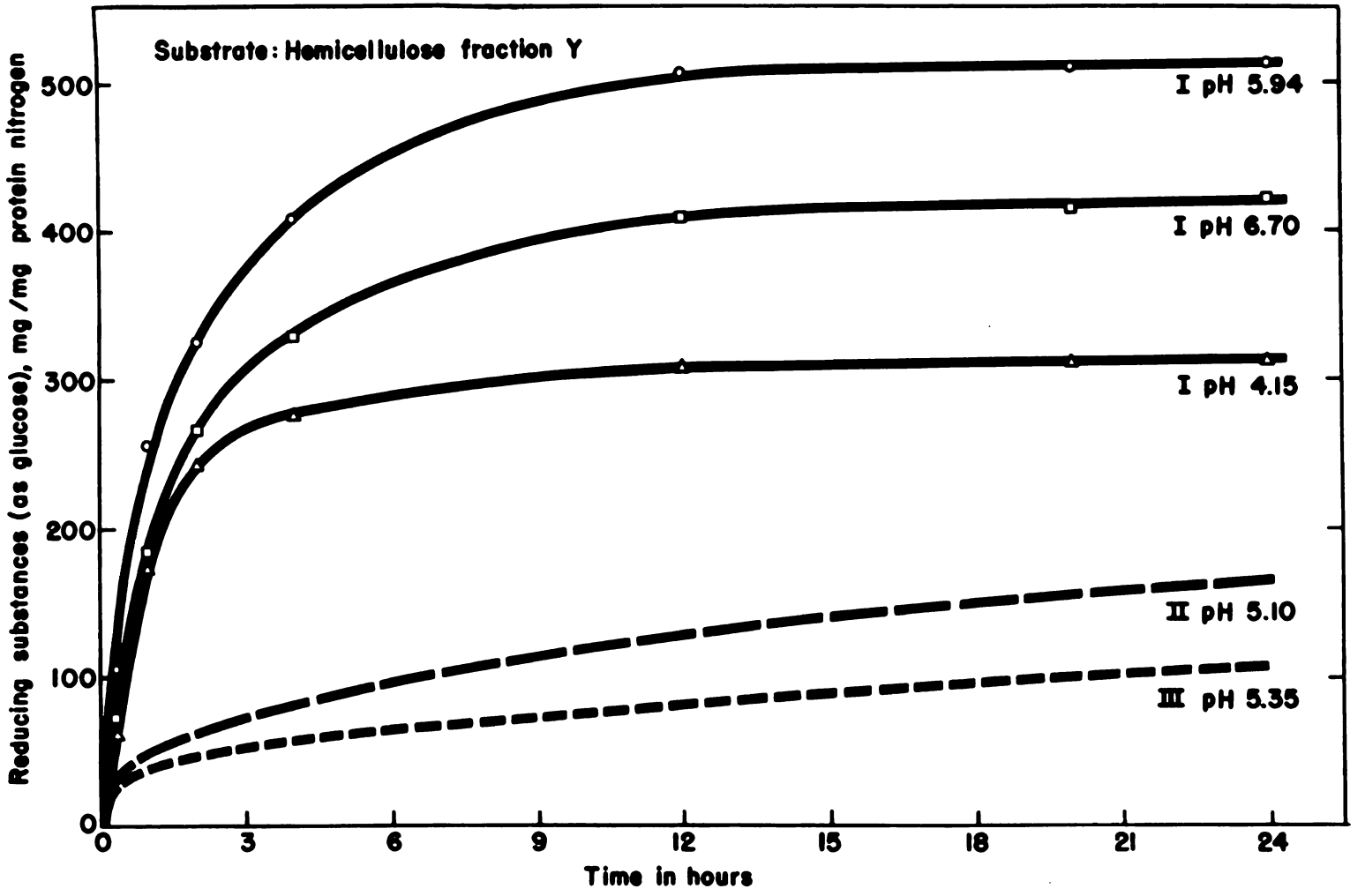

Figure 19. Progress curves for the enzymatic hydrolysis of hemicellulose Y at three pH levels. Enzyme preparation I, 16.7 micrograms of protein nitrogen per 10 ml. of reaction mixture. Curves for enzyme preparations II and III are included for comparison.

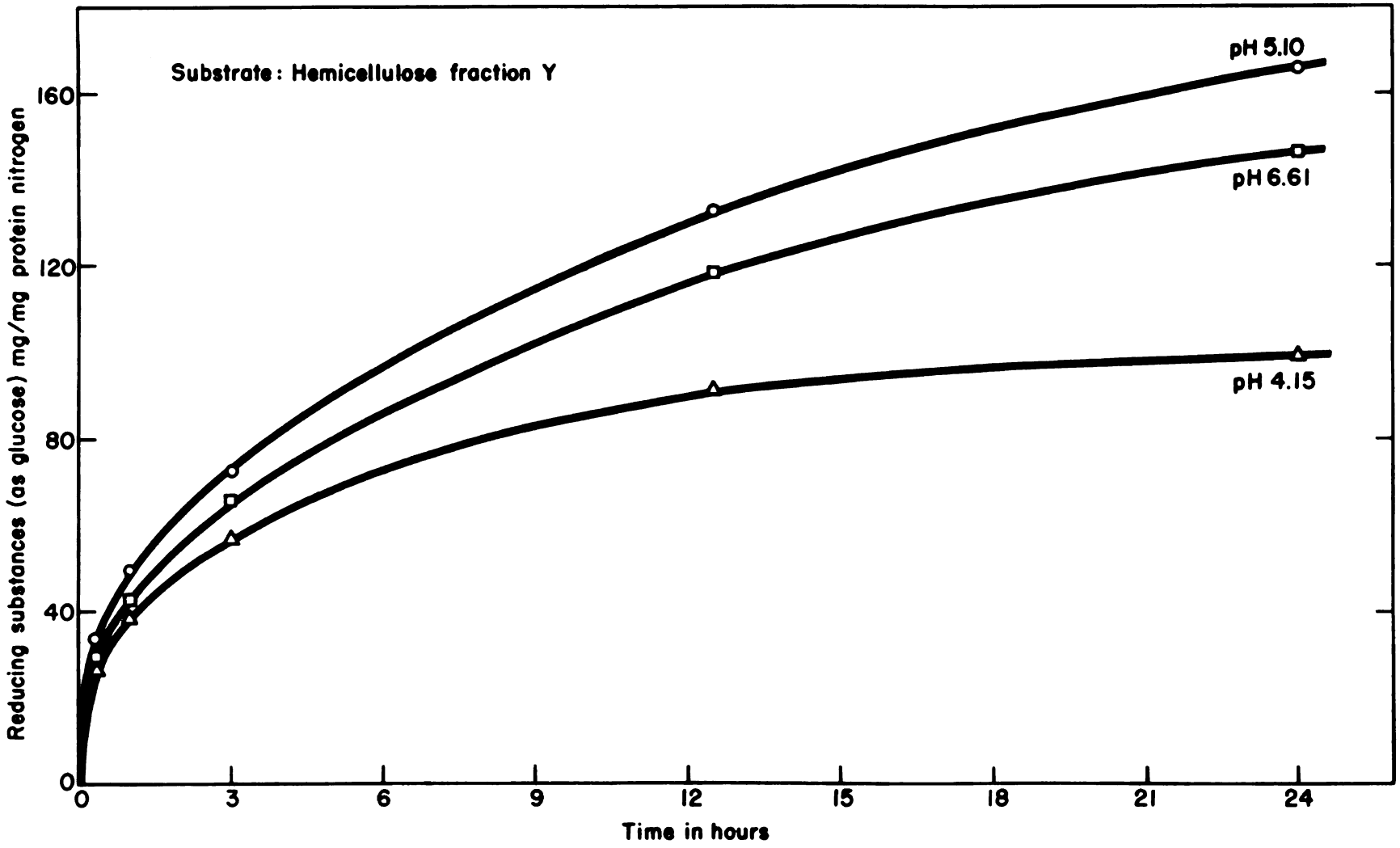

Figure 20. Progress curves for the enzymatic hydrolysis of hemicellulose Y at three pH levels. Enzyme preparation II, 37.8 micrograms of protein nitrogen per 10 ml. of reaction mixture.

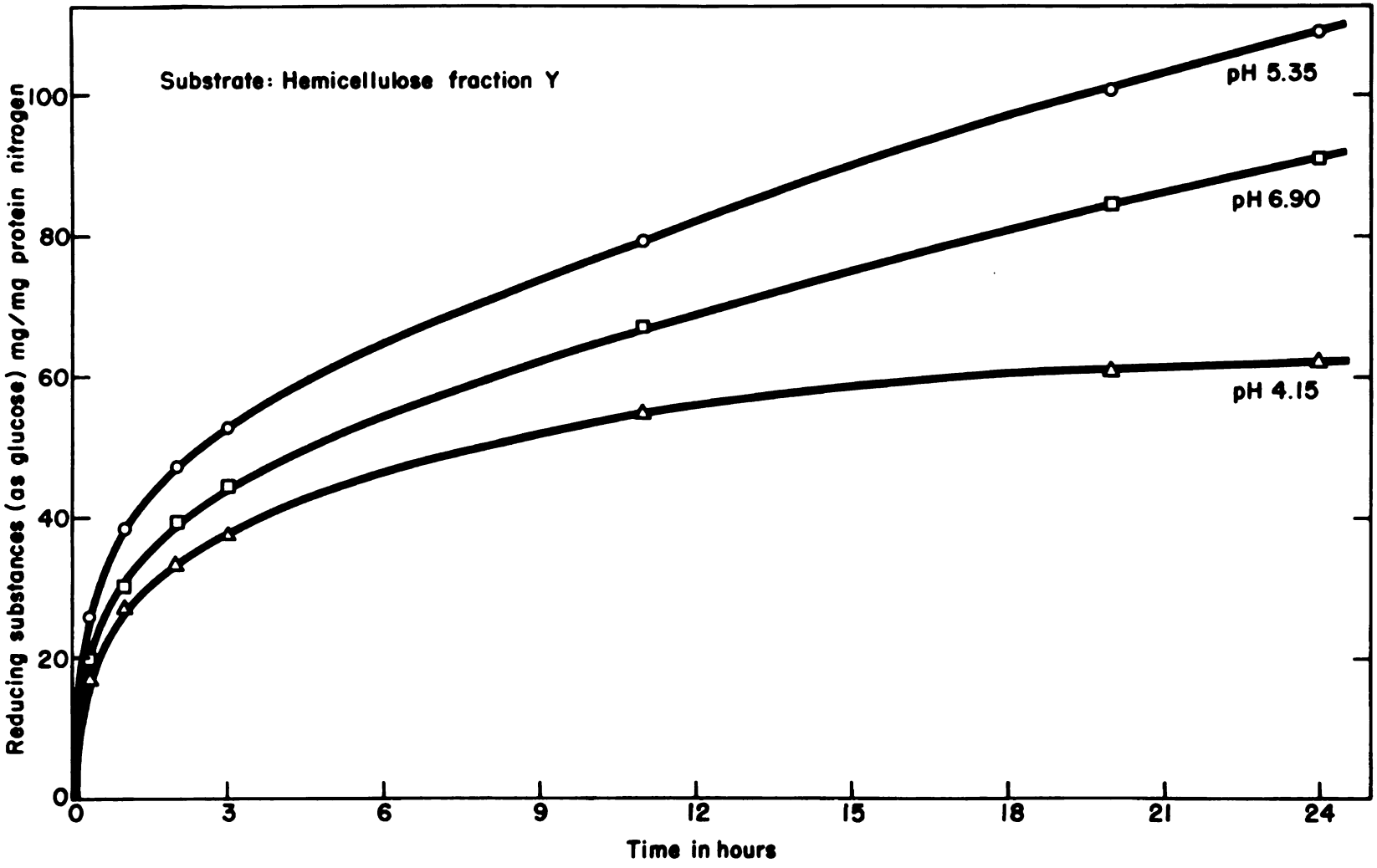

Figure 21. Progress curves for the enzymatic hydrolysis of hemicellulose Y at three pH levels. Enzyme preparation III, 67.2 micrograms of protein nitrogen per 10 ml. of reaction mixture.

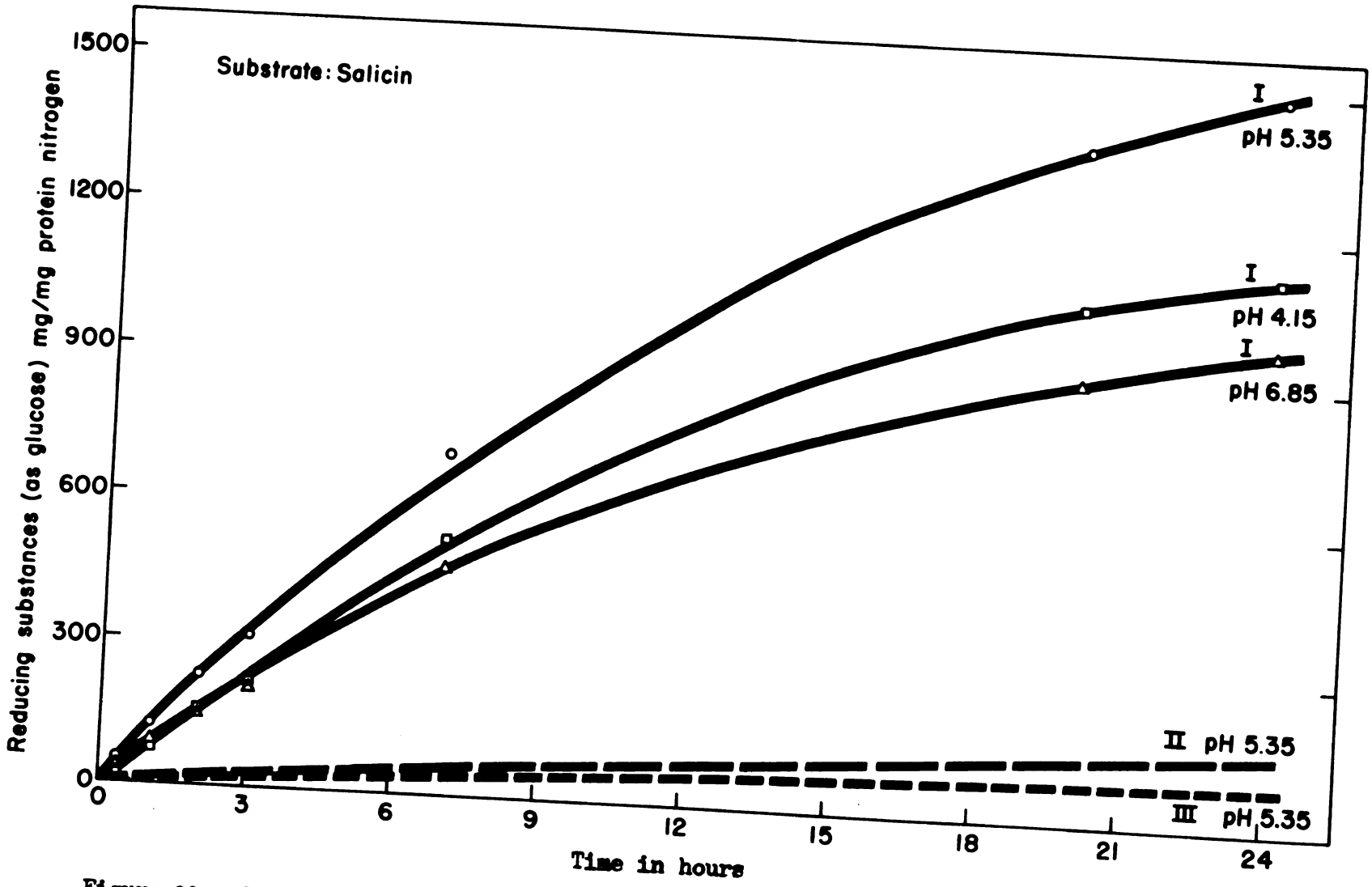

Figure 22. Progress curves for the enzymatic hydrolysis of salicin at three pH levels. Enzyme preparation I, 41.7 micrograms of protein nitrogen per 10 ml. of reaction mixture. Curves for enzyme preparations II and III are included for comparison.

