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By

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Exhaustive attempts have been made to detect changes in the fine structure of cotton cellulose brought about by the individual and combined action of the C_1 and C_x components of the cellulase complexes from Trichoderma viride, T. koningii, and Pestalotiopsis westerdijkii. None of the techniques used to date, which include measurement of the accessibility of the cotton to water vapour, infrared deuteration studies of the disordered hydroxyl groups, measurement of the disposition and accessibility of the hydroxyl groups on the surface of the crystalline elementary fibrils by repeated methylation with diazomethane,

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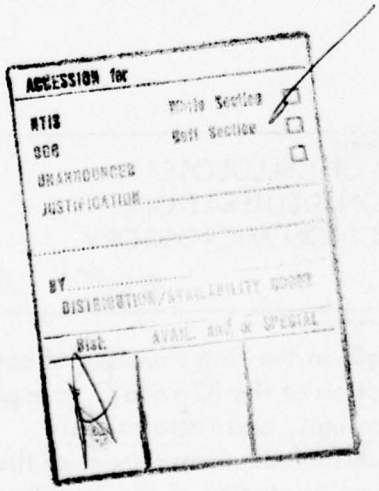
assessment of morphological changes by SEM, low-angle scattering X-ray crystallography and measurement of water imbibition and fibre density, has succeeded in detecting any significant changes in the substrate following treatment with each enzyme component alone, suggesting perhaps that highly-ordered native cotton is unaffected unless both enzyme components are present.

Reduction in the accessibility to water vapour following initial attack by the complete enzyme systems is probably associated with removal of less aggregated elementary fibrillar material. The average cross-section of the elementary fibrils in the untreated substrate has been shown to have 101 and 101 planar sides of 10 x 16 a.g.u; Wiley milling causes partial disaggregation of the elementary fibrils leading to structural units with an average cross-section around 10 x 12 a.g.u. Measurement of the distribution of the methyl substituents on the D-glucopyranosyl units at successive stages of methylation with moist ethereal diazomethane has indicated that the combined action of the C_1 and C_x components is confined to the pair of faces of the elementary fibrils containing the 2,6- and 2,3,6 hydroxyl groups accessible on alternate anhydroglucose units, suggesting that these cellulase enzymes, (possibly in the form of a unique complex), can only bond to cellulose molecules which have an appropriate and specific combination of their hydroxyl groups accessible. Action by the total enzyme system can lead to residual material containing elementary fibrils with either smaller or larger average cross-sections, depending on the extent of enzymolysis, the source of the enzyme and the temperature of treatment, and whether the system is shaken or not.

The report contains a significant bibliography.



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Abstract

Exhaustive attempts have been made to detect changes in the fine structure of cotton cellulose brought about by the individual and combined action of the C_1 and C_x components of the cellulase complexes from Trichoderma viride, T. koningii, and Pestalotiopsis westerdijkii. None of the techniques used to date, which include measurement of the accessibility of the cotton to water vapour, infrared deuteration studies of the disordered hydroxyl groups, measurement of the disposition and accessibility of the hydroxyl groups on the surface of the crystalline elementary fibrils by repeated methylation with diazomethane, assessment of morphological changes by SEM, low-angle scattering X-ray crystallography and measurement of water imbibition and fibre density, has succeeded in detecting any significant changes in the substrate following treatment with each enzyme component alone, suggesting perhaps that highly-ordered native cotton is unaffected unless both enzyme components are present.

Reduction in the accessibility to water vapour following initial attack by the complete enzyme systems is probably associated with removal of less aggregated elementary fibrillar material. The average cross-section of the elementary fibrils in the untreated substrate has been shown to have $10\bar{1}$ and 101 planar sides of 10 x 16 a.g.u; Wiley milling causes partial disaggregation of the elementary fibrils leading to structural units with an average cross-section around 10 x 12 a.g.u. Measurement of the distribution of the methyl substituents on the D-glucopyranosyl units at successive stages of methylation with moist ethereal diazomethane has indicated that the combined action of the C_1 and C_x components is confined to the pair of faces of the elementary fibrils containing the 2,6- and 2,3,6 hydroxyl groups accessible on alternate anhydroglucose units, suggesting that these cellulase enzymes, (possibly in the form of a unique complex), can only bond to cellulose molecules which have an appropriate and specific combination of their hydroxyl groups accessible. Action by the total enzyme system can lead to residual material containing elementary fibrils with either smaller or larger average cross-sections, depending on the extent of enzymolysis, the source of the enzyme and the temperature of treatment, and whether the system is shaken or not.

1. Statement of the Problem

It is now well established that the cellulase system which attacks highly ordered 'crystalline' cellulose is a group of enzymes acting in concert.

This project is concerned with the mechanism of the synergistic action between the C_1 and C_x components in the cellulase complex and the detection of any incipient changes in the fine structure of native cotton cellulose brought about by either the C_1 or C_x enzymes acting independently of each other.

2. Background

Much of the background to this project has already been discussed in an earlier report[1].

In the light of the increasing interest[2] in the utilisation of municipal and agricultural waste materials by the enzymic hydrolysis of their cellulosic components to produce glucose, which may then be used either as a chemical feedstock or fermented to fuel (ethanol), other useful chemicals, or microbial protein, it is important to develop a thorough understanding of the factors affecting the susceptibility of the various forms of cellulose to enzymolysis. Although it has been technically feasible to utilise waste cellulose via enzymolysis for at least the past 15 years, the economics remain somewhat unattractive because of the prolonged reaction times necessary to achieve efficient conversion to glucose of many of the more intractable forms of cellulose. Certainly, some types of native cellulose can be quite resistant to enzymic degradation, with dried cotton fibres among the most difficult to breakdown. The relatively slow rate of enzymolysis of native cotton is undoubtedly associated with its highly ordered, hydrogen bonded, fine structure and this material is generally recognised as being the most searching substrate for evaluating the efficacy of any cellulase enzyme system.

Comparative studies of large numbers of organisms[3,4] in the early 1950's demonstrated that, whereas many organisms can metabolise degraded forms of cellulose, comparatively few can also utilise the highly crystalline forms. Likewise, although some organisms produce large amounts of extracellular enzymes, others produce little or none. Indeed, it has been pointed out by B.v.Hoften[5] that many microorganisms degrade cellulose by direct contact with the substrate and it is possible that the occurrence of cellulase activity in culture supernatants after prolonged incubation under rather artificial laboratory conditions is due to autolysis rather than active secretion. He proposed that cellulases are more effective when cell-bound because they then occur in high concentration and are favourably aligned on the substrate. Nevertheless, most studies of the enzymic breakdown of cellulose have been carried out with extracellular enzymes.

Whilst some extracellular cellulases can degrade both crystalline cellulose (as typified by cotton) and soluble cellulose derivatives, others can degrade only the latter and are without effect on cotton. It is now well established that cellulases

capable of degrading cotton are multicomponent enzyme systems. As long ago as 1950 Reese et al[3] proposed that the cellulase complex consists of the following three enzymes; (i) a C_1 component, so named because it was believed to act first on the crystalline cellulose, de-aggregating the cellulose chains in preparation for subsequent attack by the hydrolytic enzymes, viz. (ii) the β - (1→4) glucanases (generally referred to as C_x enzymes, the 'x' reflecting the fact that normally there are several of these components in culture filtrates of cellulolytic microorganisms), which can hydrolyse soluble derivatives of cellulose or swollen and partially degraded celluloses, but cannot attack highly-ordered substrates on their own, and finally (iii) the β -glucosidases, which hydrolyse cellobiose and short-chain cello oligosaccharides to glucose. Whereas most culture filtrates of cellulolytic microorganisms contain the last two types of enzymes, comparatively few contain significant amounts of the C_1 component.

Among the most active producers of this C_1 enzyme are Trichoderma viride[6-12], T. koningii[13-18], Penicillium funiculosum[19], Fusarium solani[16,20-22], and Sporotrichum pulverulentum[23-26]. Considerable effort over the past decade has been devoted to the isolation, purification, and characterisation of the three classes of cellulase enzymes, viz. C_1, C_x, and β -D-glucosidases, elaborated by these microfungi. These three components of the cellulase complex achieve the breakdown of cotton when they act in concert; they lose this ability when separated but recover it again when recombined in their original proportions. There are now several examples of the C_1 component of one fungus acting synergistically with the C_x of a different fungus in solubilising native cotton. Selby reported 'cross synergism' of this type with the C_1 component of P. funiculosum and the C_x of T. viride[19]. Wood[27] has also observed similar effects with different combinations of C_1 and C_x from T. koningii and F. solani.

Particular attention has been directed to the C_1 component but its mode of action remains obscure. The original hypothesis of Reese et al.,[3] whereby C_1 is considered to have a prehydrolytic swelling action which causes loosening of the cellulose chains in the highly ordered, crystalline regions of the cotton, prior to attack by the hydrolytic C_x-enzymes, received support at one time by several investigators[6, 13, 19, 28, 29]. But the development of improved fractionation methods has more recently led to the isolation of more highly purified components, one of which has been shown to be a cellobiohydrolase[7-9, 12, 14-17, 22-24].