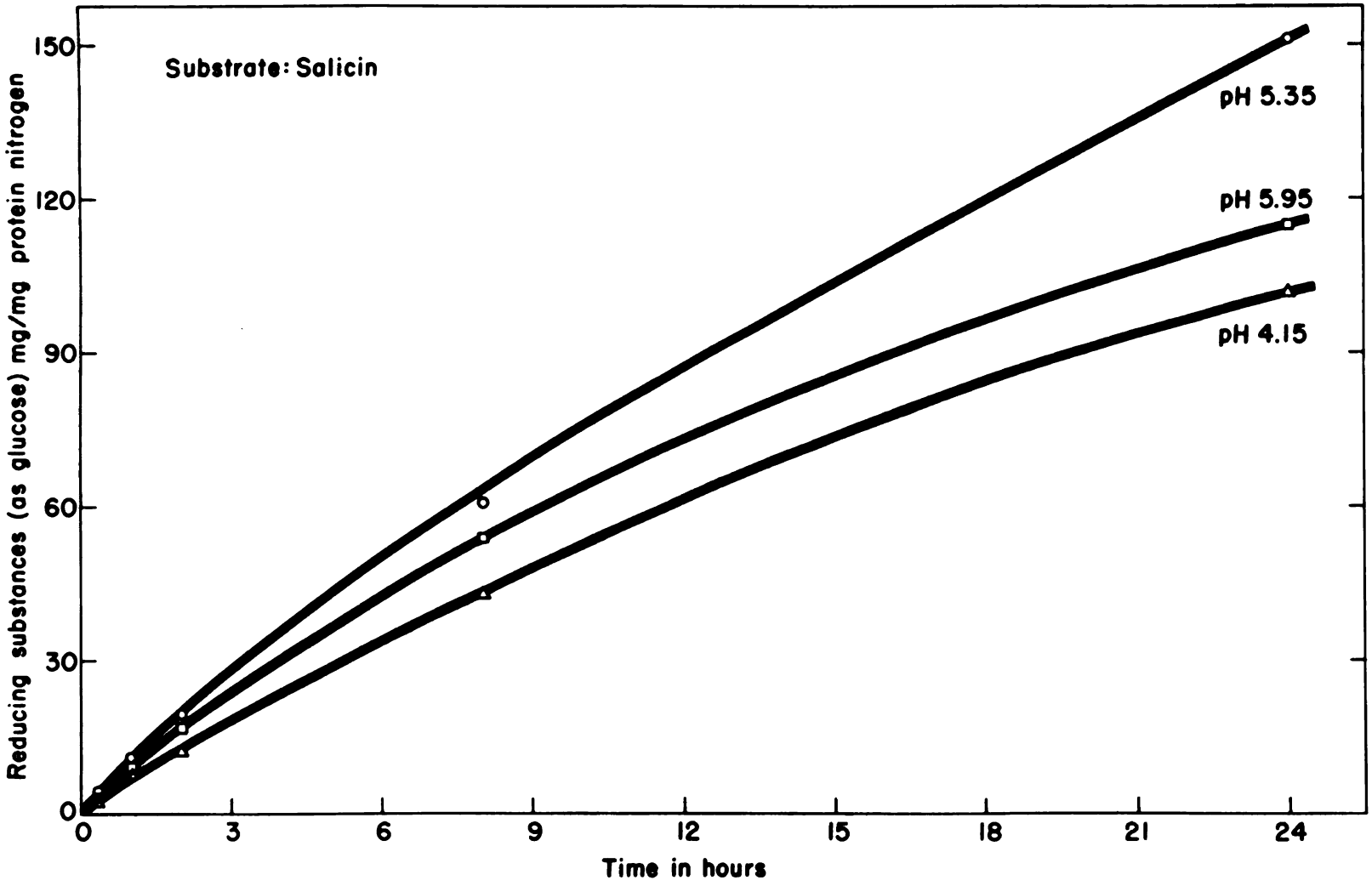

Figure 23. Progress curves for the enzymatic hydrolysis of salicin at three pH levels. Enzyme preparation II, 237 micrograms of protein nitrogen per 10 ml. of reaction mixture.

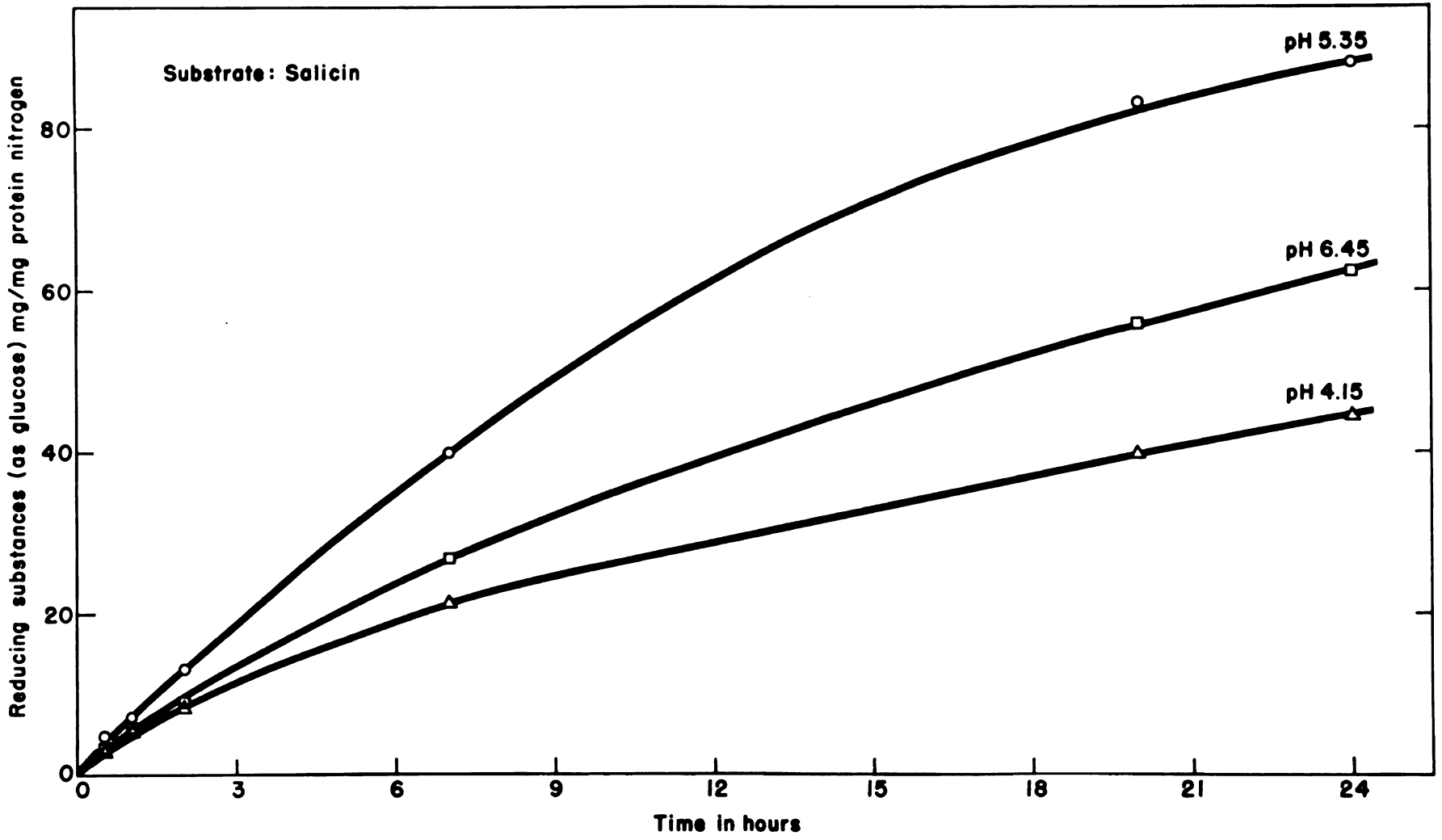

Figure 24. Progress curves for the enzymatic hydrolysis of salicin at three pH levels. Enzyme preparation III, 336 micrograms of protein nitrogen per 10 ml. of reaction mixture.

of phosphoric acid-swollen cellulose by A. niger cellulase preparations. His evidence suggested that the rapid primary phase was due to hydrolysis of the more accessible amorphous regions, and the slower secondary phase to the hydrolysis of the more crystalline areas of the substrate. The same explanation probably does not apply to the hydrolysis of the hemicellulose preparations. In the first place, hemicellulose X was completely dispersed. Secondly, both hemicellulose preparations used in this study were probably mixtures. Furthermore, they were made up of several different sugar residues and very likely contained more than one type of linkage. Branched chain structures were probably present. Some of the polysaccharides in the mixture, and certain portions of other components would probably be more susceptible to enzymatic hydrolysis than others.

The curves for the hydrolysis of salicin (Figures 22-24) resembled none of the others. The initial rate of hydrolysis decreased so slowly that the curves approach the straight line characteristic of zero-order reactions.

To study the effect of pH more effectively, the milligrams of reducing substances per milligram of protein nitrogen at specified times were taken from the curves presented in Figures 10-24 and from similar curves (not shown) for every pH level. These values are presented in Tables XXV-XXIX. Because of the relatively large differences between the absolute values for each of the enzyme preparations, graphical comparisons of the results in absolute terms would be difficult. To make comparison easier, the maximum value for each enzyme preparation with each substrate was arbitrarily given a value of 100 percent and the specific activities at the other pH levels in relation to the maximum

TABLE XXV

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF CELLULOSE SODIUM SULFATE BY THREE DIFFERENT ENZYME PREPARATIONS

Enzyme preparation	pH	Reducing substances (as glucose)					
		20 minutes		2 hours		24 hours	
		mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.
I	3.78	110	30.6	630	38.4	3120	57.6
	4.24	240	66.7	1150	70.1	4440	81.9
	4.80	320	88.9	1480	90.2	5280	97.4
	5.10	360	100.0	1640	100.0	5420	100.0
	5.34	345	95.8	1550	94.5	5370	99.1
	5.95	297	82.5	1410	86.0	4840	89.3
	6.45	290	80.6	1360	82.9	4380	80.8
	6.98	160	44.4	920	56.1	3260	60.1
	7.37	125	34.7	615	37.5	2420	44.6
	7.55	90.0	25.0	450	27.4	2200	40.6
II	4.15	67.0	62.0	263	77.8		
	4.75	94.0	87.0	334	98.8		
	5.05	108	100.0	338	100.0		
	5.35	106	98.1	325	96.1		
	5.90	103	95.4	322	95.3		
	6.45	89.0	82.4	285	84.3		
	6.95	72.6	67.2	239	70.7		
III	4.15	25.0	54.2	104	64.6	280	72.2
	4.75	38.0	82.4	156	96.9	402	104
	5.08	46.1	100.0	161	100.0	388	100.0
	5.37	45.5	98.7	157	97.5	378	97.4
	5.65	45.0	97.6	151	93.8	358	92.3
	6.00	44.0	95.4	148	91.9	318	81.9
	6.51	42.6	92.4	141	87.6	295	76.0
	6.90	39.9	86.6	114	70.8	231	59.5

TABLE XXVI

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF WHEAT STRAW CELLULOSE BY THREE DIFFERENT ENZYME PREPARATIONS

Enzyme preparation	pH	Reducing substances (as glucose)					
		2 hours		8 hours		24 hours	
		mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.	mg./mg. prot. N	% of max.
I	4.15	28.6	75.3	45.5	66.5	48.6	50.4
	4.75	37.7	99.2	67.3	98.4	90.6	94.0
	5.10	38.0	100.0	68.4	100.0	96.4	100.0
	5.33	37.3	98.1	68.0	99.4	94.7	98.2
	5.95	27.2	71.6	48.9	71.5	55.9	58.0
	6.45	24.0	63.1	49.7	72.7	61.9	64.2
	6.81	19.4	51.0	44.0	64.3	51.1	53.0
II	4.14	12.3	74.1	23.7	66.8	39.2	64.3
	4.72	14.7	88.5	32.9	92.7	57.0	93.4
	5.10	16.6	100.0	35.5	100.0	61.0	100.0
	5.35	16.6	100.0	36.1	102	59.2	97.0
	5.92	15.1	91.0	32.2	90.7	56.1	92.0
	6.43	11.6	70.0	25.6	72.1	46.5	76.2
	6.80	9.8	59	22.6	63.7	38.3	62.8
III	4.20	3.0	71	7.0	78	11.9	73.9
	4.80	4.0	95	8.7	97	15.8	98.1
	5.10	4.2	100.0	9.0	100.0	16.1	100.0
	5.40	4.0	95	8.7	97	15.5	96.3
	6.00	3.6	86	8.4	93	13.7	85.1
	6.50	2.9	69	7.0	78	11.6	72.0
	6.85	2.5	60	6.0	67	10.4	65.0

TABLE XXVII

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF HEMICELLULOSE X BY THREE DIFFERENT ENZYME PREPARATIONS

Enzyme preparation	pH	Reducing substances (as glucose)					
		2 hours		8 hours		24 hours	
		mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.
I	4.05	204	68.2	368	62.4	480	52.3
	4.64	286	95.6	580	98.3	984	107
	4.94	296	99.0	583	98.8	998	109
	5.25	299	100.0	590	100.0	918	100.0
	5.55	281	94.0	570	96.6	904	98.5
	5.86	290	97.0	575	97.4	961	105
	6.22	275	92.0	561	95.1	948	103
	6.47	271	90.6	546	92.5	816	88.9
II	4.09	34.3	62.6	60.4	53.9	93.2	49.8
	4.65	46.5	84.8	85.3	76.2	159	85.0
	4.95	48.6	88.7	91.0	81.3	166	88.8
	5.25	50.2	91.6	97.2	86.8	173	92.5
	5.80	54.8	100.0	112	100.0	187	100.0
	6.20	48.0	87.6	97.0	86.6	179	95.7
	6.40	46.6	85.0	93.0	83.0	168	89.8
III	4.15	23.2	59.9	39.6	59.5	54.8	52.2
	4.75	33.4	86.3	57.0	85.7	93.6	89.1
	5.10	38.3	99.0	63.9	96.1	101	96.2
	5.35	38.7	100.0	66.5	100.0	105	100.0
	5.95	38.5	99.5	65.8	98.9	104	99.0
	6.45	38.0	98.7	65.0	97.7	102	97.1
	6.90	36.0	93.0	62.3	93.7	96.4	91.8

TABLE XXVIII

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF HEMICELLULOSE Y BY THREE DIFFERENT ENZYME PREPARATIONS

Enzyme preparation	pH	Reducing substances (as glucose)					
		2 hours		8 hours		24 hours	
		mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.
I	4.15	244	74.8	293	61.3	314	61.2
	4.70	323	99.1	460	96.2	506	98.6
	5.30	304	93.2	443	92.7	482	93.9
	5.94	326	100.0	478	100.0	513	100.0
	6.40	297	91.1	445	93.1	477	93.0
	6.70	270	82.8	386	80.7	420	81.9
II	4.15	50.6	79.6	80.0	73.4	99.0	58.9
	4.70	61.2	96.2	106	97.2	162	96.4
	5.10	62.0	97.5	109	100.0	166	98.8
	5.35	61.4	96.5	108	99.1	164	97.6
	5.83	63.6	100.0	109	100.0	168	100.0
	6.40	56.8	89.3	101	92.7	160	95.2
	6.61	55.2	86.8	96.9	88.9	146	86.9
III	4.15	33.2	71.4	50.6	71.4	62.2	57.1
	4.75	43.8	94.2	62.5	88.1	102	93.6
	5.10	45.4	97.6	69.1	97.5	107	98.2
	5.35	46.5	100.0	70.9	100.0	109	100.0
	5.90	45.0	96.8	66.0	93.1	105	96.3
	6.40	42.6	91.6	61.5	86.7	99.2	91.0
	6.90	39.0	83.9	59.7	84.2	91.0	83.5

TABLE XXIX

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS
FROM THE DEGRADATION OF SALICIN
BY THREE DIFFERENT ENZYME PREPARATIONS

Enzyme preparation	pH	Reducing substances (as glucose)					
		20 minutes		2 hours		24 hours	
		mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.	mg./mg. prot. N.	% of max.
I	4.15	28.3	62.9	153	67.1	1120	75.2
	4.75	43.0	95.6	218	95.6	1470	98.6
	5.35	45.0	100.0	228	100.0	1490	100.0
	5.95	40.0	88.9	195	85.5	1360	91.3
	6.46	33.0	73.3	174	76.3	1180	79.2
	6.85	30.0	66.7	155	68.0	988	63.1
II	4.15	2.4	63	12.6	64.6	102	67.5
	5.35	3.8	100	19.5	100.0	151	100.0
	5.95	3.6	95	17.0	87.2	115	76.2
III	4.15	1.8	69	8.5	65	44.6	50.6
	4.73	2.0	77	9.5	73	78.4	89.0
	5.35	2.6	100	13.0	100	88.1	100.0
	5.94	1.9	73	9.7	75	82.9	94.1
	6.45	2.0	77	9.6	74	62.2	70.6
	6.85	1.2	46	7.5	58	44.9	51.0

were calculated. These relative values, also presented in Tables XXV-XXIX, were used to construct the pH-activity curves shown in Figures 25-29 The curves for three incubation times were compared.

The curves in Figure 25 for Preparation I acting on cellulose sodium sulfate, show two distinct maxima at 20 minutes and 2 hours. The larger peak occurred at pH 5.1. The smaller one at pH 6.2-6.3 was no longer definitely in evidence after 24 hours. Preparation II also had two activity peaks, the major one occurring at pH 5.1 at 20 minutes, the secondary one at pH 5.8. After 2 hours, the major peak had shifted to pH 4.9. For Preparation III, there was only one distinct peak (pH 5.1) during the early stages of the reaction. Later, the activity optimum shifted to pH 4.75. On the alkaline side of the optimum pH, the curve was at first rather flat, with some indication of a peak at pH 6.2-6.5.

When cellulose was used as the substrate (Figure 26) only one distinct maximum was evident for each enzyme preparation at 2 hours. The maxima occurred at pH 4.95, 5.25, and 5.10 for Preparations I, II, and III, respectively. A second peak at pH 6.4 was noted for Preparation I at 8 hours; it became more distinct at 24 hours. At the same time the pH of the major maximum had changed to 5.1. Preparation II showed only one peak at 8 hours, but at 24 hours there was some indication of a peak at about pH 5.9. The pH of the major maximum changed from 5.25 at 2 hours to 5.1 at 24 hours. Preparation III showed a second peak at pH 5.9 at 8 hours, but it did not persist at 24 hours.

Two almost equal maxima may be seen in the pH-activity curves for Preparation I and hemicellulose X. The first shifted from pH 5.2 at 2 hours to 4.9 at 24 hours. The second optimum was stationary at pH 5.9.

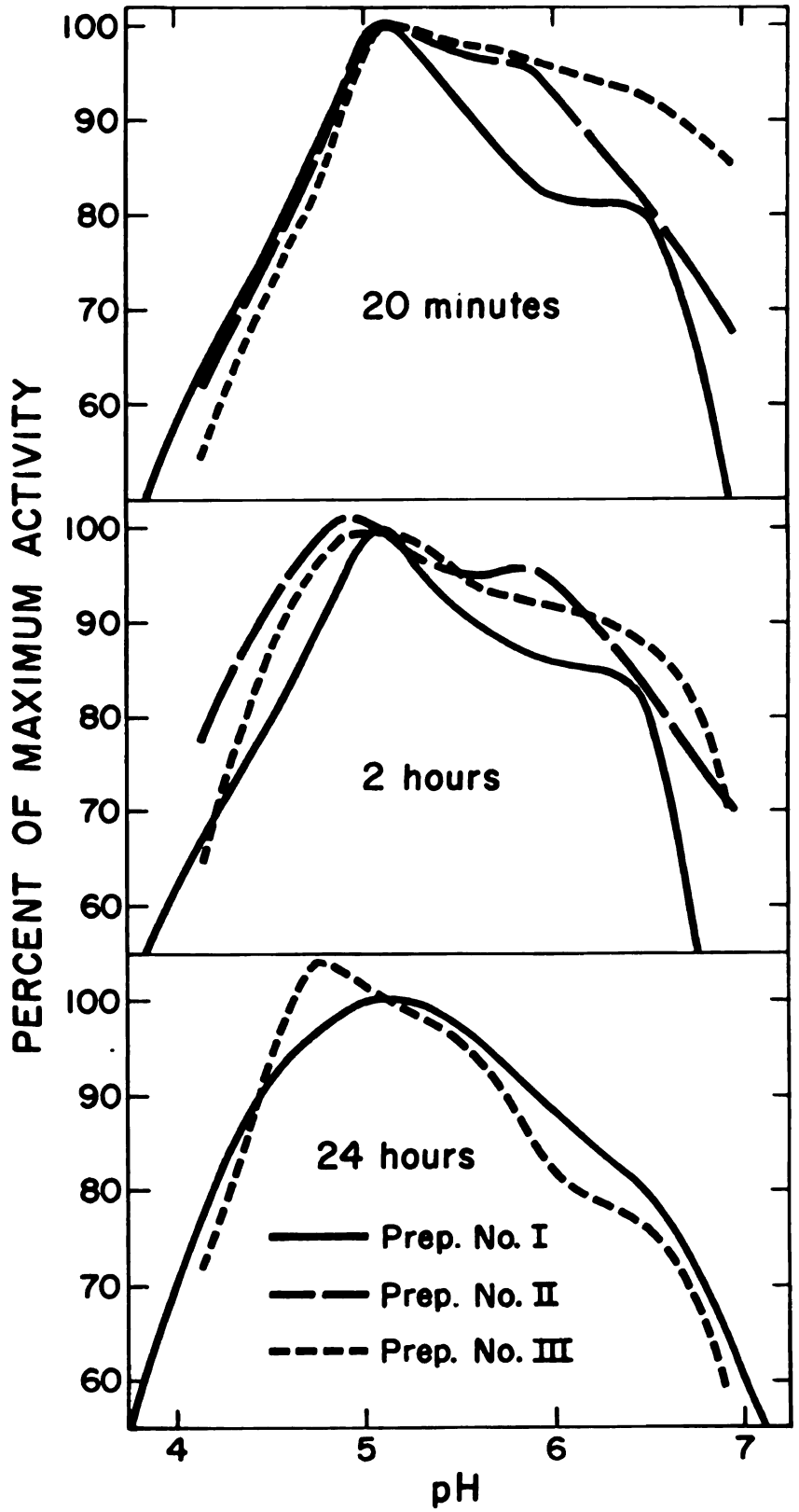

Figure 25. The effect of pH on the hydrolysis of cellulose sodium sulfate by three enzyme preparations. The relative activities at other pH levels were calculated by assigning a value of 100 percent to the maximum activity of each enzyme preparation.

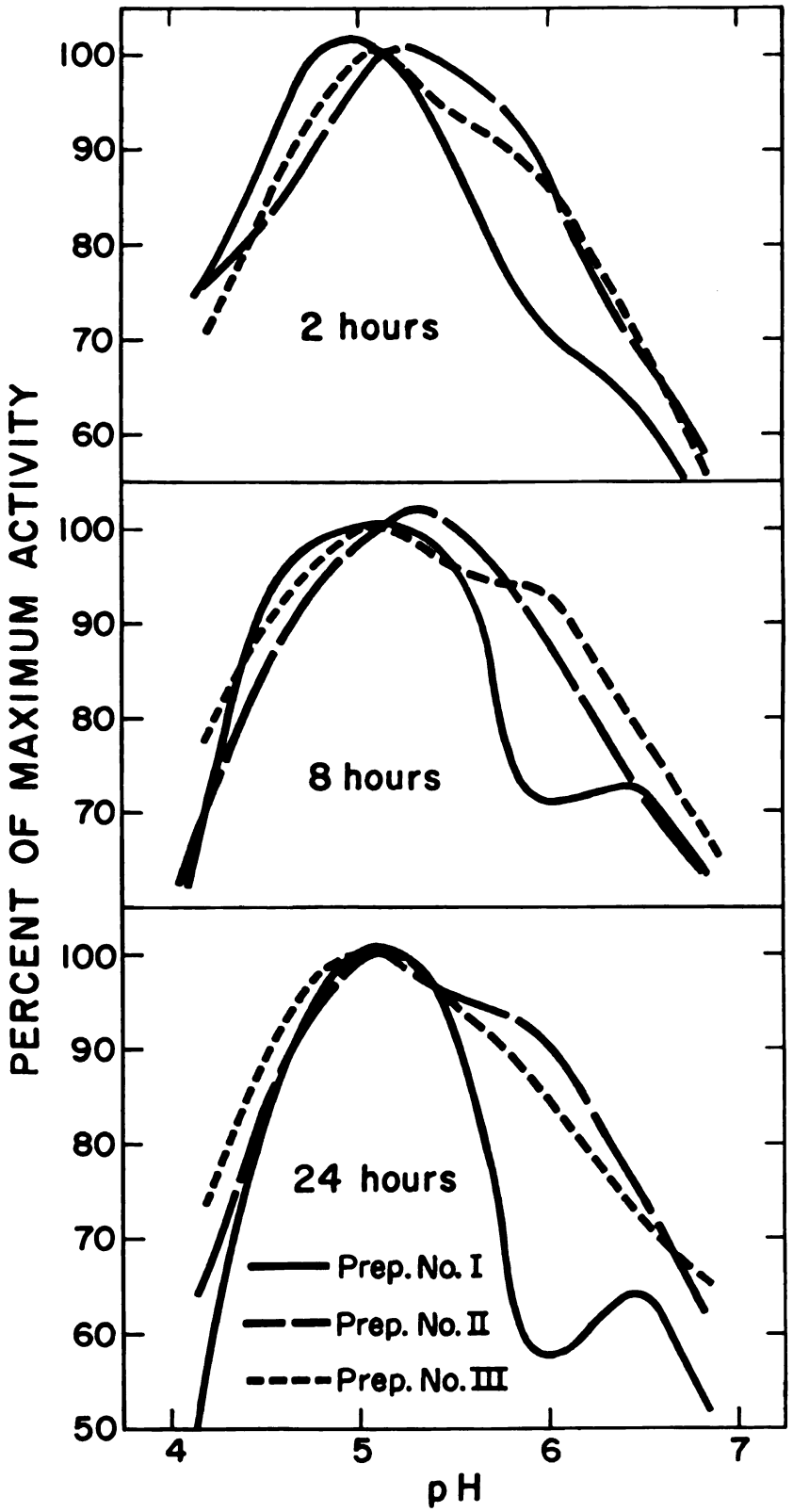

Figure 26. The effect of pH on the hydrolysis of wheat straw cellulose by three enzyme preparations. The relative activities at other pH levels were calculated by assigning a value of 100 percent to the maximum activity of each enzyme preparation.