In these careful fractionations the elution of the cellobiohydrolase component coincided with the optically dense component previously accepted as being C_1 (defined as the enzyme acting synergistically with the randomly acting C_x enzymes to effect the solubilisation of highly ordered cellulose), and there is now an increasing consensus of opinion that this exo-glucanase and C_1 are one and the same.

When highly purified and essentially free from C_x endo-glucanase activity, the exo-glucanase/C_1 components of the cellulase complexes isolated by Wood from T. koningii[13-16] and from F. solani[16, 20-22], by Selby and Maitland[6], and Berghem and Pettersson[9] from T. viride, by Selby[19] from P. funiculosum, and by Eriksson and Petterssen[23] from Sporotrichum pulverulentum appears to be similar in that they all show an inability to attack native cellulose but they readily hydrolyse disordered cellulose, such as phosphoric acid-swollen cellulose, and the soluble cello-oligosaccharides. Cellobiose is the principal product in each case. Wood[22] and Pettersson et al[8] have shown that the rate of decrease of the degree of polymerisation of phosphoric acid-swollen cellulose with components of this type from F. solani and T. viride respectively is very slow compared with that effected by the randomly acting C_x endo-glucanases. The evidence cited by various authors indicates, therefore, that β -1,4-glucan cellobiohydrolases, capable of removing cellobiose from the non-reducing end of the cellulose chain, are produced by several organisms.

The relative resistance of highly ordered substrates to these exo-enzymes is presumed to be associated with the comparatively few non-reducing chain ends available for attack but some slight degradation of substrates such as cotton or Avicel will inevitably also result from the presence of varying residual amounts of C_x enzymes in the separate cellobiohydrolase component. Complete absence of randomly acting endo-glucanase activity towards carboxymethylcellulose has been reported[6, 23] for two of these cellobiohydrolase components when assayed by a viscosimetric method. This technique is very sensitive to the presence of endo-glucanase activity since only one or two random scissions along the chain results in a relatively large change in viscosity, whereas enzyme attack from the end of the chain would produce little change in viscosity. Limited production of reducing sugars from water soluble cellulose derivatives is generally observed but this ceases once the available unsubstituted cellobiose residues have been removed from the chain ends.

In many reports of the separation of the components of cellulase complexes, however, the fractionation appears to be incomplete. Thus, the so-called C_1 type components isolated by the Japanese workers[7, 11] from T. viride still contained appreciable carboxymethyl-cellulase activity and possessed the ability to attack the crystalline cellulose, Avicel, when acting alone; the potentiation of this 'Avicelase' activity when mixed with isolated fractions containing the C_x endo-glucanase components was not particularly marked. Likewise, Halliwell and Griffin[17] reported that a purified cellobiohydrolase component isolated from T. koningii did not require addition of the C_x component of this cellulase system for the solubilisation of cotton. The action of this cellobiohydrolase component towards cotton and bacterial cellulose was found to be inhibited by the cellobiose produced, explaining the apparent synergism with the cellobiase isolated from this cellulase complex. Similar results have recently been reported by Pettersson[12] who separated four different cellulolytic enzymes from T. viride, one of which, an exo-glucanase, apparently catalysed alone the hydrolysis of microcrystalline cellulose up to 80% and native cotton cellulose up to 40% solubilisation by removing cellobiose units from the chain ends. These experiments were carried out in equipment in which the cellobiose could be continually removed.

Several workers have reported the disintegration of cotton fibres into small insoluble fragments by various components of the cellulase complex. This fragmentation phenomenon highlights the difficulties associated with the purification of the individual components. Thus, earlier separations of the C_1 component by Selby and Maitland[6] and by Wood[13] apparently possessed this short fibre forming activity but subsequent preparations obtained by Wood[27] no longer show this activity after further purification by isoelectric focussing. Pettersson[12] demonstrated that two endo-glucanases separated from the T. viride cellulase complex possessed this property, as does the endo-cellulase isolated by Halliwell[18]. Fragmentation of this type is now thought to be a characteristic of purified C_x enzymes, and not of C_1 .

A feature of much of the fractionation work carried out on cellulase systems is the multiplicity of components that have been isolated, particularly of the components showing C_x type activity. For example, recent separations by Eriksson, Pettersson, and colleagues[24, 30, 31] have led to the isolation of five endo-1,4-β-glucanases and an exo-1,4-β-glucanase from culture solutions of Sporotrichum pulverulentum. There were only small differences in the amino acid composition of the endo-glucanases.

However, all but one of these isoenzymes were shown to be glycoproteins, with appreciable differences in their carbohydrate content varying between 0 and 10.5%. A strong synergistic effect was observed between each of these endo-glucanases and the exo-glucanase, but all five gave rise to different degradation products from Avicel, they exhibited differences in the preferred mode of attack on cellodextrins and differences in their synthetic activities were observed. The two endo-1,4-β-glucanases isolated from T. viride by Pettersson[12] also contained different amounts of carbohydrate (12% and 21%). Wood and McCrae[15] actually separated 8 highly purified components from the cellulase complex elaborated by T. koningii, viz. a cellobiohydrolase, five C_x components, and two β-glucosidases. Likewise, Nisizawa and his co-workers[32] separated a multitude of extracellular cellulase components of different molecular weight from the culture medium of Pseudomonas fluorescens var. cellulosa. Several C_1-type components have also been recently isolated from cellulase complexes from different organisms. Wood[16, 27] has resolved the C_1 component in T. koningii into a major and minor protein component by isoelectric focussing. Either component acted in synergism with the C_x component to the same extent in solubilising cotton fibres. The only apparent difference between these two C_1 isoenzymes was in their carbohydrate content (9% in the major and 32% in the minor component). The C_1 from F. solani was also easily resolved into 2 enzyme fractions with apparently identical specificities[16] but associated with different amounts of carbohydrates (11% and 20%). Using a similar isoelectric focussing technique Berghem and Pettersson[9] isolated four C_1-type components from a T. viride cellulase. The separation of these isoenzymes with essentially the same specificities is undoubtedly an added complication, but the role of the individual enzymes in the cellulase complex can only be confidently established where scrupulous attention is paid to their purity.

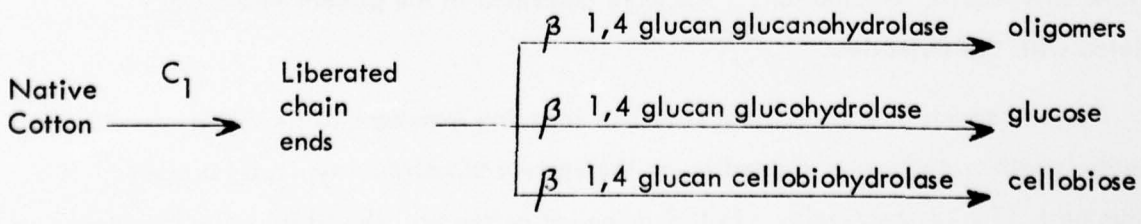
The original concept of Reese et al[3], involving the C_1 enzyme in initiating attack on highly ordered substrates and modifying the cellulose structure to make it more accessible to attack by the C_x enzymes, has recently been reversed by several groups of workers following the characterisation of the C_1 component as having cellobiohydrolase activity. According to this latest hypothesis it is the endo-1,4-β-glucanases which attack first, in a random manner, to produce chain ends for subsequent attack by the exo-enzyme. This theory is now generally supported by Eriksson and Pettersson[23], Wood and McCrae[14], Berghem and Pettersson[9], and Nisizawa[33]. However, Reese[34, 35] has critically questioned this interpretation and he does not

believe C_1 and cellobiohydrolase are one. He gives two pertinent reasons for claiming that the cellobiohydrolase cannot be the old C_1: (i) Cellobiohydrolase has been previously characterised in a cellulolytic bacterium; both C_x and cellobiohydrolase were shown[36] to be present in culture filtrates of Cellvibrio gilvus, yet this combination of enzymes hydrolysed native cellulose at barely detectable rates and obviously lacks the true C_1 component. (ii) Since there are several C_xs of differing specificities capable of acting on the liberated chains, together also with the exo-enzyme, glucohydrolase, which can act independently from the non-reducing end, why is there the special need for a cellobiohydrolase, and why cannot the reaction go rapidly to completion without the intervention of cellobiohydrolase if C_x does indeed act first?

Reese's implication that C_1 and cellobiohydrolase are two different protein components having similar net charges and molecular weights and therefore inseparable by current techniques has been extensively tested[15, 22, 23] but no evidence for their non-identity has been found to date.

Wood has pointed out[15] that it is not unreasonable for the C_x enzymes to be the ones that initiate the attack, since other early manifestations of attack on cotton *fibres, such as changes in tensile strength*[15, 19, 27], *fragmentation into short* fibres[12, 18, 27], and enhancement of alkali swelling[13, 14, 16, 20] appear to be associated with C_x enzymes acting alone, whereas carefully purified C_1 alone produces no such changes. Be that as it may, all of these effects are enhanced by C_1 and C_x acting together. But the mechanism describing the hydrolysis of highly ordered cellulose in terms of initial action by endo-glucanase (C_x) followed by a cellobiohydrolase (C_1) does not explain why these two enzyme types are incapable of solubilising cotton when acting alone yet individually both are capable of hydrolysing swollen cellulose.