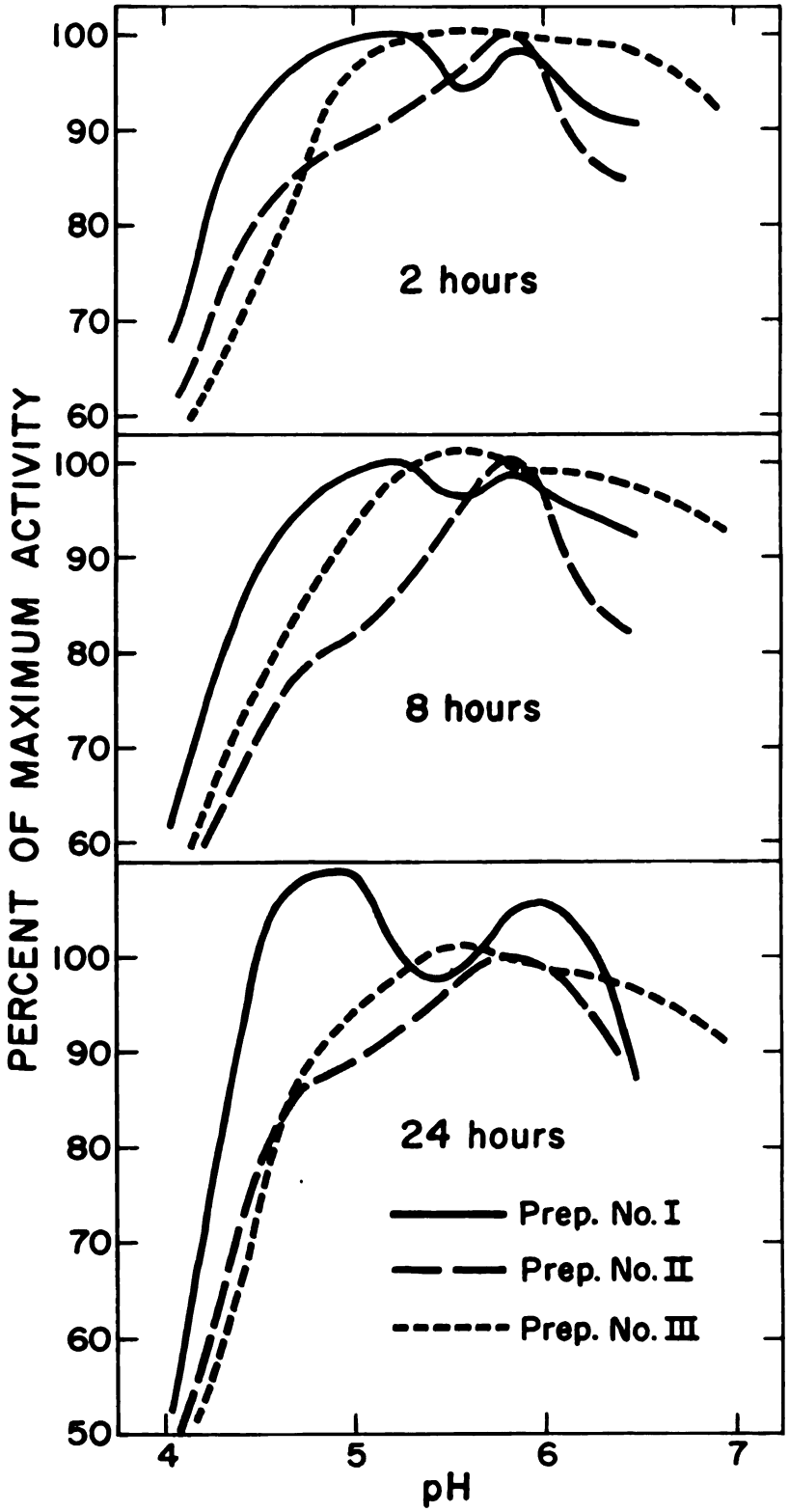

Figure 27. The effect of pH on the hydrolysis of hemicellulose X by three enzyme preparations. The relative activities at other pH levels were calculated by assigning a value of 100 percent to the maximum activity of each enzyme preparation.

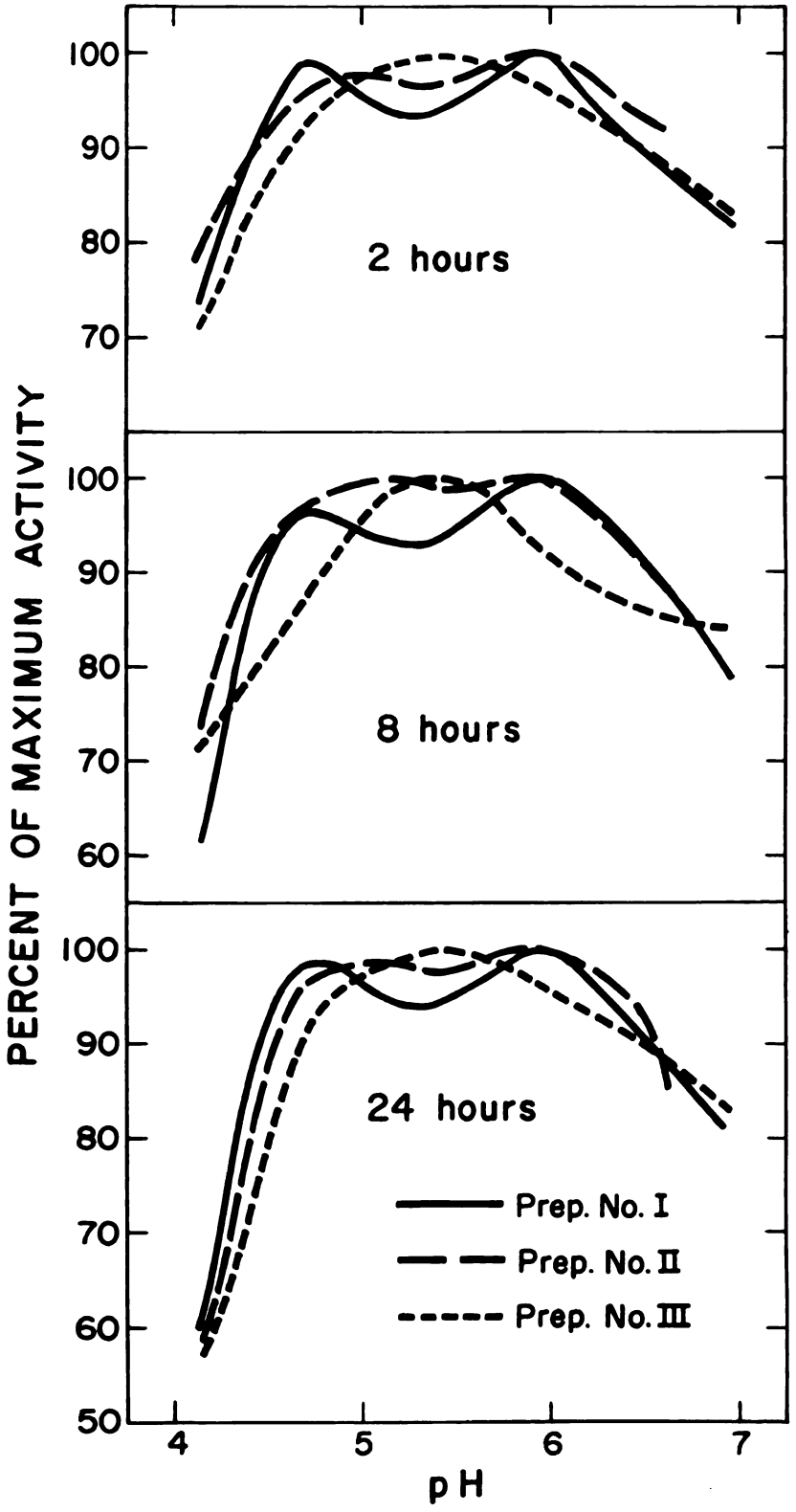

Figure 28. The effect of pH on the hydrolysis of hemicellulose Y by three enzyme preparations. The relative activities at other pH levels were calculated by assigning a value of 100 percent to the maximum activity of each enzyme preparation.

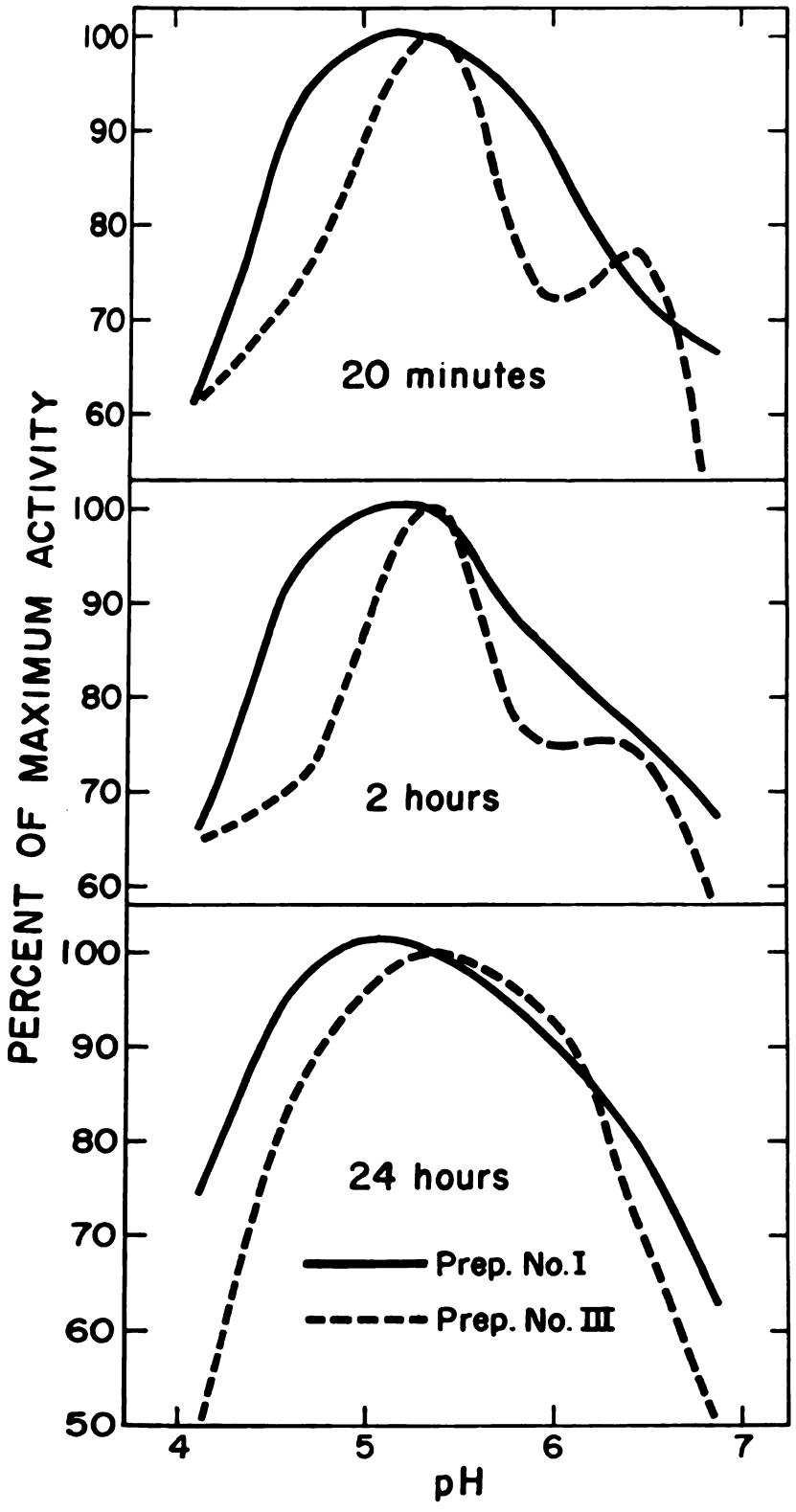

Figure 29. The effect of pH on the hydrolysis of salicin by two enzyme preparations. The relative activities at other pH levels were calculated by assigning a value of 100 percent to the maximum activity of each enzyme preparation.

Preparation II also had two activity peaks, but in this case one of the peaks was not very pronounced. The pH of the major maximum was 5.8, coinciding nicely with the secondary optimum of Preparation I. The minor peak of Preparation II came at about pH 4.7, somewhat lower than the major peak of Preparation I. The activity of Preparation III showed only one optimum pH range at 5.4-5.8. Above pH 5.4, the activity showed little tendency to decline until pH 6.5. The result was a broad, flat curve.

The results with hemicellulose Y were similar to those with hemicellulose X. Both Preparations I and II had two distinct maxima. The peaks at the higher pH coincide very well at pH 5.95. The smaller peak of Preparation I, at pH 4.7, held constant throughout the 24-hour reaction period. The secondary peak of activity of Preparation II was at pH 5.0-5.1. The activity of Preparation III was highest at pH 5.4; no other peak was evident.

The single optimum for the action of Preparation I on salicin was at pH 5.1-5.2. The main optimum for Preparation III was at pH 5.35; a smaller peak at pH 6.4 was quite distinct at 20 minutes, less so at 2 hours, and had disappeared entirely at 24 hours. Only three pH levels were used with Preparation II, so pH-activity curves could not be constructed.

The results described above are summarized in Table XXX and are in eneral agreement with the interpretation given to the comparison of ecific activities of the enzyme preparations at a constant pH. The idence suggests that at least two of the protein components were capable catalyzing the hydrolysis of each of the substrates used in this study. the case of wheat straw cellulose, most of the activity appeared to be

TABLE XXX

pH OF ACTIVITY MAXIMA OF THREE ENZYME PREPARATIONS

(The pH of the main peak is given first.)

Substrate	Enzyme preparation		
	I	II	III
Cellulose sodium sulfate	5.1, 6.3	4.9-5.1, 518	5.1, --
Wheat straw cellulose	5.1, 6.4	5.1-5.3, 5.9?	5.1, 5.9?
Hemicellulose X	4.9-5.2, 5.85-5.95	5.8, 4.7	5.4-5.8, --
Hemicellulose Y	5.95, 4.7	5.95, 5.0-5.1	5.4, --
Salicin	5.1-5.2, --		5.35, 6.4

associated with a single component, but at least one other probably contributed a small part of the activity.