It seems to us that attack on highly ordered cellulose must be initiated by a chain-separating enzyme. Unfortunately this is difficult to prove. The original concept[3] has now been modified by Reese[34, 35] to include the possibility that C_1 randomly splits the covalent bonds of the cellulose chains at the crystallite surface. Thus:-



This expanded concept makes C_1 a member of the C_x random-acting enzymes, albeit a special member having properties not possessed by any previously defined C_x component. Its action is confined to crystalline cellulose; unlike the endo-C_x β -1,4-glucan glucanohydrolase, it has no action on cellulose derivatives. Furthermore, since it produces no soluble reducing sugars, it is obviously unable to act on the products of its own action.

The fact that the requirement for the C_1 enzyme is considerably reduced when crystalline cellulose is rendered more accessible by physical methods such as ball-milling, swelling, or reprecipitation from solvents lends support to this original hypothesis. Millett et al[37] have reviewed the influence of chemical and mechanical pretreatment techniques on enzymic hydrolysis rates and recently Tassinari and Macy[38] have described differential speed two roll milling as an effective pretreatment for increasing the susceptibility of cellulose to enzymic hydrolysis. Such processes are generally supposed to disrupt the crystalline structure of the substrate.

The C_1 component has a very low activation energy. Whilst this is apparently in accordance with the argument for C_1 being a 'hydrogen bondase', it has been pointed out by Goksøyr et al[39] that the rate-determining step might well be the release of the enzyme involved from the products and it is this step that determines the activation energy. This possibility is supported by the fact that cellobiose is a strong inhibitor of C_1 .

Yet another possible mechanism for making the crystalline parts of the cellulose more accessible has been suggested by Eriksson et al[40] who have discovered an oxidising enzyme in the culture filtrate from S. pulverulentum which gives at least twice the extent of degradation given by the same mixture of endo- and exo-glucanases when the oxidising enzyme is absent. It is postulated that the role of this oxidising enzyme is to oxidise a small number of anhydroglucose units to uronic acid moieties thus breaking the regular hydrogen bonds between the cellulose chains and making the crystalline regions more accessible to the hydrolysing enzymes.

Clearly, the precise mechanism of the synergistic action between the C_1 and C_x enzymes still requires elucidation. The work described in the present report was initiated with this objective.

Cotton consists of a unicellular seed hair tapering from base to tip, with variable length and diameter depending on the variety of cotton - typically around 2.5 cm and 15 μ respectively. In the unopened cotton boll the fibres are cylindrical

in shape with a lumen containing protoplasm and other cell fluids, but when the boll opens and the fibres dry out they become flattened and convoluted. The exterior surface of raw cotton is a coherent membrane, the cuticle, consisting of wax and pectic substances. The cuticle is removed by kier treatment or boiling in peroxide to expose the primary wall consisting of a loose and somewhat felt-like assembly of microfibrils and bundles of microfibrils which are formed during the extension of the cell and define its size. The bulk of the cellulose in the cotton fibre is contained in the secondary wall lying immediately inside the primary wall and which is deposited in layers on the inside of the established cell. The secondary wall cellulose of cotton is a tightly packed and well oriented assembly of fibrils with no evidence of branching having ever been found. The fibrils seen with the optical microscope are at least 2000 Å thick but these are clearly bundles of much smaller units and the electron microscope reveals single elementary fibrils with a spread of diameter below 75 Å i.e. with dimensions that are no wider than the width of a single crystallite as deduced from X-ray measurements. Aggregation of elementary fibrils into microfibrils (200 - 300 Å thick) and then into macrofibrils (1000 Å) is evident in electron micrographs.

The nature of the elementary fibrils has been the subject of much research. Although early X-ray work suggested that there was a 30% non-crystalline fraction in cotton, more recent interpretations of the X-ray fibre diagram indicates that scoured cotton is almost completely crystalline, or rather that all the material present contributes to the cellulose I X-ray diffraction; some disorder on the surface of the fibrils can arise from chains that are nevertheless near enough to their lattice positions to contribute to coherent X-ray diffraction.

Virtually all the hydroxyl groups in cotton cellulose are hydrogen bonded and infrared studies on the hydroxyl-stretching band in the 3 μ region indicates that there appears to be no free hydroxyl groups present. The hydroxyl groups are either bonded in a regular, ordered pattern, and are almost inaccessible to deuterium oxide (with the result that exchange to OD is slow and incomplete) or hydrogen-bonded in a somewhat more random, disordered manner (these OH groups are completely accessible to D_2O and exchange very rapidly to OD). Infrared deuteration studies have shown[41] that 58-60% of the hydroxyl groups in cotton are hydrogen-bond ordered i.e. 40 - 42% are disordered; this is reasonably consistent with a 'crystalline' elementary fibril of lateral dimensions 50 Å x 40 Å (i.e. about 10 a.g.u. x 8 a.g.u. - see below) which would have about 40% of its cellulose chains on the surface of the elementary fibril; since only about half of the hydroxyl groups on these surface cellulose molecules will be pointing outwards it is necessary to postulate some

slight hydrogen-bond disorder throughout the outermost layer of chains.

It is evident on the basis of a fringed fibril structure for cotton that the important factor in determining the reactivity of the cellulose is the accessibility of the surfaces of the elementary fibrils. The hydrogen-bond disordered component of cotton (consisting of the total hydroxyl groups on the surface of the elementary fibrils, plus some additional hydroxyls close to the surface) is accessible to water molecules and thus is responsible for the sorption of water vapour by the fibre. The moisture regain of the cotton is therefore directly related to the hydrogen-bond disorder determined by infrared deuteration[41]. It has been shown[42], however, that aqueous solutions do not have access to as many hydroxyl groups as does water vapour and comparison of accessibilities obtained from moisture regains with those measured by acid hydrolysis and periodate oxidation suggest that about one-third of the hydroxyl groups in cotton cellulose are accessible to water vapour but not to the active species in aqueous acid or periodate solutions; penetration of the interface between the elementary fibrils is thought to be more difficult for these active species than for molecules of water[43].

There are three hydroxyl groups (at C(2), C(3), and C(6)) on each anhydroglucose unit (a.g.u.) of the cotton cellulose molecules which are potentially available for entering into chemical reactions. From studies of repeated reactions between a solution of dimethyl sulphate in dimethyl sulphoxide and cotton fabric impregnated with sodium hydroxide (2N), followed by total hydrolysis of the partially methylated cellulose and g.l.c. analysis of the derived partially methylated glucoses in the hydrolysates after trimethylsilylation[44] we deduced several years ago[45] a model for the elementary structural unit in cotton cellulose having a surface of D-glucopyranosyl units with accessible 2-, 3-, and 6-hydroxyl groups in the proportions indicated by the relative amounts of the various methyl glucoses at the end of the initial rapid reaction. When the amounts of the individual sugars present in the hydrolysates are examined in relation to the number of cycles of methylation it is clear that the methylation occurs in two stages, an initial rapid reaction (on the surfaces) and a slower reaction (limited penetration) which proceeds steadily over successive treatments[45]. According to the derived model, alternate D-glucopyranosyl units along the cellulose chains in one pair of parallel surfaces of a unit of rectangular cross section gives rise to 2- and 6-O-methyl-D-glucose respectively on methylation and total hydrolysis, whilst those on the other pair of surfaces yields 2,6-di-O-methyl-

and 2,3,6-tri-O-methyl-D-glucose. *Intramolecular hydrogen bonding between* C(3) hydroxyl and the C(5^1) oxygen atom[46] was assumed to explain the production of approximately equal amounts of 2- and 6-O-methyl-D-glucose, and of 2,6-di-O-methyl- and 2,3,6-tri-O-methyl-D-glucose respectively from each pair of surfaces, with very little 3-O-methyl-, 2,3-di-O-methyl-, or 3,6-di-O-methyl-D-glucose.

A structural unit containing a bundle of 80 cellulose chains in a block 8 x 10 a.g.u. cross section was finally indicated by the observed distribution of methoxyl groups. Complete methylation of the outside only of such a bundle containing 32 accessible a.g.u. on the surface and 48 inaccessible units inside (in the cross section), would give 60 moles % of D-glucose and 10 moles % each of 2- and 6-O-methyl-, 2,6-di-O-methyl-, and 2,3,6 -tri-O-methyl-D-glucose after complete hydrolysis. The observed results agreed well with these figures, only small amounts of other 3-O-methylated glucoses being detected. The elementary fibrillar unit indicated by these results would have a cross section about 40 x 50 Å, closely similar to the smallest elementary fibrils observed by electron microscopy (30 - 60 Å)[47].

The presence of larger units was indicated, however, by a similar examination of the methoxyl distribution in cotton methylated with diazomethane in ether saturated with water[45], suggesting that these elementary fibrils must be aggregated under these conditions. Indeed, in the dry state cotton must be almost completely aggregated since dry cotton is scarcely methylated at all by dry ethereal diazomethane[48]. Water must therefore lead to extensive disaggregation, but complete breakdown to elementary fibrils does not apparently occur unless dilute alkali (2N NaOH) is present. These techniques were subsequently used to measure the accessibility of cotton chemically swollen in 5N sodium hydroxide, 62% sulphuric acid, or 81% phosphoric acid[49]. Observed increases in accessibility are in line either with the opening up of new surfaces by further subdividing the aggregated elementary fibrils or with progressive penetration of the outer layers by the swelling agents.

More recently a somewhat different chemical approach has been used by Rowland et al at the Southern Regional Research Laboratories[50-54], to study the disposition of D-glucopyranosyl units on the surfaces of crystalline elementary fibrils of cotton cellulose and the results compared, correlated, and rationalised[55] with the approach of the group at the Shirley Institute described above.

We believe that the uniqueness of the C_1 *enzyme is characterised by its ability*

to catalyse the hydrolysis of β-1,4-glucan bonds in cellulose molecules that are situated on the surface of crystalline structural elements in native cellulose, where the spatial disposition of the hydroxyl groups is quite different from that obtaining on other cellulose molecules in disordered structures or in aqueous solutions of cellulose derivatives of low DS, because of the close packing of the perfectly aligned polymer molecules throughout these strongly hydrogen-bonded regions. On the 101 planar surfaces, to which the planes of the D-glucopyranosyl units are perpendicular, the hydroxyl groups at C(2) and C(3) will protrude on alternate D-glucopyranosyl units while the hydroxyl groups at C(6) will protrude on the others. The hydroxyl groups at C(3) are probably unavailable for enzyme coupling, however, because of involvement in intramolecular hydrogen bonds between C(3) hydroxyl and the $C(5^1)$ oxygen atom. On the $10\bar{1}$ planar surfaces, to which the planes of the D-glucopyranosyl units are parallel, the hydroxyl groups at C(2) and C(6) are thought to be equally available. Half of the D-glucopyranosyl units in this surface will have the $O(3)\ H\text{--}\ O(5^1)$ hydrogen bonds oriented above the plane of the surface whereas in the other half these hydrogen bonds will be oriented below the plane of the surface. In contrast, the hydroxyl groups at C(2), C(3), and C(6) positions of D-glucopyranosyl units of disordered cellulose may be expected to be equally available for bonding with the C_x enzymes.

Whilst C_x components with CM-cellulase activity are able to break randomly the chains of water-soluble cellulose derivatives or highly swollen cellulose, they are unable to act on cellulose molecules uniquely situated on the surface of highly-ordered, 'crystalline' regions in native cellulose until these molecules are released from the elementary fibrillar surfaces by some special enzyme which we shall continue to call C_1. The liberated chain ends provide additional sites for attack by the cellobiohydrolase component. A combination of actions by C_x and cellobiohydrolase is suggested to complete the removal of the disordered cellulose molecules on the surface of the elementary fibrils before C_1 can act on the next underlying layer of highly ordered cellulose molecules.

Some support for these ideas is afforded by the work of Rautela and King[56] who have shown that the crystal structure of cellulose I, II and IV is a significant determinant of the effectiveness of cellulase systems.

3. Experimental Methods

3.1 Moisture regain and water retention measurements

Measurements of moisture regain and water retention (water imbibition) were carried out on the same specimens, both in the 'never-dried' state and also after drying over P_2O_5. 'Never-dried' specimens (0.2 - 0.4g) of the enzyme treated and control samples were soaked for 60 minutes in distilled water, centrifuged at 1200g for 15 mins and weighed. They were then successively conditioned to constant weight over saturated salt solutions in evacuated desiccators in a room thermostatted at 25°C; the first salt solution was zinc sulphate (88% r.h.) and this was followed by saturated sodium nitrite (65% r.h.). The dry weights were then determined after drying for 14 days in vacuo over P_2O_5, to give the water retention and moisture regain values expressed as a percent of the dry weight. The specimens were then conditioned again and the moisture regain re-measured first at 65% r.h. and then 88% r.h. Finally, the water retention was redetermined by soaking the 88% r.h. conditioned specimens in distilled water and centrifuging as before.

Gibbons'[57] values for the moisture regain of fully accessible cellulose at 65% r.h. (1.53 moles water/D-glucopyranosyl unit) and at 88% r.h. (2.6 moles water/D-glucopyranosyl unit, by extrapolation) were used to calculate accessibilities to water vapour:

$$\% \text{ Accessibility } (A_{H_2O} \text{ vap.}) = \frac{100 \text{ M.R.}_{65\% \text{ r.h.}}}{1.53} = \frac{100 \text{ M.R.}_{88\% \text{ r.h.}}}{2.5}$$

where M.R. is expressed in moles water per polymer unit.

3.2 Infrared deuteration

The basis of the infrared deuteration technique and its significance in relation to structure have been described previously and this technique has been used to measure hydrogen-bond order and disorder in several types of cellulose[41, 58-61]. Its application to cotton, however, has been limited in the past because of difficulties in obtaining satisfactory infrared spectra from cotton fibres. These difficulties were overcome in a previous study[62] and a method was devised that gave specimens of satisfactory transmission characteristics. In this method the cotton fibres are milled, sieved onto the bottom plate of a high-pressure die in an even layer of suitable

thickness and pressed at 145,000 lb/in^2 for 20 minutes. The resulting coherent film is lifted off the die with a razor blade and mounted in a metal foil holder. The fibre films obtained in this way have good infrared transmission characteristics and are thin enough for quantitative measurements; although there are usually some holes they are rarely more than 10% of the area of the film.

Initial problems encountered in the present project with moist, 'never-dried' fibre assemblies have been discussed in an earlier report[1] where it was pointed out that the fibres must be air dried in order to obtain satisfactory pressed discs. To avoid underestimating the number of accessible hydroxyl groups present in the original moist samples, due to any recrystallisation that may occur on drying, they were deuterated with a large excess of liquid D_2O before drying and prior to preparation of the pressed fibre discs. This initial treatment ensures that all the hydroxyl groups which are accessible to water in the moist enzyme-treated cotton fibres are exchanged with deuterium before there can be any structural changes associated with drying. After pressing the dried, deuterated fibres in air the pressed discs were re-deuterated in D_2O vapour in a special cell prior to infrared examination.

The experimental procedure described previously[1] was subsequently modified as follows: Moist fibres (about 10 mg) were cut with sharp scissors to a fibre length of about 1 mm and immersed in liquid D_2O (3 ml) in a sealed tube for 30 minutes. The D_2O was removed under vacuum (10^{-3} mm) at ambient temperature and dried fibres were carefully sprinkled on to the bottom surface of a polished stainless steel die (13 mm diameter) to give a uniform, thin layer of fibres weighing about 1 to 2 mg. The die was assembled and pressed in a hydraulic press for 30 minutes with a pressure of 30,000 lb. The pressed fibre disc, which should be uniform, coherent, semi-translucent, and free from holes, was gently removed from the die face with a razor blade. It was then mounted in a special holder and placed in a sealed brass cell fitted with CaF_2 windows, which was then evacuated (10^{-3} mm) for 30 minutes. The accessible O-D groups in the specimen are largely re-hydrogenated during the preparation of the disc, which was carried out in air at ambient humidity. The specimen was therefore next re-deuterated for about 30 minutes by connecting the evacuated cell to a 57% r.h., D_2O vapour, atmosphere over a saturated solution of sodium bromide in D_2O. After re-evacuating the cell through a cold trap to remove all the D_2O the infrared spectrum was recorded between 2.5 and 5 microns using a Grubb-Parsons 'Spectromaster' Mk.II, with a scanning speed

of 0.25 microns/minute.

The fraction of accessible hydroxyl groups in the original 'never-dried' sample was obtained as a percent of the total hydroxyl groups by measuring the intensity of the O-D band at 2530 cm^{-1} and the C-H band near 2900 cm^{-1} and multiplying the O-D/C-H intensity ratio by 24. This factor of 24, which was obtained from calibration data relating to previous work, is appropriate for the deuterated, disordered material; a different factor (16) has been shown to apply to the ordered fraction[63]. The C-H band is not affected, of course, by the deuteration and constitutes an internal measure of the thickness of the pressed disc.

In calculating the intensities of the O-D and C-H bands errors may arise due to the uncertainty as to the exact position of the 100% transmission level on the infrared spectrum. The true 100% level can be calculated on the basis of the known band shape of the O-D band. If the apparent absorption at the band peak intensity maximum is A, the apparent absorption at the 'half-height' of the band is B (the 'half-height' position is identified from the two frequencies $\left[2600cm^{-1} \text{ and } 2435cm^{-1}\right]$ previously characterised from spectra of known band shape), and the apparent absorption at the base of the band at the peak frequency ($2530cm^{-1}$) is C, then it can be shown[63] that the true 100% transmission level is given by $\frac{B^2 - AC}{2B - (A+C)}$.

These experiments showed a poor level of reproducibility and it was necessary to make several measurements on each prepared specimen. However, repeated exposure of the pressed disc to D_2O leads to a slow steady deuteration of the crystalline, hydrogen-bond ordered, structure over a period of several days. Allowance was made for this slight penetration by D_2O of the outer layers of the elementary fibrils by recording the change in accessible hydroxyl group content with time and extrapolating the uniform part of the deuteration rate curve to zero time.