The results with Preparation III, especially with respect to its action on cellulose and cellulose sodium sulfate, deserve further comment. The evidence suggests that the maximum which occurred at pH 5.1 with Preparations I and II was due to component 2. Preparation III supposedly contained no component 2, yet the only clear-cut maximum obtained with this preparation was at pH 5.1. Two different enzymes could, of course, have the same optimum pH. However, a second possibility which should not be neglected is that, in spite of the electrophoretic evidence to the contrary, Preparation III may have contained a small amount of component 2. On the other hand, the pH optima for Preparation III with hemicelluloses X and Y, and salicin do not coincide with any of the optima of the other two preparations.

It should also be emphasized that the occurrence of two peaks in pH-activity curves does not necessarily constitute proof that two enzymes are involved, especially with substrates such as the hemicellulose preparations. With these substrates, the occurrence of two peaks could be interpreted on the basis of different pH optima for the hydrolysis of two types of linkage.

End Products of Enzymatic Hydrolysis

Chromatograms of the cleared, concentrated hydrolysates of cellulose sodium sulfate and wheat straw cellulose are shown in Figure 30. The R_g value of each of the spots is given in Table XXXI, in which the spots are numbered downward from the spot corresponding to glucose (the largest, densest spots).

The major end product in each case was D-glucose. Two unidentified spots can be seen in the chromatogram of cellulose sulfate hydrolysates obtained with Preparation I (columns A and B, Figure 30). The R_g values of these spots, 0.40 and 0.14, do not correspond to the R_g value of any of the known sugars used in later chromatograms. With Preparation II a prominent spot at R_g 0.52 was found. The same spot occurred with the hydrolysate from Preparation III (R_g 0.54) as well as a spot (R_g 0.69) corresponding to the position of cellobiose (R_g 0.71).

With wheat straw cellulose and enzyme Preparation I (column F, Figure 30), the spot corresponding to glucose appears to be elongated. The top portion of this spot was a bright lemon-yellow color in contrast to the brown color characteristic of glucose. Its R_g of 1.08 corresponded fairly well to that of fructose in a later chromatogram. There was also an indication of the presence of fructose in the hydrolysates of cellulose sodium

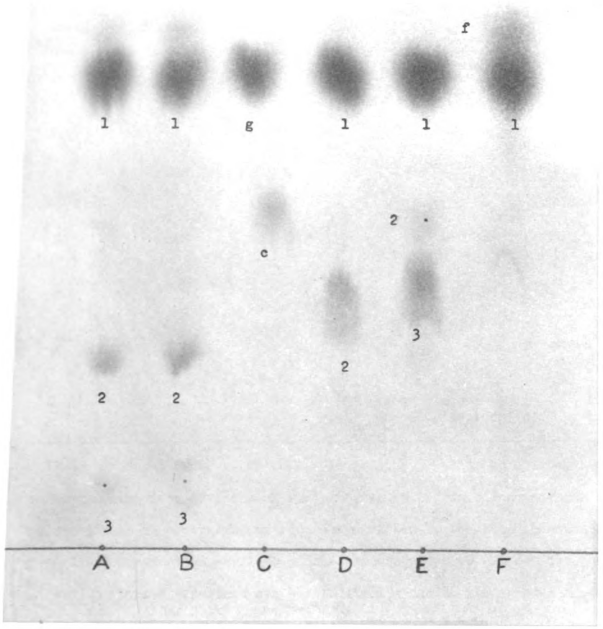

Figure 30. Products of hydrolysis of cellulose sodium sulfate and cellulose by three enzyme preparations at pH 5.1.
A and B. CSS, Preparation I, 24 and 96 hr., resp. D. CSS, Preparation II, 96 hr. E. CSS, Preparation III, 96 hr. F. Wheat straw cellulose, Preparation I, 96 hr. C. Glucose (g) and cellobiose (c).

TABLE XXXI

R_g VALUES OF SPOTS ON CHROMATOGRAMS OF ENZYMATIC HYDROLYSATES OF CELLULOSE SODIUM SULFATE AND WHEAT STRAW CELLULOSE

(See Figure 30)

Column	Substrate	Enzyme prep.	Time of hydrolysis	Spot No.	R_g
			hours		
A	CSS	I	24	1	0.99
				2	0.39
				3	0.13
B	CSS	I	96	1	0.99
				2	0.40
				3	0.14
D	CSS	II	96	1	1.00
				2	0.52
E	CSS	III	96	1	1.00
				2	0.69
					0.54
F	cellulose	I	96	f	1.08
				1	0.99
C			Known glucose	g	1.00
			Known cellobiose	c	0.71

sulfate produced by Preparation I. To be more certain, an eluate of the corresponding area of a streak chromatogram of the solution used for column B was concentrated and rechromatographed by the two-dimensional technique previously described. Some glucose was also eluted, but the identification of fructose was quite definite due to its greater concentration and more intense spot, and because its R_g values with the butanol-pyridine-water solvent (1.11) and phenol-water (1.25) matched those of an authentic sample of fructose (1.11 and 1.28).

Eluates of the unknown spots of columns B, D, and E were also obtained from streak chromatograms. Samples were analyzed for reducing sugars (as glucose) and tested qualitatively for free sulfate before and after acid hydrolysis. The eluate corresponding to R_g 0.36 (enzyme Preparation I) gave a doubtful test for free sulfate (barium chloride) before, and a positive test after hydrolysis. Hydrolysis increased the reducing sugar content by 60 percent. The material eluted from the R_g 0.14 area (Preparation I) gave a positive test for sulfate only after hydrolysis, and a three-fold increase in reducing sugar content accompanied the liberation of sulfate. The eluate from the area suspected of containing cellobiose (enzyme Preparation III, column E) contained no free sulfate either before or after acid hydrolysis. Its reducing power increased 2.1 times after hydrolysis. The material causing the spot at R_g 0.54 (Preparations II and III) increased in reducing power by 60 percent after acid hydrolysis and contained sulfate ion only after hydrolysis. Thus, aside from cellobiose, the unknown spots all contained bound sulfate. Whether their reducing power after acid hydrolysis was a consequence of the loss of the sulfate radical or was due to the liberation of new reducing groups by hydrolysis of β-1, 4 glucosidic linkages is not known.

The most important finding was that, except for glucose, the end products of hydrolysis of cellulose sulfate with Preparation I were different from those obtained with Preparation II and III. This suggests the existence of more than one enzyme acting either on the original substrate or on the intermediate products.

Fructose appeared only in the hydrolysates prepared with enzyme Preparation I. Its appearance, therefore, can be correlated only with the presence

of component 1. If the reasonable assumption is made that fructose was not an original constituent of cellulose sodium sulfate and wheat straw cellulose, than it must also be assumed that component 1 in some way brings about the formation of fructose, probably from glucose. The concentration and clearing treatments were the same for all the hydrolysates, so it is unlikely that fructose was formed after the enzymatic reaction was stopped.

A very faint spot above the glucose spot in column F (wheat straw cellulose) indicated the presence of a small amount of xylose in this substrate.

Chromatograms of the 80 percent ethanol soluble end products of the enzymatic hydrolysis of hemicellulose fractions X and Y are presented in Figures 31 and 32, respectively. The areas in which spots occurred were numbered on the completed chromatogram, starting with the spot having the highest R_g. The R_g values of the unknown and known spots are given in Tables XXXII and XXXIII.

The solutions used for A and B in Figure 31 were fractions obtained from the same 48-hour enzymatic hydrolysate of hemicellulose X, and those used for C and D were obtained from a 96-hour hydrolysate produced by enzyme preparation I. The fractions represented by A and C were from the 80 percent ethanolic extracts of the residues obtained by evaporation of the alcoholic filtrates of the original hydrolysates to dryness. Fractions C and D were obtained by dissolving in water the residues remaining after extraction with 80 percent ethanol. All the fractions were deionized as previously described. It can be seen that the same constituents were present in the two fractions of both hydrolysates. However, undesirable

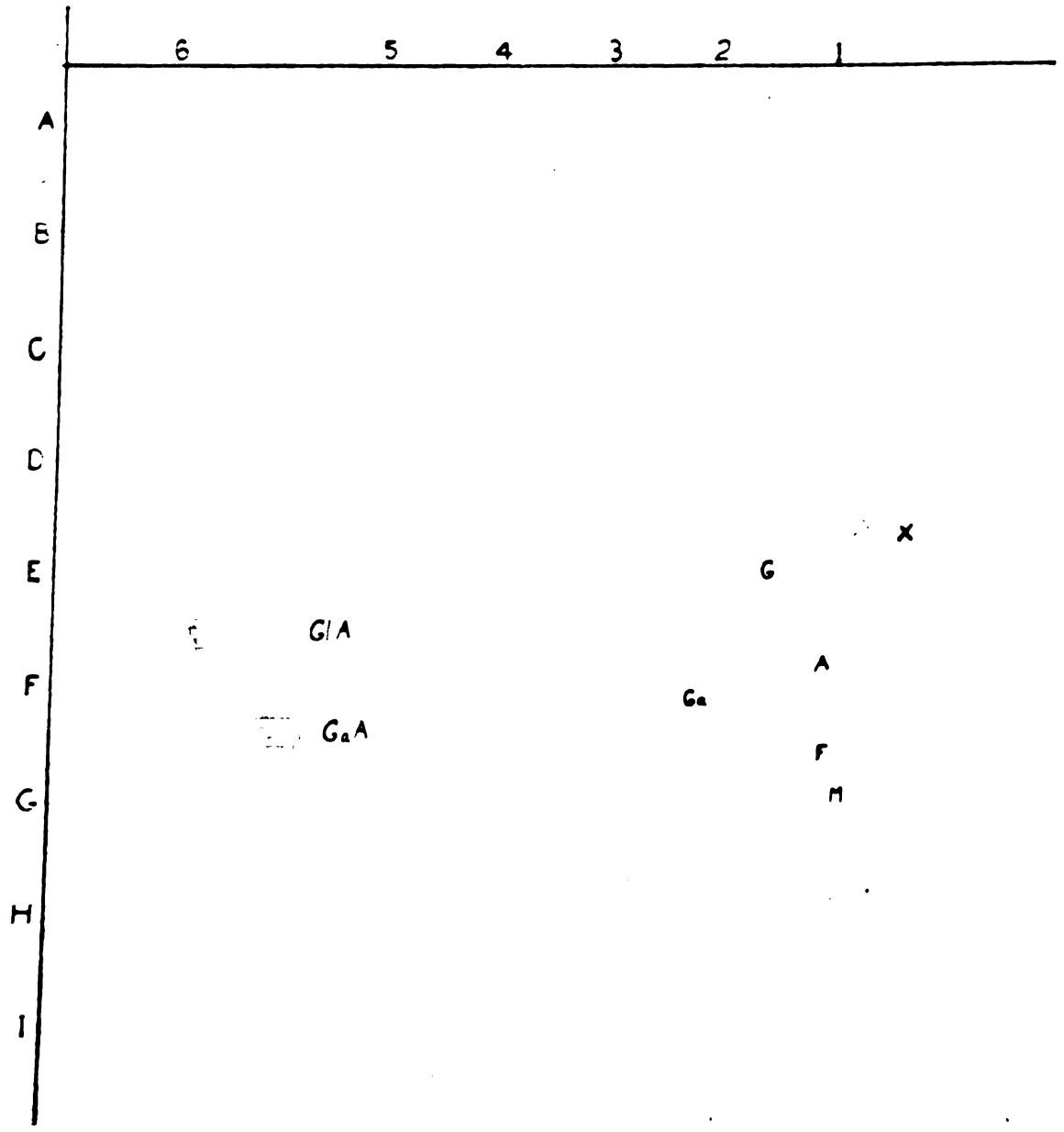

Figure 31. Products of hydrolysis of hemicellulose X by three enzyme preparations at pH 5.35.
A and B. Preparation I, 48 hr. C and D. Preparation I, 96 hr.
H. Preparation II, 96 hr. I. Preparation III, 96 hr.
E. Xylose (X), glucose (G), and glucuronic acid (GlA).
F. Arabinose (A), galactose (Ga), and galacturonic acid (GaA).
G. Mannose (M) and fructose (F).

TABLE XXXII

R_g VALUES OF SPOTS ON CHROMATOGRAMS OF ENZYMATIC HYDROLYSATES OF HEMICELLULOSE X

(See Figure 31)

Column	Spot number 1	2	3	4	5	6
A	1.20	1.03	0.84	0.69	0.50	0.21
B	1.19	1.02	0.84	0.65	0.49	0.0-0.23
C	1.20	1.04	0.87	0.71	0.51	0.17
D	1.20	1.02	0.84	0.67	0.51	0.0-0.23
H	1.19	1.06 0.99	0.83		0.47	0.20
I	1.20	1.08 1.01	0.86		0.50	0.21
E	X: xylose 1.20		G: glucose 1.00		GIA: glucuronic acid 0.20-0.34	
F	A: arabinose 1.08		Ga: galactose 0.88		GaA: galacturonic acid 0.17-0.38	
G	F: fructose 1.08		M: mannose 1.11			

trailing of the spots was noted with the aqueous extracts. No qualitative differences between the end products of the 48-hour and 96-hour hydrolysates were found.

The spot in area 1 was probably xylose. The spots in area 2 of A-D were elongated; the leading edge had the dark brown color of the aldopentoses and the trailing edge had the light brown color characteristic of the aldohexoses. The R_g, calculated from the estimated center of the spot, was slightly higher than 1.00 in each case. It seemed probable that spot 2

TABLE XXXIII

R_g VALUES OF SPOTS ON CHROMATOGRAMS OF ENZYMATIC HYDROLYSATES OF HEMICELLULOSE Y

(See Figure 32)

Column	Spot number					
	1	2	3	4	5	6
A	1.15	0.98	0.83		0.48	0.03-0.16
B	1.17	1.01	0.87	0.71	0.53	0.18
G	1.13	1.07 1.01	0.88			0.18
H	1.18	1.07 1.00	0.87			0.18
C	x: xylose	1.18				
D	g: glucose	1.00				
E	a: arabinose	1.07				
F	ga: galactose	0.91				

was made up of two incompletely resolved sugars.

The spot in area 3 had an R_g slightly lower than that of galactose, but its color was considerably darker. It was found later that this spot was not galactose.

Spots 4, 5, and 6 could not be identified with any of the known sugars used.