3.3 Diazomethane methylation procedure

The diazomethane methylation procedure described previously[45,49] has been used with only minor modification. The moist fibres (as received) were washed, centrifuged, and then steeped in the moist 0.25M ethereal diazomethane solution (100 ml/g) at 4°C. The diazomethane concentration was seriously depleted during the initially fast reaction in the first 24 hours but thereafter a large excess of reagent was maintained for the remainder of the 6 weeks' reaction period by changing the ethereal solution after day 1, 2, 4, 7, 10, 15, 21, and 28. In later experiments the period of methylation was extended to 35 and 42 days to improve the accuracy of the extrapolation of the steady, slow-rate part of the curves to zero time. Small specimens (about 100 mg) of

the partially methylated fibres were collected each time the reagent was changed, for subsequent analysis by hydrolysis, trimethylsilylation, and g.l.c. determination of the partially methylated glucose derivatives.

3.4 Acid hydrolysis of methylated fibres

The procedure for hydrolysis of the partially methylated cellulose samples closely followed that described by Croon and Lindberg[64] and used previously by Haworth, Roberts, and Sagar[44], with suitable scaling down for application to fibre samples weighing only ca. 15 mg. Air dry methylated cotton fibre (15 mg) was dissolved in 72% (v/v) sulphuric acid (0.6 ml) at room temperature over about 30 minutes. The solution was diluted with water (4.4 ml) and heated for 4 hr. at 100°C. The sulphuric acid was neutralised overnight with an excess of finely ground solid barium carbonate and the barium salts removed by filtration and washed with water (ca. 6 ml) followed by ethanol (ca. 3 ml). The combined filtrate and washings were concentrated on a rotary evaporator under reduced pressure and the concentrate (ca. 0.5ml) transferred to a small glass vial (5cm x 1cm). After freezing in liquid nitrogen the solution was evaporated to dryness by connecting the tube to a high vacuum line.

3.5 Gas-liquid chromatographic analysis of the hydrolysates

The procedure described by Haworth, Roberts, and Sagar[44] for determination of the distribution of methoxyl groups in partially methylated cotton samples, based on the gas-liquid chromatographic examination of the trimethylsilyl derivatives of the series of methyl ethers of glucose produced on hydrolysis, was closely followed.

The vacuum dried hydrolysate was dissolved in pyridine (0.2 ml) (previously dried over potassium hydroxide pellets), hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.1 ml) were added, and the tube stoppered and shaken well. Fuming of the reaction mixture when the stopper is removed indicates an adequate excess of trimethylsilylating reagents. The mixture was allowed to settle overnight before taking samples of the supernatant solution for g.l.c. analysis.

The modified Pye Argon Chromatograph described previously[44] was used for analysis of these trimethylsilylated hydrolysates. Satisfactory resolution of the sixteen possible trimethylsilylated sugars in these hydrolysates, viz. α - and β - anomers of glucose, 2-,3-, and 6-O-methyl-D-glucose, 2,3-,2,6- and 3,6-di-O-

methyl-D-glucose, and 2,3,6-tri-O-methyl-D-glucose, was achieved using two columns. Column 1 was packed with glass micro-beads, 105-125 μm diameter, coated with n-hexatriacontane, 0.204% by weight, total weight of stationary phase 0.0441g. Column 2 was packed with 60-100 mesh Kieselguhr (M & B Embacel acid-washed) coated with poly (ethylene glycol succinate) m.p. 103-104°, 10.0% by weight, total weight of stationary phase 0.369g. Column temperatures were 135°. Samples (0.1 μl) of the trimethylsilylated hydrolysates were introduced onto a plug of silica-glass wool on top of the column packing by means of capillary pipettes.

Peak areas were measured by planimeter and the relative area attributed to individual components was computed for each column in turn, assuming an anomeric ratio for all components of 1:1, which is a reasonable first approximation based on measured amounts of each anomer over many determinations. Individual values for α - and β - anomers were summed to give the figures for the respective sugars; final values are means of the results obtained on the two columns.

Attempts were made to measure changes in the cellulose chain-end groups by gas-liquid chromatographic estimation of the 2,3,4,6 tetramethyl-D-glucose content in the trimethylsilylated hydrolysates of the extensively methylated samples. A Model 64 Pye 104 Gas Chromatograph fitted with dual flame ionisation detectors, heated detector ovens, and twin columns was used to carry out this investigation. Standard Pye coiled glass columns (5 ft in length and 4 mm i.d.) were used in an attempt to separate the trace amounts of tetra methyl glucose from the much larger amounts of unmethylated glucose and the tri-,di-, and mono-methyl glucoses. The columns were silanised before packing by passing through them a 2% solution of dimethyl dichlorosilane in carbon tetrachloride ('Repelcote' - Hopkins and Williams) and then drying at 100°C. The treated columns were packed either with 60 - 100 mesh kieselguhr ('Embacel', May and Baker, treated with Repelcote) coated with methyl silicone gum rubber, SE-30, 1.75% by weight, total weight of stationary phase in the column, 70 mg, or 100 - 120 mesh kieselguhr ('Embacel' acid and alkali washed) coated with Apiezon L, 2.30% by weight, total weight of stationary phase in the column, 35 mg. Silanised silica glass wool plugs were placed on the top of the column packing after filling. The new packed columns were conditioned before use by heating at 250°C for 48 hours with an argon flow of 60 ml/minute. Samples (1.0 μl) of the trimethylsilylated solutions of the hydrolysates of the methylated cotton

fibres were injected through a septum with a 1 µl hypodermic syringe. The gas chromatographic operating conditions were as follows: Argon was used as the carrier gas with a flow rate close to 60 ml/minute; the SE-30 column temperature was programmed after a 20 minute hold from 125 to 210°C at 4°C/min, and the Apiezon L column after a 10 minute hold from 150 to 250°C at 2°C/min; the detector ovens were thermostatted at 250°C.

3.6 Electron microscopy

Longitudinal specimens for examination by scanning electron microscopy were scattered on an adhesive conducting surface on brass stubs (1 cm diameter). The specimens were then vacuum coated with gold to render them electron-conducting. The prepared specimens were examined in a Jeol JSM 1 scanning electron microscope at an accelerating voltage of 10kV, typical areas from each specimen being selected for photography.

Most of the 'never-dried' samples examined in the later stages of this work were prepared for vacuum coating by drying the moist fibres in a Model E3000 Critical Point Drying Apparatus (Polaron Equipment Ltd) to avoid the possibility of changes in the fibre surface associated with the large surface tension forces created in the micro-cavities when a liquid/gas interface is allowed to develop during normal drying under vacuum. The moist specimens were exchanged with acetone and the acetone replaced with liquid CO_2 (the critical point drying liquid) and the final drying run carried out inside the pressure vessel by passing hot water (40°C) through the water jacket to raise the temperature above the critical point. At this stage the liquid/gas meniscus disappears and the chamber contains only gas. The vent valve can then be opened slightly and the gas bled off slowly to atmosphere avoiding condensation, to leave the specimens dry.

3.7 X-ray measurements

Moist fibre samples were critical point dried and then compressed into pellets about 1mm. thick with the minimum amount of pressure sufficient to ensure cohesion of the fibres. The pellets were mounted in a small-angle X-ray camera with two cassettes, one at 5cm and the other at 17cm distance from the specimen. X-ray photographs were taken over a period of several hours with nickel-filtered copper K α radiation, a wide-angle diffraction pattern being recorded with the cassette at 5 cm and a small-angle diffraction pattern with the second cassette positioned at 17 cm from the specimen.

3.8 Fibre density measurements

The method described by Orr et.al.[65] for determining the specific volume of cotton fibres using density-gradient columns was suitably modified. Two columns (I and II) were prepared, respectively in 1 l and 500 ml. graduated measuring cylinders fitted with ground-glass stoppers. Column I was prepared from three stock solutions A, B and C consisting of A, xylene (91.6 ml) and carbon tetrachloride (208.4 ml), 1.37 S.G.; B, xylene (93.2 ml) and carbon tetrachloride (506.8 ml), 1.48 S.G.; and C, pure carbon tetrachloride, 1.594 S.G. The stock solutions were dried over type 5A molecular sieves before making a series of mixtures of A with B, and B with C in the ratios 1:9 through to 9:1. The column was then slowly filled with 50 ml. of each mixture, putting the densest liquid (pure CCl_4) in first and finishing with the least dense mixture (stock solution A). The second column, II, covering a narrower range of densities, was prepared in a similar manner from two of the above stock solutions (B and C). After drying, a series of 50 ml. mixtures was made ranging from 9:1 through to 1:9 of these two liquids and the column slowly filled, starting as before with the pure CCl_4 and finishing with the least dense mixture (50 ml. of stock solution B).

A sample dropping device containing P_2O_5 was fitted to the top of the columns, which were immersed in a thermostat bath maintained at 20°C. The prepared columns were equilibrated for 48 hours before use to allow a uniform density to be established. The columns were calibrated with small glass floats of known density. The density at any point in the columns was obtained by plotting the density of the floats against their position opposite the graduated scales of the column. The columns were calibrated each day that measurements were taken since diffusion gradually altered the gradient.

The moist fibres were conditioned at 65% r.h. over a saturated solution of sodium nitrite in an evacuated desiccator at 25°C before being prepared for the density gradient columns either by tying knots in small assemblies of fibres or by pressing into small pellets in a hydraulic press under a pressure around 3000 lb/in^2. The prepared specimens were reconditioned and then introduced immediately into the gradient columns where they were left for 24 hr. to reach equilibrium, before measuring their flotation heights. Floats and samples were removed from the column by means of a small fine-mesh wire cup attached to a rod without noticeably affecting the density gradient.