The hydrolysates of hemicellulose X obtained with enzyme Preparations II and III gave spots in areas 1, 3, 5, and 6, probably identical with those obtained with enzyme Preparation I. The amount of the substance responsible for spot 4 was relatively smaller than in the hydrolysates produced with enzyme Preparation I, and area 2 was resolved into two distinct

spots with the R_g values and colors of arabinose and glucose.

The results with hemicellulose Y hydrolysates were qualitatively much the same as those described for hemicellulose X. With this substrate also, area 2 was resolved into 2 spots corresponding to arabinose and glucose only in the hydrolysates produced by enzyme Preparations II and III (Figure 32 and Table XXXIII). Spots 4 and 5 were not detected with certainty in the hydrolysates from enzyme Preparations II and III.

The unknown spots were investigated further by isolating the substances from the appropriate areas of streak chromatograms as described in the section on methods. Portions of the eluates were analyzed for reducing power before and after acid hydrolysis, and other portions were rechromatographed before and after acid hydrolysis. The results with the end products of hemicellulose X hydrolysis are shown in the chromatograms in Figures 33 and 34. The R_g values and color of the spots, and the percentage change in the reducing power brought about by hydrolysis are presented in Table XXXIV.

The only eluate unaffected by acid hydrolysis was that from area 1. The spot appeared at the same location before and after treatment with 0.4 N hydrochloric acid at 120° C. All of the other acid-hydrolyzed eluates gave one or more new spots. The hydrolyzed eluate from area 2 gave spots corresponding to xylose and glucose, that from area 3 gave a single spot corresponding to xylose, and the reducing substances in the eluates from areas 4 and 5 were apparently made up of three constituents; (a) a relatively large proportion of xylose, (b) smaller quantities of arabinose, and (c) a uronic acid. (The very faint uronic acid spots, identified mostly by their distinctive red color, were indicated on the

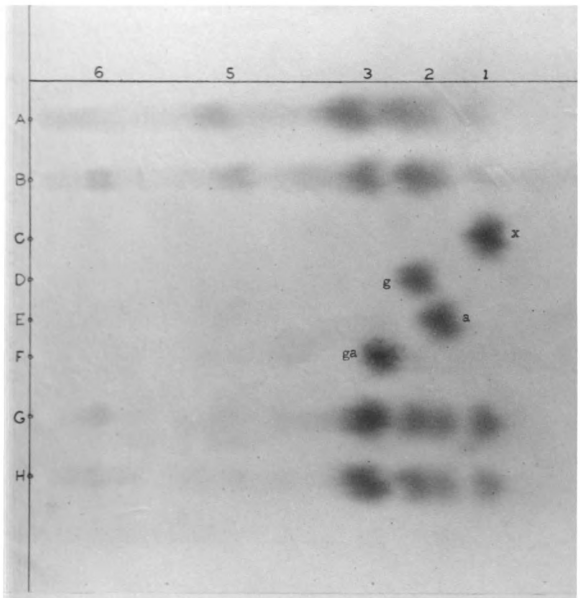

Figure 32. Products of hydrolysis of hemicellulose Y by three enzyme preparations at pH 5.35.
A and B. Preparation I, 48 and 96 hr., respectively.
G. Preparation II, 96 hr. H. Preparation III, 96 hr.
C. Xylose (x). D. Glucose (g). E. Arabinose (a). F. Galactose (ga).

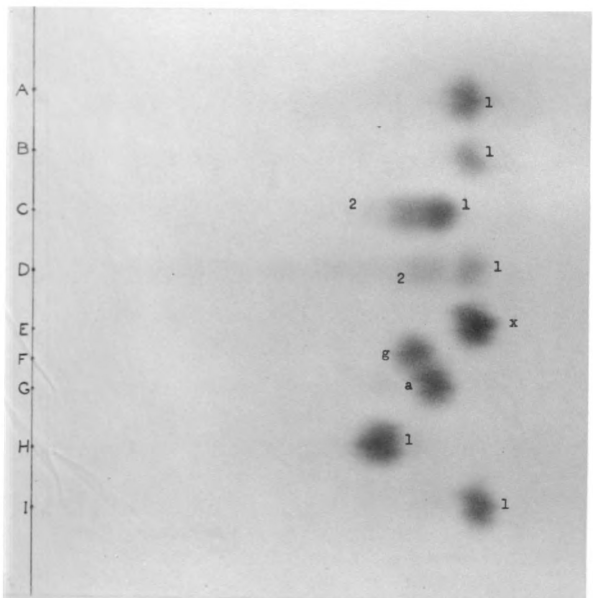

Figure 33. Effect of acid hydrolysis on the isolated end products in the enzymatic hydrolysates of hemicellulose X.
A, C, and H. Eluates of areas 1, 2, and 3, respectively, before hydrolysis (Figure 31). B, D, and I. Eluates of areas 1, 2, and 3, respectively, after hydrolysis. E. Xylose (x). F. Glucose (g). G. Arabinose (a).

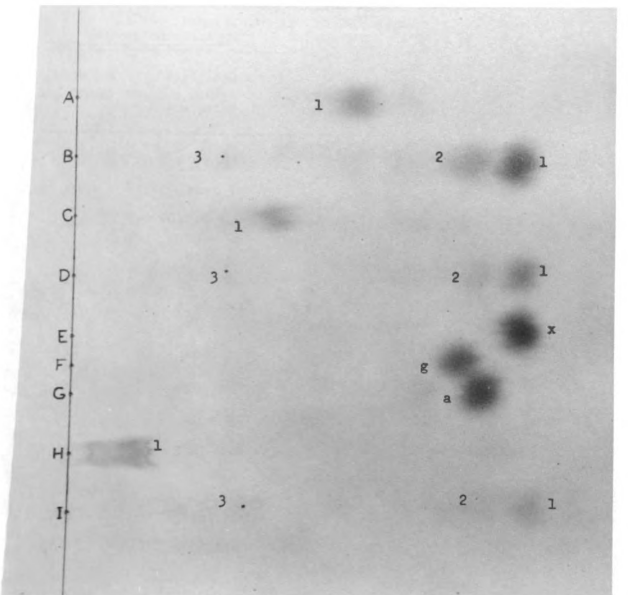

Figure 34. Effect of acid hydrolysis on the isolated end products in the enzymatic hydrolysates of hemicellulose X.

A, C, and H. Eluates of areas 4, 5, and 6, respectively, before hydrolysis (Figure 31). B, D, and I. Eluates of areas 4, 5, and 6, respectively, after hydrolysis. E. Xylose (x). F. Glucose (g). G. Arabinose (a).

TABLE XXXIV

EFFECT OF ACID HYDROLYSIS ON THE END PRODUCTS OF ENZYMATIC HYDROLYSIS OF HEMICELLULOSE X

(Data from Figures 33 and 34. Source of eluates: streak chromatograms of solution used for C, Figure 31.)

Figure No. and column	Description of eluate*	Change in reducing power	Spot No.	Color**	R_g
		%			
33A	spot 1		1	P	1.14
33B	spot 1-H	-5	1	P	1.14
33C	spot 2		1	P	1.06
			2	H	0.99
33D	spot 2-H	-20	1	P	1.14
			2	H	1.02
33H	spot 3		1	P	0.91
33I	spot 3-H	70	1	P	1.16
34A	spot 4		1	P	0.74
34B	spot 4-H	100	1	P	1.16
			2	P	1.04
			3	U	0.39
34C	spot 5		1	P	0.52
34D	spot 5-H	140	1	P	1.16
			2	P	1.07
			3	U	0.40
34H	spot 6		1	?	0.04-0.19
34I	spot 6-H	140	1	P	1.17
			2	P	1.06
			3	U	9.44
33E			x xylose		1.15
34E			x xylose		1.16

TABLE XXXIV concluded

Figure No.	Description of eluate*	Change in reducing power	Spot No.	Color**	R_g
33F			g	glucose	1.00
34F			g	glucose	1.00
33G			a	arabinose	1.05
33G			a	arabinose	1.06

*The letter "H" after the number indicates the acid hydrolyzed portion of the eluate.

**Colors are indicated as follows: P, the dark brown color characteristic of aldopentoses; H, the lighter brown color given by aldohexoses; U, the dark red color given by uronic acids.

chromatogram, before it was photographed, by a pencil mark.) The hydrolyzed eluate from area 6 also contained the spots corresponding to xylose and uronic acid, but showed no trace of the arabinose spot, possibly because the concentration was too low.

The reducing equivalent of all the eluates except the ones from spots 1 and 2 were increased by acid hydrolysis. Because of the empirical and non-specific nature of the method used to estimate reducing substances, the results can be used only in a qualitative sense. Thus it is probable that the material responsible for spot 6 was a higher oligosaccharide than that of spot 3, but the number of residues per molecule cannot be estimated from the results.

The chromatograms obtained with the hydrolyzed and unhydrolyzed eluates containing the end products of hydrolysis of hemicellulose Y are shown in Figures 35 and 36. The R_g values and color of the spots and the percentage increase of reducing power of the acid hydrolysates are presented in Table XXXV.

The results with the eluates from areas 1, 2, and 3 were the same as before[1] (Figure 35). The results with the remaining eluates were different (Figure 36). No uronic acid was detected in the hydrolyzed eluates of areas 4 and 5. Moreover, xylose and glucose, instead of xylose and arabinose, were identified in the hydrolyzed eluates. The hydrolyzed eluate of area 6 yielded spots corresponding to xylose, glucose, and uronic acid. The amount of glucose in all three cases was small in comparison to the amount of xylose.

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[1]A small amount of area 2 was evidently eluted with the xylose of area 1, and a small amount of spot 3 with area 2.

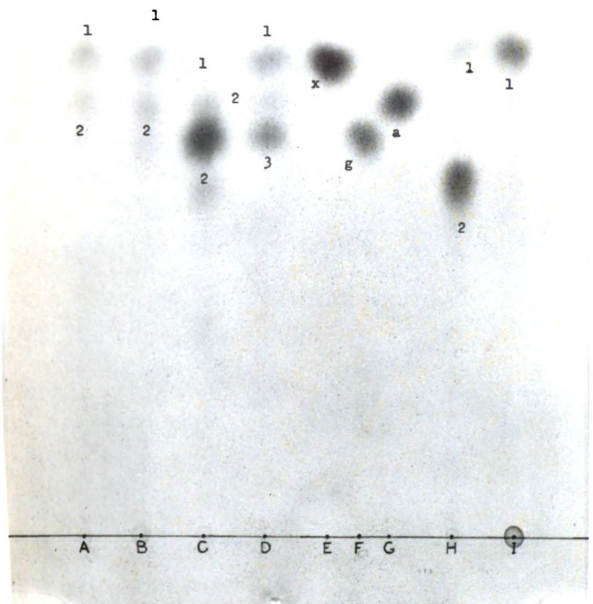

Figure 35. Effect of acid hydrolysis on the isolated end products in the enzymatic hydrolysates of hemicellulose Y.

A, C, and H. Eluates of areas 1, 2, and 3, respectively, before hydrolysis (Figure 32). B, D, and I. Eluates of areas 1, 2, and 3, respectively, after hydrolysis. E. Xylose (x). F. Glucose (g). G. Arabinose (a).

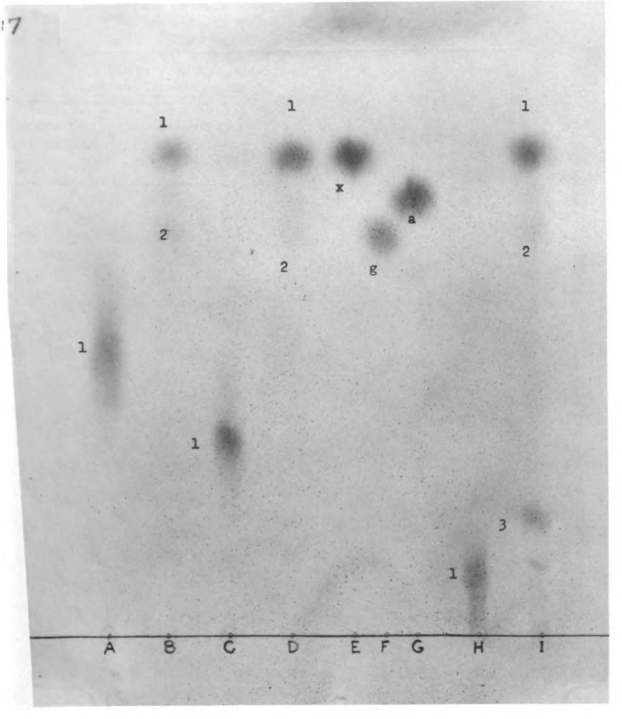

Figure 36. Effect of acid hydrolysis on the isolated end products in the enzymatic hydrolysates of hemicellulose Y.

A, C, and H. Eluates of areas 4, 5, and 6, respectively, before hydrolysis (Figure 32). B, D, and I. Eluates of areas 4, 5, and 6, respectively, after hydrolysis. E. Xylose (x). F. Glucose (g). G. Arabinose (a).

TABLE XXXV

EFFECT OF ACID HYDROLYSIS ON THE END PRODUCTS OF ENZYMATIC HYDROLYSIS OF HEMICELLULOSE Y

(Data from Figures 35 and 36. Source of eluates: streak chromatogram of solution used for B, Figure 32)

Figure No. and column	Description of eluates*	Change in reducing power	Spot No.	Color**	R_g
35A	spot 1		1	P	1.21
			2	H	1.09
35B	spot 1-H	-5	1	P	1.20
			2	P	1.08
35C	spot 2		1	H	0.99
			2	P	0.87
35D	spot 2-H	-7	1	P	1.20
			2	P	1.09
			3	H	1.01
35H	spot 3		1	P	1.22
			2	P	0.90
35I	spot 3-H	40	1	P	1.22
36A	spot 4		1	P	0.72
36B	spot 4-H	86	1	P	1.22
			2	H	1.01
36C	spot 5		1	P	0.50
36D	spot 5-H	112	1	P	1.21
			2	H	0.99
36H	spot 6		1	P	0.15
36I	spot 6-H	124	1	P	1.22
			2	H	1.01
			3	U	0.30
35E			x	xylose	1.20
36F			x	xylose	1.21

TABLE XXXV concluded

Figure No.	Description of eluates*	Change in reducing power	Spot No.	Color**	R_g
35F			g	glucose	1.00
36F			g	glucose	1.00
35G			a	arabinose	1.09
36G			a	arabinose	1.10

*The letter "H" after the number indicates the acid-hydrolyzed portion of the eluate.