4. Samples Investigated

The work described in this report has been carried out on three separate series of enzyme treated samples. Two of these sets of samples were prepared by Dr Elwyn Reese, U.S. Army Natick Development Center, Natick, Massachusetts, while the third set was prepared by Dr Tom Wood, Rowett Research Institute, Aberdeen, Scotland.

The first series of samples prepared by Dr Reese relates to 'University' plugging cotton and consisted of four samples of Wiley milled material and four samples of unground fibres. 'University' plugging cotton is a non-absorbent non-sterile, long staple white cotton, (used primarily for plugging test tubes) manufactured by the Rock River Manufacturing Corporation, Jamesville, Wisconsin, The product is a 50:50 blend of Punjab Deshi cotton from India and American Comber. Neither cotton has any additives or has undergone any pretreatments other than double cleaning for the Deshi.

The second series of samples prepared by Dr Reese and the samples prepared by Dr Wood are based on the same batch of well-scoured American upland cotton, species Acala 4-42, taken from a selected bale grown several years ago in the San Joaquin Valley, California, and prepared at the Shirley Institute. The suppliers (Calcot Ltd) chose a bale that was of good middling quality, free from spot, staple length $1^{3}/32$" with a micronaire value of 4.5. The sample of cotton was given minimum processing. The bale was opened and blown, passed through a porcupine beater and made into a lap. The lap was sandwich-blended and then 30 lb of fibre selected at random from this blended cotton. Two pounds of the selected cotton were wet out at 60°C in water containing 1 ml/l of Calsolene Oil H.S., and then boiled for 3 hours under 35 lb/sq.in. excess pressure in an aqueous solution of caustic soda (1%) containing castor oil soap (0.15%). The treated cotton was thoroughly washed in distilled water and dried in air. Microscopical examination of the scoured cotton showed it to be free from the pectinous outer layer but the primary cellulose wall was intact. Viscosity determinations showed that the cotton had undergone almost no degradation during the processing. Unground fibres were used for all enzyme treatments of Acala cotton.

Dr Reese's samples included fibres treated with isolated C_x and C_1 components from Trichoderma viride, the complete enzyme system from this organism, with and without inhibition by Methocel, and the complete enzyme system from Pestalotiopsis westerdijkii (C_x only since no C_1 component is reputed to be produced by this organism).

Treatments of the unground cotton fibres (10g for complete enzyme systems, 4g where enzyme fractions were used) were carried out on a shaker (except for one sample, H, treated with the complete enzyme system from T. viride, which was unshaken), in pH 4.5 citrate buffer at 50°C over a period of 2.9 to 5.0 days. Weight losses due to the various enzyme treatments were calculated from the amount of reducing sugars produced in the supernatant solution. The treated cotton fibres were filtered and washed with water containing merthiolate (0.01%) as a preservative.

A second set of Acala samples was prepared by Dr Wood by incubating the scoured Acala cotton with enzyme fractions separated on a column of DEAE - Sephadex from the cellulase complex obtained from a 4 week fermentation of Trichoderma koningii on cotton fibres in an inorganic salts medium.[13] Acala cotton fibres (5g) were suspended in 200 ml of approximately 0.2N sodium acetate/acetic acid buffer (pH 4.8) containing sodium azide (0.005%) and 40 mg C_1 enzyme protein (Folin-Lowry) and/or 39.5 mg C_x enzyme protein and incubated at 37°C for 11 days. The extent of hydrolysis of the cotton was determined as before by measuring the reducing sugars (expressed as glucose) in the filtrates. The cotton was filtered and washed with water containing sodium azide (0.02%).

Details of the various enzyme treatments and the extent of enzymolysis for the sixteen samples prepared by Dr Reese (designated 2C, 2XP, 2E, 2EM, 2C-F, 2X-F, 2E-F, and 2EM-F and A to H) and the four samples prepared by Dr Wood (designated C_o, C_1, C_x, and C_{1+x}) are given in Table 1. All the samples were kept moist to prevent any changes due to drying. The 'never-dried' samples containing either methiolate or sodium azide as preservatives were stored at 4°C until required for examination to further inhibit microbiological action; no sign of any fungal growth was ever observed on these stored samples.

5. Results and Discussion

Several techniques have been used in an attempt to detect changes in the fine-structure of cotton cellulose brought about by the individual and combined action of the C_1 and C_x components of the cellulase complex from T. viride, Pestalotiopsis westerdijkii, and T. koningii. The approaches investigated included measurement of changes in accessibility of the cotton to water vapour, infrared deuteration studies of changes in the disordered hydroxyl groups, studying any changes in the disposition and accessibility of the hydroxyl groups on the surface of the crystalline elementary fibrils of the cotton by repeated methylation with diazomethane, examination of morphological changes of the fibres by water of imbibition measurements and scanning electron microscopy, following any changes in the fibre density, attempts to detect changes in the number of cellulose chain-ends, and low-angle scattering X-ray crystallography to probe possible changes in the size and packing of the elementary fibrils after enzyme treatment.

5.1 Accessibility to water vapour

Moisture regain and water imbibition measurements were carried out on the various undried moist fibres and after drying the same specimens over P_2O_5. The values obtained for the number of molecules of water per cellulose polymer unit in equilibrium at 65% and 88% relative humidities for the undried samples (Table 2) and after drying over P_2O_5 (Table 3) confirm our earlier observations (ref.1 p.17) that the accessibility to water vapour at 25°C is significantly reduced after extensive hydrolysis catalysed by the complete cellulase systems from cellulolytic micro-organisms. The relationship between accessibility and the extent of enzyme hydrolysis, however, is seen (Fig.1) to be appreciably different for the scoured Acala cotton treated by the enzyme system from T. viride and the enzyme system from T. koningii. Thus, the proportion of anhydroglucose units accessible to water vapour in the undried material was reduced by only 2.2% after treatment at 37°C by Dr Wood with the combined C_1 and C_x enzyme fractions from T. koningii, corresponding to the removal of about one-third of the weight of the cellulose, whereas similar weight losses produced by the complete enzyme system from T. viride acting at 50°C over a shorter period of time were associated with a reduction in accessibility (4.0%) nearly twice this level. A similar decrease in accessibility (around 4%) was previously observed (ref.1, Table 2 and Fig.1) for the undried, unground University plugging cotton treated under similar conditions with the complete enzyme system from T. viride, but a Wiley milled sample of this University plugging cotton exhibited an even greater drop in water vapour

accessibility (5.0 - 5.3%) after a period of enzymolysis which led to the removal of around 30% of the initial weight of cotton.

It seems clear that these reductions in the water-vapour accessibilities of cotton fibres are brought about by the preferential enzymic removal of cellulosic material from those parts of the cotton fibre which are generally more accessible (both to water vapour and to the enzymes themselves). Kassenbeck[66] has distinguished four zones in the secondary wall which differ in their accessibilities to reagents and he attributed these differences to local variations in the packing density of the fibrillar structure caused by the asymmetry of the mechanical forces produced in the cotton hair during its very first drying from the form of a swollen cellular tube to a collapsed fibre. The more accessible regions are found in the collapsed, concave part of the fibre cross-section; even more accessible are the limited areas located at the boundaries between this zone and the zones of highest density of packing of elementary fibrils corresponding to the two highly convex extremes of the characteristic bean-shaped cross-section. But in order to rationalise the difference in the water-vapour accessibilities of the residual materials left after removal of about one-third of the cellulose by the two complete enzyme systems we must now assume that the relatively slower attack by the T. koningii enzyme system at 37°C is probably somewhat more uniformly distributed throughout the fibre structure compared with the more rapid, presumably more selective, attack by the T. viride enzyme system at 50°C.

Of the eight samples prepared by Dr Reese, the sample treated with the complete enzyme system in the presence of Methocel (Sample D) showed a lower accessibility to water vapour than might have been expected on the basis of its measured extent of enzymic hydrolysis, whilst the sample treated with the enzyme from P. westerdijkii showed a higher accessibility than expected in relation to the general correlation between water-vapour accessibility and extent of enzymic hydrolysis for this series of treatments (Fig.1).

All the fibres showed much lower accessibility to water vapour after drying over P_2O_5 due presumably to collapse of part of the cellulose structure. Whilst the accessibilities of fibres extensively degraded by the complete enzyme systems from these two cellulolytic micro-fungi were still significantly lower after drying than the accessibility of the corresponding dried, control materials, the level of this reduction in accessibility was now much greater (4.6%) for the fibres treated by Dr Wood, compared with a reduction in accessibility of only 2.5% for the fibres treated at 50°C by Dr Reese (Fig.1). This reversal in the magnitude of the loss in water-vapour

accessibility produced by the two enzyme systems between 'never-dried' and dried fibres is hard to explain but clearly the cotton treated at 37°C with the T. koningii system has shown a much greater collapse of its structure (greater aggregation of the elementary fibrils?) on drying, compared with the fibre treated at 50°C with the T. viride enzyme system.

This effect, i.e. on drying, appears to apply also to the samples treated with the C_1 and C_x fractions. None of these enzyme components isolated from the two enzyme systems produced signigicant changes in the water-vapour accessibility of the 'never-dried' treated cotton, whereas both the C_1 and C_x component from T. koningii appeared to cause a small reduction in accessibility when measured on the dried fibres.

5.2 Water of imbibition

The water of imbibition (i.e. water retention after soaking the samples in distilled water for 60 minutes followed by centrifuging at 1200 g for 15 minutes) data determined on undried specimens and after drying over P_2O_5 are given in Tables 2 and 3 respectively and plotted against the extent of enzymolysis in Fig. 2. Water of imbibition values for undegraded fibres are generally considered to be a measure of the internal porosity of the fibre. The marked increase in the water of imbibition levels brought about by enzyme degradation may be partly associated with the opening-up of the internal fibre structure but, more importantly, it is probably a reflection of the micro-cracks and crevasses introduced into the surface of the fibres. Scanning electron photomicrographs of degraded fibres presented in a previous report (ref.1, Figs. 10-17)and later in this report show extensive development of microholes, resulting from the preferential removal of material from the collapsed concave part of the fibre cross-section, and of spiralling microcracks between lamellar sheets of secondary cell-wall material. These disruptions in the fibre surface probably have the power of exerting considerable capillary attraction leading to the retention of increased amounts of liquid water against the standard centrifugal force used in these measurements; this force is normally large enough to remove any liquid water from the much larger capillaries formed at the contact points between individual fibres in the fibre mat. It is suggested, therefore, that water retention values serve as an indication of the extent of any morphological changes in the structure of the secondary cell-wall of the cotton fibres. The fact that the most extensively degraded fibre (H) had a somewhat lower percent water retention than sample B, which has a lower extent of enzymolysis (Fig.2), may simply indicate that much of the porous (i.e. to liquid water) internal structure of the fibre has been removed in sample H and/or that the surface crevasses have become so large that they can no longer retain

water against the centrifugal force.

After drying over P_2O_5 all the fibres showed a marked reduction in the amount of water retained in this test; presumably this is caused by the general collapse of the internal structure on drying and the partial closure of some of the cracks on the fibre surface.

The behaviour of the fibres treated with the complete T. koningii cellulase by Dr Wood and of fibres treated by Dr Reese with T. viride cellulase is again quantitatively different, with the latter materials exhibiting a greater propensity to retain water in this test (Fig. 2), indicating, perhaps that the distribution of enzyme attack, on a morphological scale, must be different in these two cases. Whether the different relationship between water retention and extent of enzymolysis for these two groups of materials is associated with differences between the molecular size/shape of the two enzyme systems or due perhaps to differences in the fibre accessibilities at the two incubation temperatures (37 and 50°C) cannot be answered at this stage.

Interestingly, the water retention values for the two samples treated with the separated C_x and C_1 components (samples E and F respectively) from T. viride were above the two curves (undried and dried, Fig. 2) connecting water retention and extent of enzymolysis for the remainder of the second set of samples prepared by Dr Reese, except for sample G, treated with the enzyme system from P. westerdijkii, which fell below these curves. This latter observation is particularly confusing in view of the fact that SEM examination showed (see later) that this sample (G) had been degraded, with about 10% of the fibres exhibiting extensive cracking typical of $C_1 + C_x$ enzyme attack;but the magnitude of the 95% confidence limits for these water retention values suggest that caution should be exercised in the interpretation of these results.

5.3 *Infrared deuteration studies*

It now seems clear that previous difficulties (ref.1, p.19) experienced in making suitable pressed discs from control fibres of the 'University' plugging cotton (2C-F) for infrared deuteration work were associated with the cuticle still being present on these fibres. Photomicrographs (approx 130x) of the residual material from the control and enzyme treated fibres after dissolving the cellulose in cuprammonium hydroxide are shown in Fig.3. The amount of residual cuticle appears to be progressively

reduced in the order 2C-F, 2X-F, 2EM-F, and 2E-F, confirming that all three enzyme treatments must be responsible for the partial removal of cuticle material; satisfactory pressed discs were easily made from these three enzyme treated fibres.

These observations imply that all of the enzyme preparations used in these experiments contain enzyme activity capable of destroying at least part of the cuticle/primary cell wall material. The primary wall is generall considered[67] to consist of a wax/pectic layer in which network systems of cellulosic fibrils are embedded. Evidence has been presented[68] that indicates that the pectic substance is present as the insoluble salt of polyvalent ions rather than as a pectin-cellulose compound. Results obtained by Tripp, Moore, and Rollins[69] indicate that the primary wall contains protein, determined as %N x 6.25 (14%), pectic substance, determined as anhydrogalacturonic acid (9%), wax, determined as alcohol-solubles (8%), ash (3%) and cutin or suberin (4%). Kling and Mahl[70] showed in 1952 that the pectin layer can be destroyed by pectinase ('Siltrajol', Bayer) and by cellulase ('Luizyme', Luit Poltwerker of Munich). No difficulties have been experienced in making pressed discs, suitable for infrared examination, from any of the present range of enzyme-treated samples based on scoured Acala cotton, presumably because the cuticle and non-cellulosic components of the primary wall were effectively removed by the preliminary alkaline scour. Nevertheless, the poor reproducibility of this technique has remained a major problem.

Repeated measurements on the same pressed disc leads to a slow steady deuteration of the crystalline, hydrogen-bond ordered structure over a period of several days. Typical rate curves (Fig. 4) show the initial rapid deuteration of the accessible hydroxyl groups on the surface of the elementary fibrils within the first hour or so, followed by a slow steady increase in the apparent accessibility associated with the penetration of the outer layers of the elementary fibrils by the D_2O. Allowance was made for this slow deuteration of the crystalline structure in assessing the results obtained from repeated measurements (carried out on each pressed disc over a period of several days to improve the precision of the method), by extrapolating the steady part of the deuteration rate curves to zero time (see Fig. 4).

Experimental values for the percent total disordered cellulose, i.e. the proportion of hydroxyl groups readily accessible to D_2O, obtained by computing the intercept of the linear regression lines using the 'least squares' criterion, are given in Table 4 for each of the samples prepared by Drs Reese and Wood; at least two separate series of experiments were carried out on each control and enzyme treated sample. But despite the fact that each of the values recorded in Table 4 are based on several infrared spectra recorded over periods up to five days, the agreement between replicate values was still quite poor and, indeed, the mean results obtained for Dr Reese's samples do not follow any logical pattern. The mean values obtained for Dr Wood's samples, based on a greater degree of replication, indicate a reduction in accessibility to D_2O for the sample extensively degraded by the complete enzyme system, little change due to the action of the C_x fraction but a marked increase in accessibility resulting from the action of the C_1 fraction.

It might be anticipated that the accessibility values obtained by this infrared-deuteration technique should show some correspondence with the water-vapour accessibility values and indeed the infrared accessibilities of the C_o, C_x and C_{1+x} samples were approximately midway between the corresponding water-vapour accessibility values obtained on the 'never-dried' and dried specimens. But absence of any change in water-vapour accessibility (or diazomethane methylation - see next section), associated with the action of this C_1 component, makes the results obtained for this sample by infrared deuteration rather suspect.

The significance, if any, of the apparent differences in the rate of deuteration of the crystalline O-H in these various enzyme-treated samples (Table 4) is uncertain.

At the present time it seems unlikely that the precision of this infrared deuteration method can be improved upon and the limit of its useful development would seem to have been reached.

5.4 Diazomethane methylation studies

A comprehensive investigation has been carried out in an attempt to detect any changes in the microstructural features of the undried cotton fibres, variously treated by Drs Reese and Wood, by following the course of successive methylation reactions which ultimately methylate all the accessible hydroxyl groups on the crystalline

surfaces of the elementary fibrillar units. In this technique a relatively rapid initial reaction is followed by a much slower reaction at constant rate corresponding to the slow penetration of the outer layers of cellulose molecules on the surface of the elementary fibrils. In the earlier stages of this work it was shown[1] that a sodium hydroxide/dimethylsulphate/toluene methylation technique is unsuitable for measuring changes in the numbers and distribution of accessible hydroxyl groups brought about by the various enzyme treatments, owing to disruption of the structure of the ground and/or enzyme-degraded fibres during the methylation process leading to extensive loss of methylated material. Previous work[45, 49] carried out at the Shirley Institute on the methoxyl distribution of cotton methylated with diazomethane in ether saturated with water indicated that there is much less disaggregation of the elementary fibrillar units with this reagent, compared with the effects of methylating with dimethyl sulphate in the presence of dilute sodium hydroxide solutions. Since diazomethane methylation in ethereal solution appears to take place with minimum disruption of the cotton fine structure and is largely confined to the accessible regions these reaction conditions have now been used in an attempt to detect the putative changes in the accessibility of the cotton fibres after treatment with the different individual enzyme components of the cellulase complexes from T. viride and T. koningii.