**Colors are indicated as follows: P, the dark brown color characteristic of aldopentoses; H, the lighter brown color given by aldohexoses; U, the dark red color given by uronic acids.

No evidence of a significant extent of hydrolysis of the oligosaccharides of spots 3-6 for hemicellulose X was obtained when small portions were incubated at 40° C. with enzyme Preparation I. The reducing equivalent of each eluate remained essentially unchanged after 24 hours of incubation. Thus it is probable that the spots represent end products and not intermediate products.

Chromatograms of hydrolysates of hemicellulose X and hemicellulose Y taken at 1.5, 6, 20, and 48 hours are shown in Figures 37 and 38, respectively. R_g values are presented in Tables XXXVI and XXXVII. Enzyme Preparation I was used in both cases. The concentrated hydrolysates were not deionized. The amount of reducing sugars (as glucose) applied at each starting spot (except A) was kept constant at about 65 micrograms in the case of the hemicellulose X hydrolysates, and at about 70 micrograms with the hydrolysates of hemicellulose Y. The volumes of the original hydrolysate represented in spots A-E were 0.1, 0.1, 0.04, 0.02 and 0.015 ml., respectively, in the case of the hemicellulose X hydrolysates, and 0.04, 0.04, 0.032, 0.022, and 0.016 ml. for the hemicellulose Y hydrolysates. Care was taken to treat all samples alike in order that a constant proportion of each component would be carried through the procedure. The experiment was undertaken to determine the order of appearance or disappearance of the end products or intermediate products of hydrolysis.

The results with the two substrates were similar. The major exception was the occurrence of spots in areas 3 and 5 in the solution of hemicellulose X before the enzyme was added (A, Figure 37). These spots did not occur with the suspensions of hemicellulose Y (A, Figure 34). Another difference, noted previously, was the relatively small amount of the

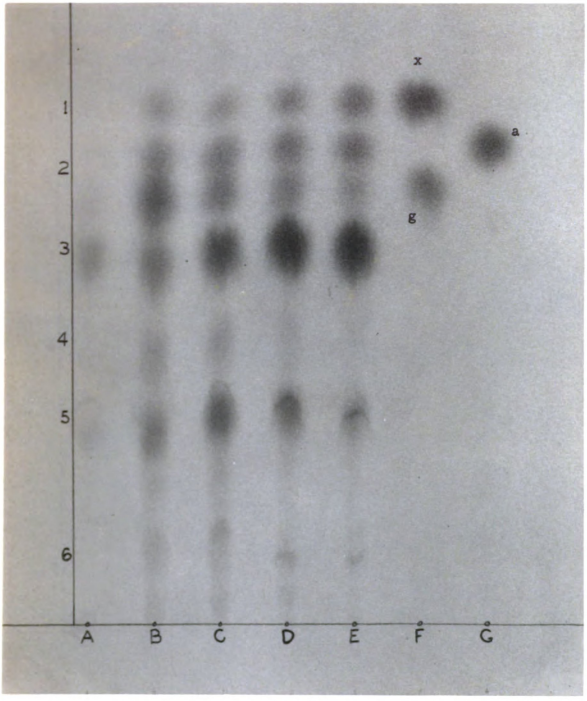

Figure 37. Effect of time of reaction on the relative proportions of end products obtained by the enzymatic hydrolysis of hemicellulose X. Enzyme Preparation I, 16.7 µg. of protein nitrogen per milliliter of reaction mixture, pH 5.3.

A. Before enzyme was added. B. 1.5 hr. C. 6 hr. D. 20 hr. E. 48 hr. F. Xylose (x) and glucose (g). G. Arabinose (a).

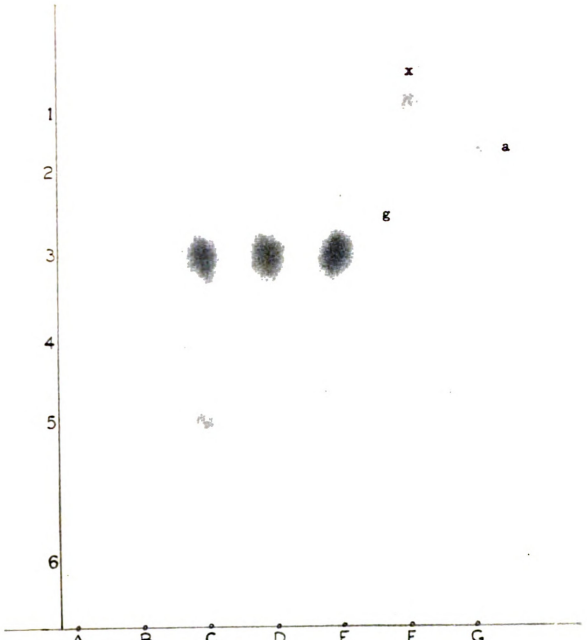

Figure 38. Effect of time of reaction on the relative proportions of end products obtained by the enzymatic hydrolysis of hemicellulose Y. Enzyme Preparation I, 16.7μg. of protein nitrogen per milliliter of reaction mixture, pH 5.3.
A. Before enzyme was added. B. 1.5 hr. C. 6 hr.
D. 20 hr. E. 48 hr. F. Xylose (x) and glucose (g).
G. Arabinose (a).

TABLE XXXVI

R_g VALUES OF SPOTS ON CHROMATOGRAMS OF ENZYMATIC HYDROLYSATES OF HEMICELLULOSE X

(See Figure 37)

Column	Spot number						
	1	2_1*	2_2*	3	4	5	6
A				0.84		0.45	
B	1.18	1.06	0.97	0.82	0.61	0.46	
C	1.18	1.07	0.99	0.86	0.65	0.49	0.21
D	1.19	1.09	1.00	0.86	0.64	0.49	0.15
E	1.19	1.09	0.99	0.85		0.47	0.14
F	xylose 1.19; glucose 1.00						
G	arabinose 1.09						

*2_1 indicates the upper spot of area 2, and 2_2 indicates the lower spot of the same area.

TABLE XXXVII

R_g VALUES OF SPOTS ON CHROMATOGRAMS OF ENZYMATIC HYDROLYSATES OF HEMICELLULOSE Y

(See Figure 38)

Column	Spot number						
	1	2_1*	2_2*	3	4	5	6
B	1.20	1.10	1.00	0.86	0.64	0.48	0.17
C	1.19	1.08	0.99	0.85	0.66	0.48	0.17
D	1.19	1.08	0.99	0.85		0.47	
E	1.19	1.08	0.99	0.86		0.48	
F	xylose 1.20; glucose 1.00						
G	arabinose 1.09						

*2_1 indicates the upper spot of area 2, and 2_2 indicates the lower spot

substances causing spots 4 and 6 in the hydrolysates of hemicellulose Y.

With both substrates, the relative concentrations of xylose (spot 1), arabinose (top spot of area 2), and the substance causing spot 3 increased throughout the course of the reaction. The relative intensity of the glucose spot (lower spot of area 2) and of spots 4 and 6 decreased as the reaction proceeded, whereas the intensity of spot 5 increased during the first part of the reaction and then decreased. It should be emphasized that the changes in concentration are relative; only positive changes may be interpreted as evidence that the substance was increasing in absolute concentration. The concentration of the substances which were represented by the spots with decreasing intensities may have remained constant or actually increased. On this basis, it appears that the reactions which resulted in the appearance of glucose and spots 4, 5, and 6 approached completion more rapidly than those which resulted in the appearance of xylose, arabinose, and the material of spot 3. It is probable that the substance represented by spot 3 was an end product, refractive to further enzymatic hydrolysis. The results are inconclusive with respect to the status (end product or intermediate) of the oligosaccharides of spots 4, 5, and 6.

Spot 5 in these chromatograms exhibited a peculiar characteristic not found with the corresponding spots on previous chromatograms. A crescent-shaped area can be seen in the photographs, especially in Figure 38. The color of the crescent was the dark red color characteristic of the uronic acids. The remainder of the spot was the dark brown color characteristic of pentoses.

Some of the material of spot 5 was isolated by elution from the

appropriate area of a streak chromatogram. The eluate was rechromatographed by the two dimensional technique, using the usual butanol:pyridine:water mixture in one direction, and phenol:water in the second direction. Two spots, one having the red color of a uronic acid and the other the color produced by pentoses, were found. The R_g of the red spot was 0.23 and that of the brown spot was 0.48 in the first solvent.

It will be recalled that the solutions used for the chromatograms in Figures 37 and 38 were not treated with the ion-exchange resins, whereas those used for the previous chromatograms were treated. On the basis of the results obtained with the unhydrolyzed eluate of spot 5, it may be concluded that uncombined uronic acid occurred in the hydrolysates. The abnormally rapid migration of the free uronic acid into the area occupied by spot 5 was an artifact, caused, perhaps, by the presence of inorganic ions. It is also possible that free uronic acid was originally present in the hydrolysates used for previous chromatograms, but was removed by the anion-exchange resin.

In summary, evidence was obtained which indicated that D-xylose, L-arabinose, D-glucose, uronic acid, and an unknown di- or trisaccharide of D-xylose were end products of the hydrolysis of both hemicellulose fractions by M. verrucaria enzyme preparations. Inconclusive evidence was also obtained suggesting that three other oligosaccharides found in enzymatic hydrolysates of the hemicelluloses were end products rather than intermediate products. The last-mentioned oligosaccharides appeared to be different in the hydrolysates of the two hemicelluloses.

DISCUSSION OF RESULTS

DISCUSSION OF RESULTS

The primary objectives of this study were (1) to determine whether the cellulase activity of M. verrucaria culture filtrates is the property of a single or of several of the protein components present, and (2) to study the relationships among the cellulase, hemicellulase, and β-glucosidase activities of the culture filtrates. The results will be discussed in relation to these objectives. Certain observations, perhaps interesting and important in themselves, but not contributing directly to the settlement of the inquiry, were discussed in the previous section.

Evidence for the multiplicity of enzymes having cellulolytic activity was manifold. Some of the observations listed below cannot, by themselves, be considered conclusive, and others are merely suggestive, but considered together, they present a strong argument for the multiple enzyme hypothesis.

(1). The biphasic nature of the activity (CSS) accumulation curve during the growth of the organism could be the result of an increased production of a second enzyme at the time of the upward inflection. The possibility was not specifically investigated, and other explanations are admittedly possible. Jermyn (1953) found indications of a variable production of Cx activity by Stachybotrys atra, and Reese and Gilligan (1953) found it possible, by changing the conditions of culture, to influence the production of one of the Cx components by M. verrucaria.

(2). Although other factors were involved, the failure to obtain fractions enriched in cellulase activity (CSS) by salt and solvent precipitation may have been due, in part, to the existence of two or more enzymes with different physical properties.

(3). It was evident during fractionation by electrophoresis convection that protein component 2 was the most active cellulase when cellulose sodium sulfate was the substrate, but statistical correlation of the specific activities of some of the fractions with their electrophoretic composition strongly suggested that more than one of the components was involved. Comparison of the specific activities of the three final enzyme preparations with cellulose sodium sulfate and wheat straw cellulose led to the same conclusion, but indicated further that the proportion of the total activity attributable to component 2 was greater when cellulose was used as the substrate than when cellulose sodium sulfate was used.

The results with respect to the soluble cellulose derivative are in agreement with the findings of Reese and Gilligan (1953), who presented evidence indicating the presence of at least two separate enzymes in M. verrucaria culture filtrates capable of hydrolyzing carboxymethylcellulose. It will be recalled that these workers separated the protein components by paper chromatography and incubated transverse sections of the chromatograms directly with the substrate. Whitaker's (1953) results, on the other hand, indicated that practically all the cellulase activity of M. verrucaria culture filtrates can be attributed to a single component. Whitaker used only insoluble cellulose substrates. The results obtained in the present investigation, showing that the ratio of activities of the constituent enzymes apparently depends on the particular substrate employed, may offer a partial explanation for the seemingly contradictory results reported from different laboratories. Since no pure fractions were obtained, the results offer no evidence for or against

the hypothesis advanced by Reese et al. (1950) that two enzymes, C_1 (which in some as yet unexplained manner frees linear polyanhydroglucose chains from the crystalline matrix of native cellulose) and Cx (which hydrolyzes the β-1, 4-glucosidic linkages), are necessary for the hydrolysis of native cellulose.

(4). The differences displayed by the three final enzyme preparations in response to changes in pH constitute supporting evidence for the multiple enzyme hypothesis.

(5). The difference in end products obtained from the hydrolysates of the three enzyme preparations acting on cellulose sodium sulfate can be interpreted on the basis of the presence of more than one enzyme.

The occurrence of multiple components in the system studied in this investigation is not unique. The work of Jermyn (1952b) with the cellulase and β-glucosidase systems of A. oryzae has already been mentioned. Giri et al. (1952) found indications of multiple components in amylase systems, and Reed (1950) obtained similar results with a polygalacturonase system.

The most convincing evidence for the ability of at least one enzyme to catalyze the hydrolysis of at least portions of the hemicelluloses, as well as cellulose, was obtained by comparing the ratio of specific activity of the three final enzyme preparations on both types of substrate. The comparison indicates that component 2 apparently is the most active hemicellulase as well as cellulase. Moreover, the relationship between specific activity ratios of the three enzyme preparations with hemicellulose X (soluble) and hemicellulose Y (insoluble) were qualitatively similar to the ratios obtained with cellulose sodium sulfate and wheat

straw cellulose, indicating that the physical state of the substrate may have the same effect on hemicellulase as on cellulase activity.

The pH-activity curves for the hydrolysis of the hemicellulose preparations by the three enzyme solutions give further evidence for the multiplicity of enzymes acting on the hemicelluloses. Although the occurrence of two peaks for a single enzyme preparation, with substrates such as the hemicelluloses, cannot be interpreted as evidence of two enzymes, the marked difference between the curves for the three enzyme solutions makes this interpretation more sound.

The similarity of the end products of hydrolysis of the hemicelluloses by the three enzyme preparations might appear to be contradictory to the multiple enzyme hypothesis as applied to the hemicellulases. Aside from this, however, the similarity in the relative proportions of end products does serve to validate the comparison of specific activities. If the end products were different or were present in widely different proportions in the hydrolysates produced by the three enzyme preparations, then comparisons of specific activity would be less useful due to the nonspecificity of the copper reduction method of estimating reducing sugars.

The glucose found with the end products of hydrolysis of the hemicelluloses may have been due to partial degradation of cellulose during the extraction of the hemicelluloses with strong alkali, or it might actually be a part of a hemicellulose molecule. In any case, the relatively small amount of glucose present gives assurance that the activity measured by the copper reduction method was due mostly to cleavage of bonds other than the β-1, 4-glucosidic bonds.

Since a large share of the total activity with both hemicelluloses and cellulose was apparently contributed by component 2, it appears probable that the cellulase of M. verrucaria is not absolutely specific. These results are contrary to those of Grassman et al. (1933) who found that the xylanase activity of A. oryzae preparations could be separated from the cellulase activity. However, the difference could be due to the use of preparations from different sources.

The results with salicin as the substrate indicate clearly that nearly all of the β-glucosidase activity was confined to component 1. Since component 1 apparently had some action on cellulose sodium sulfate, and component 2 may have had some action on salicin, it appears that, although their specificities are not absolute, a complete separation of the two enzymes for purposes of classification is justified in the case of this system. These results do not agree entirely with those of Jermyn (1952b) who obtained evidence with A. oryzae preparations for the existence of several enzymes capable of hydrolyzing both carboxymethylcellulose and several β-glucosides.

SUMMARY

The present investigation was carried out to study (1) the possible multiplicity of cellulolytic enzymes and (2) the relationships among the cellulase, hemicellulase, and β-glucosidase activities in culture filtrates of M. verrucaria.

The culture filtrates from thirteen different 12-liter cultures of the organism were concentrated five to ten times by slow partial freezing. During this process an unexplained increase in the total activity of most of the filtrates was observed. Some loss of activity occurred in the water removed as ice.

Attempts to obtain fractions enriched in cellulase activity by means of fractional ammonium sulfate, acetone, or ethanol precipitation of the proteins, and by chromatography on powdered cellulose columns, ended in failure.

After the material was concentrated further by precipitation of the proteins with 75 percent acetone or saturated ammonium sulfate, fractionation by electrophoresis convection was found to be successful.

Fractionations were controlled by the determination of protein nitrogen, cellulase activity (using cellulose sodium sulfate as the substrate), and by electrophoretic analysis.

The unfractionated enzyme preparations had six electrophoretic components. Determinations of specific activity, in conjunction with electrophoretic analysis of fractions obtained during the course of the fractionation, showed that most of the activity with cellulose sodium

sulfate accompanied the three protein components having the lowest mobilities (components 1, 2, and 3). Three preparations with different ratios of the protein components were obtained by means of electrophoresis convection. The first preparation contained only components 1, 2, and 3, but the relative concentration of component 3 was low. The second preparation contained all the proteins except component 1, and the third had all the proteins except components 1 and 2.

The specific activities of the three enzyme preparations with cellulose sodium sulfate, two hemicellulose preparations from wheat straw, cellulose from wheat straw, and salicin were determined and compared. pH-activity curves for all of the enzyme preparations with each substrate were constructed and compared. The end products of enzymatic hydrolysis of cellulose sodium sulfate and the hemicellulose preparations were investigated by means of paper chromatography.

Considered in toto, the results indicate that the ability to catalyze the hydrolysis of each of the polysaccharide substrates is shared by at least two enzymes. Component 2 appeared to be the most active enzyme with all four polysaccharides, but the proportion of the total activity attributable to component 2 varied with the substrate. The evidence suggests that component 1 has some (variable) activity with all the substrates with the possible exception of wheat straw cellulose. The distribution of the remaining activity was not clearly indicated by the results.

Component 1 was found to be the most effective enzyme for the hydrolysis of salicin.

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APPENDIX A

TABLE XXXVIII

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF CELLULOSE SODIUM SULFATE BY THREE DIFFERENT ENZYME PREPARATIONS

(Results are expressed as the mg. of reducing substances (as glucose) per mg. of protein nitrogen)

pH	Enzyme Preparation I						
	Time in hours						
	1/3	1	2	4	8	20	25
3.78	110	348	610	1090	1800	2920	3180
4.24	264	640	1120	1880	2850	4250	4480
4.80	283	863	1480	2370	3570	5070	5320
5.10	360	890	1640	2510	3730	5240	5470
5.34	355	887	1610	2450	3670	5160	5420
5.95	297	820	1400	2280	3360	4650	4870
6.45	297	743	1360	2240	3260	4240	4410
6.98	156	535	920	1560	2290	3130	3290
7.37	129	348			1630		2450
7.55	101	228			1330		2230

pH	Enzyme Preparation II				
	Time in hours				
	1/4	1/2	2	4	8
4.15	52.2	89.7	262	392	523
4.75	75.3	122	334	473	631
5.05	82.6	144	338	483	629
5.35	83.7	144	325	453	598
5.90	82.5	135	322	446	584
6.45	77.2	118	285	409	530
6.95	65.0	100	239	328	424

pH	Enzyme Preparation III					
	Time in hours					
	1/3	2/3	2	4	10	24
4.15	28.3	43.0	104	157	227	280
4.75	42.3	72.6	167	226	324	402
5.08	46.1	78.6	160	226	314	388
5.37	45.5	81.0	158	222	313	378
5.65	45.5	80.1	158	203	300	358
6.00	44.9	78.0	152	187	283	318
6.51	42.6	76.2	149	180	262	295
6.90	39.9	62.5	114	154	198	231

TABLE XXXIX

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF WHEAT STRAW CELLULOSE BY THREE DIFFERENT ENZYME PREPARATIONS

(Results are expressed as the mg. of reducing substances (as glucose) per mg. of protein nitrogen)

pH	Enzyme Preparation I Time in hours					
	1	1 1/2	3	6	20	24
4.15	20.5	25.2	34.1	43.2	48.2	48.6
4.75	27.0	32.4	46.5	60.4	88.8	90.6
5.10	27.9	33.0	45.3	60.8	93.2	96.4
5.33	25.9	31.4	45.8	58.0	91.8	94.7
5.95	17.0	21.6	34.1	46.5	53.7	55.9
6.45	14.2	19.9	30.7	49.2	59.0	61.9
6.81	10.5	13.7	27.3	39.6	50.4	51.1

pH	Enzyme Preparation II Time in hours				
	1/3	1	3	10	24
4.14	3.8	9.0	15.0	26.6	39.2
4.72	5.2	9.2	18.9	37.1	57.0
5.10	5.4	11.8	20.7	40.1	61.0
5.35	5.2	11.2	20.8	40.4	59.2
5.92	4.6	9.9	18.9	35.9	56.1
6.43	3.5	7.2	15.0	28.8	46.5
6.80	2.5	6.0	13.1	25.0	38.3

pH	Enzyme Preparation III Time in hours				
	1/2	1	3	11	24
4.20	0.9	1.8	3.9	8.0	11.9
4.80	1.2	2.7	5.1	10.4	15.8
5.10	1.7	2.7	4.8	10.7	16.1
5.40	1.8	2.4	4.8	10.4	15.5
6.00	1.2	2.1	4.8	9.8	13.7
6.50	0.9	2.1	3.6	8.3	11.6
6.85	0.6	1.5	3.6	7.1	10.4

TABLE XL

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF HEMICELLULOSE X BY THREE DIFFERENT ENZYME PREPARATIONS

(Results are expressed as the mg. of reducing substances (as glucose) per mg. of protein nitrogen)

pH	Enzyme Preparation I						
	Time in hours						
	1/3	1	2	4	11 1/2	20	24
4.05	58.0	138	204	285	429	458	480
4.64	100	210	283	415	683	905	984
4.94	102	214	276	422	699	913	1010
5.25	103	214	288	422	698	874	918
5.55	102	203	281	417	667	856	904
5.86	103	212	280	412	676	893	961
6.22	100	208	264	405	671	883	948
6.47	90	190	264	393	636	778	816

pH	Enzyme Preparation II					
	Time in hours					
	1/3	1	2	3	10	24
4.09	15.9	26.1	34.3	40.1	66.6	93.2
4.65	20.6	35.2	46.5	55.5	94.3	159
4.95	22.2	37.0	48.6	57.8	102	166
5.25	22.7	39.7	49.8	61.6	109	173
5.80	23.7	38.8	55.4	62.1	132	187
6.20	22.7	37.0	48.8	57.8	110	179
6.40	22.0	38.4	46.6	58.2	104	168

pH	Enzyme Preparation III						
	Time in hours						
	1/3	1	2	4	11	20	24
4.15	8.5	17.8	21.8	30.3	44.6	50.8	54.8
4.75	14.0	27.2	33.4	44.0	63.1	84.0	93.6
5.10	17.3	30.1	38.3	48.8	72.6	93.0	101
5.35	18.5	30.4	39.2	49.9	74.1	96.8	105
5.95	17.4	30.7	39.0	49.4	75.0	96.5	104
6.45	17.9	30.4	37.2	49.0	74.1	95.0	102
6.90	17.5	27.8	35.0	47.6	69.9	91.8	96.4

TABLE XLI

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF HEMICELLULOSE Y BY THREE DIFFERENT ENZYME PREPARATIONS

(Results are expressed as the mg. of reducing substances (as glucose) per mg. of protein nitrogen)

pH	Enzyme Preparation I Time in hours						
	1/3	1	2	4	12	20	24
4.15	61.0	174	244	277	309	312	312
4.70	103	250	318	395	487	502	506
5.30	85.3	216	304	375	470	478	482
5.94	107	257	326	409	506	510	513
6.40	81.2	194	297	377	470	478	476
6.70	72.0	185	268	330	408	415	420

pH	Enzyme Preparation II Time in hours				
	1/3	1	3	12 1/2	24
4.15	26.2	38.2	56.6	91.1	99.0
4.70	30.6	48.0	71.5	128	162
5.10	32.8	49.7	72.2	133	166
5.35	32.1	48.0	69.7	133	164
5.83	33.9	50.8	69.3	133	168
6.40	31.2	47.5	65.6	125	160
6.61	29.1	42.7	65.6	118	146

pH	Enzyme Preparation III Time in hours						
	1/3	1	2	3	11	20	24
4.15	16.7	27.1	33.2	37.5	54.8	61.0	62.2
4.75	25.3	37.2	44.7	48.8	71.4	93.8	102
5.10	26.5	37.5	45.4	50.0	79.2	98.8	107
5.35	25.9	39.0	47.1	53.0	79.2	101	109
5.90	23.6	37.2	45.0	49.4	74.7	96.8	105
6.40	24.1	36.0	42.6	47.0	69.0	90.1	99.4
6.90	19.6	30.0	39.0	44.3	66.7	84.4	91.0

TABLE XLII

THE EFFECT OF pH ON THE ACCUMULATION OF END PRODUCTS FROM THE DEGRADATION OF SALICIN BY THREE DIFFERENT ENZYME PREPARATIONS

(Results are expressed as the mg. of reducing substances (as glucose) per mg. of protein nitrogen)

pH	Enzyme Preparation I						
	Time in hours						
	1/3	1	2	3	7	20	24
4.15	28.3	78.7	153	218	526	1050	1120
4.75	45.6	121	218	307	639	1350	1470
5.35	48.4	126	228	312	710	1370	1490
5.95	45.6	110	193	278	552	1230	1360
6.46	37.4	97.8	174	240	510	1080	1180
6.85	31.7	80.6	149	204	466	895	988

pH	Enzyme Preparation II				
	Time in hours				
	1/3	1	2	8	24
4.15	2.4	7.5	12.6	40.9	102
5.35	3.8	11.2	19.5	60.7	151
5.95	3.6	9.3	17.0	51.8	115

pH	Enzyme Preparation III					
	Time in hours					
	1/2	1	2	7	20	24
4.15	2.7	5.1	8.0	21.1	39.6	44.6
4.73	3.8	4.8	10.0	29.8	69.8	78.6
5.35	4.5	7.0	12.9	39.6	83.0	88.1
5.94	2.7	5.1	10.0	30.2	73.0	82.1
6.45	3.0	4.5	8.7	26.7	55.7	62.2
6.85	1.5	4.5	8.0	20.4	40.2	44.9

APPENDIX B

APPENDIX B

Preparation of Buffers and Reagents

Phosphate buffer, pH 6.9 ionic strength 0.145, used for electrophoretic analysis and electrophoresis convection.

Mix 13.0 ml. of 4 M monobasic sodium phosphate solution with 160 ml. of 0.5 M dibasic sodium phosphate solution and dilute to 2 liters with distilled water.

Phosphate buffer, pH 5.9 ionic strength 0.145, used for electrophoresis convection.

Mix 50 ml. of 4 M monobasic sodium phosphate solution with 60 ml. of 0.5 M dibasic sodium phosphate solution and dilute to 2 liters with distilled water.

Acetate buffer, pH 5.0 ionic strength 0.15, used for electrophoresis convection.

Mix 150 ml. of 2 N sodium acetate solution with 34.3 ml. of 3.5 N acetic acid solution and dilute to 2 liters with distilled water.

Citrate buffers from pH 3.5 to pH 7.5, 0.5 M, used for determination of enzymatic activity.

Dissolve 10.5 g. of citric acid (monohydrate) in a small volume of water and, using a glass electrode pH-meter, titrate with 10 M sodium hydroxide to a point a little below the desired pH. Cooling is necessary during the titration. After adjusting the volume of

solution to about 90 ml., add 100 mg. of 'Merthiolate' and titrate to the final pH with 1 or 2 N sodium hydroxide. Adjust the final volume to 100 ml.

Somogyi's (1945) alkaline copper reagent for micro determination of reducing sugars.

Dissolve 28 g. of anhydrous dibasic sodium phosphate and 40 g. of potassium sodium tartrate in about 700 ml. of water, add 100 ml. of N sodium hydroxide, followed by 80 ml. of 10 percent cupric sulfate (pentahydrate) with stirring. Add 180 g. of anhydrous sodium sulfate, dissolve, and allow to stand a day or two. Filter into a one-liter volumetric flask already containing 10 ml. of exactly 1 N potassium iodate solution. Dilute to 1 liter and mix thoroughly.

Color reagent used for paper chromatographic detection of reducing sugars (Mukherjee and Srivastava, 1952).

Dissolve 0.5 g. of p-anisidine in 2 ml. of phosphoric acid (sp. gr. 1.75), dilute to 50 ml. with absolute ethanol, and filter. Save the filtrate (A). Dissolve the precipitate in a minimum of water, dilute with an equal volume of ethanol. Add phosphoric acid to a concentration of 2 percent by volume (B). Mix A and B.