The results for the distribution of the methyl substituents in the D-glucopyranosyl units at successive stages of diazomethane methylation are given in Table 5 for a typical scoured, untreated cotton yarn and the original untreated, air-dry 'University' plugging cotton, in Table 6 for the unground, 'never-dried' 'University' plugging cotton treated with enzyme fractions from the T. viride cellulase complex, in Table 7 for the Wiley-milled 'University' plugging cotton similarly treated, in Table 8 for the samples of scoured Acala cotton fibres treated with cellulase components from T. viride and P. westerdijkii by Dr Reese, and in Table 9 for Dr Wood's samples treated with components from the T. koningii cellulase complex. In agreement with previous observations,[45, 49] the glucose produced on hydrolysis is seen to fall rapidly during the first stage of methylation but a slow steady rate is reached after about 10 - 15 days which is believed to correspond to the gradual penetration of elementary structural units by the methylating agent. The yields of the main mono- and di-methylated components, viz. 2-, 6-, and 2,6-di-O-methyl-D-glucose, rise rapidly at first to peak values (generally around 15 days) before falling slowly at constant rates associated with the production of

2,3,6-tri-O-methyl-D-glucose. No particular significance is thought to be connected with the appreciable differences in initial rates of methylation of some of these samples; these differences may be associated with varying extents of catalytic decomposition of the diazomethane during the first couple of days.

Extrapolation of (i) the rate curves showing the decline in the amount of glucose obtained on hydrolysis of samples taken after various degrees of methylation (Figs. 5-15), (ii) the rate curves showing the extent of methylation as measured by the overall degree of substitution (Figs. 16-26), and (iii) the individual rate curves showing the development of 2-, 3-, and 6-0-methyl-, 2,3-, 2,6-, and 3,6-di-O-methyl-, and 2,3,6-tri-O-methyl-D-glucose (eg. Figs 27-36), gave respectively the amounts of glucose, the DS, and the amounts of each of these partially methylated glucoses on completion of the first stage of methylation corresponding to the saturation of the readily accessible hydroxyl groups. The values derived for the various samples of unground and Wiley-milled 'University' plugging cotton are summarised in Table 10, for the scoured Acala cotton samples treated by Dr Reese in Table 11, and for Dr Wood's samples in Table 12, along with the corresponding values calculated for the crystalline elementary fibrils with average cross sections that provide the best fit to these experimental data. In making these calculations it is assumed that the average cross-section of the elementary fibrils can be represented by a rectangle of sides A and B, corresponding to the number of anhydro-glucose units in each side. Side A is assumed to contain the corner a.g.u. and to give rise to 2,6-di- and 2,3,6-tri-0-methyl-D glucose and side B to give 2- and 6-0-methyl-D-glucose. For this simple model it can be shown that the moles percent unmethylated glucose (i.e. derived from a.g.u. confined to inaccessible regions inside the elementary fibrils) is given by $100\,(AB-2A-2B+4)/AB$, the moles percent 2- and 6-0-methyl-D-glucose are each given by $100\,(B-2)/AB$, the moles percent 2,6-di- and 2,3,6 - tri-0-methyl-D-glucose by $100/B$, and the overall degree of substitution (D.S.) by $(5A+2B-4)/AB$.

Data for scoured cotton yarn (Table 10) are in reasonable agreement with values found previously[49], and indicate that the rapid stage of methylation by diazomethane is confined to about 18% of the total hydroxyl groups in the cotton cellulose, situated on 30% of the total anhydroglucose units (a.g.u.).

The results for the original 'University' plugging cotton without further treatment and for the unground control fibre (2C-F) are similar and not significantly different from those obtained on the well scoured cotton yarn. These data (Table 10) suggest that the average cross-section of the elementary fibrils, or aggregates thereof, contains of the order of 200 a.g.u., corresponding to a unit with a rectangular cross-section in which side A (assumed to contain the corner units and to give rise to G_{26} and G_{236}) equals 10 a.g.u. and B (giving rise to G_2 and G_6) equals between 16 and 20 a.g.u. Disappointingly, there seems to be no justification for believing that the very small differences observed in the total number and distribution of readily accessible hydroxyl groups between control and enzyme-treated, unground 'University' plugging cotton fibres (2C-F, 2X-F, 2EM-F, and 2E-F) are significant. It is to be anticipated that the effects of the various enzyme treatments will be quite small and some improvement in the overall level of reproducibility of this technique was sought in the subsequent work on Acala cotton (see below) by extending the period of methylation (Section 3.3).

The data in Table 10 indicate that Wiley-milling marginally increases the accessibility of the fibres to the diazomethane reagent in moist ether. The control sample (2C) appears to be composed of structural units of average cross-section around 10 x 12 a.g.u., consistent with the possibility that grinding the fibres has caused a partial disaggregation of the larger 10 x 18 units, associated with cleavage along the G_{26}, G_{236} face (side A). This suggestion is undoubtedly an over-simplification, however, since it does not account for the non-equivalence of G_{26} and G_{236} in these fibres or the significant amounts of G_{23}. The effects of enzyme treatment are again difficult to detect in this set of experiments, although Wiley-milled fibres treated with the total enzyme complex from T. viride, with or without Methocel inhibition (samples 2EM and 2E respectively) appear to have marginally reduced accessibility to diazomethane in moist ether, which is consistent with the preferential removal of the more accessible, less aggregated, regions of the ground fibre by the complete enzyme complex.

The data (Tables 11 and 12) for the scoured Acala cotton control samples (A and C_o) obtained in the two series of experiments with this cotton as substrate are in good agreement and indicate that the average cross-section of the elementary fibrils in this starting material has $10\bar{1}$ and 101 planar sides of 10 x 16 D-glucopyranosyl units. Head and Hadfield [49] concluded that the data they obtained for this same

material was very close to the calculated values for units in which sides A and B equalled respectively either 12 x 14, 13 x 14, or 11 x 15 a.g.u. and they also suggested that several other models close to these dimensions fitted the data almost as good. In fact the above 10 x 16 unit seems to fit their data somewhat better than any of their three specific suggestions.

The various samples treated to differing extents with the complete enzyme system from T. viride appear to show a progressive increase in the average number of cellulose molecules in the 101 or 'B' side of the cross-section (i.e. the side giving rise to G_2 and G_6) in the residual material after increasing extents of enzymolysis. Thus, the best fit for the experimental data from samples C and D (5 and 7% enzymolysis) is an average cross-section around 10 x 18 a.g.u., while the data for sample B (19% of the cellulose removed) requires a 10 x 24 a.g.u. cross-section. However, the average size of the elementary fibrils remaining in the most extensively degraded sample (H) (unshaken during treatment), appears to be only 10 x 12 a.g.u. These observations can be rationalised by postulating that the enzymic attack at 50°C by the complete cellulase system from T. viride is confined initially to the more accessible regions in the collapsed part of the fibre cross-section where, we suggest, the elementary fibrils are farther apart and smaller (less aggregated). Selective enzymic removal of these more accessible, smaller units, accelerated perhaps by the fragmentation associated with shaking these samples, should give residual material with a larger average size of elementary fibrillar cross-section, as observed. The observations on the extensively degraded sample H are more difficult to explain. Presumably when the digestion is unshaken, resulting in little fragmentation, a more uniform erosion of the 'A' face of the larger elementary fibrils occurs, giving residual material with the average length of the 'B' side of the cross-section significantly reduced.

The sample (C_{1+x}) treated at 37°C with the complete enzyme system from T. koningii appears (Table 12) to contain elementary fibrils of smaller average cross-section even than sample H. We have already suggested on the basis of the water vapour accessibility measurements that this enzyme system causes a more uniform level of degradation throughout the fibre and perhaps this too can be related to absence of

fragmentation associated with not shaking during the enzyme digestion; the lower temperature and longer time of digestion and differences in enzyme size may also be contibuting factors.

An important aspect of these observations is that the size of face A (containing the 2,6 and 2,3,6 hydroxyls accessible on alternate anhydroglucose units) remains unchanged, regardless of the enzyme treatment, implying that the combined action of the C_1 and C_x enzyme components is to remove successive layers of cellulose molecules from this face only. We believe that these observations support the hypothesis that one of the components of the cellulase complex (C_1?) has a specific affinity for cellulose molecules situated only in this face of the elementary fibril, because of the unique stereospecific distribution of their free (non-hydrogen bonded) hydroxyl groups at the 2,6 and 2,3,6 positions on adjacent anhydroglucose units along the cellulose chain. Disruption of the hydrogen-bond system on the surface of the elementary fibril is probably accompanied by a concomitant splitting of glycosidic bonds to produce liberated chain ends which become accessible to C_x endo- or exo-glucanases. Since the arrangement of the hydroxyl groups on the two pairs of faces of the rectangular elementary fibril is quite different, it seems reasonable to suppose that quite different enzymes would be required to bond to cellulose molecules in these faces. The interesting possibility now arises as to whether other cellulolytic micro-organisms might elaborate a C_1 enzyme with an affinity for cellulose molecules in the faces containing anhydroglucose units with alternate 2- and 6-hydroxyl groups accessible.

Samples E, F, and G treated by Dr Reese, and C_1 and C_x treated by Dr Wood gave results by this diazomethane methylation technique which were very close to those obtained on the control samples. However, if the only action of the C_1 component is to free some of the cellulose molecules from their hydrogen bonded positions on the 'A' faces of the elementary fibrils, and perhaps to break some of the glycosidic bonds in these molecules, the only changes in the pattern of methylation products would be a decrease in the amount of 2,6-di-0-methyl-D-glucose (G_{26}) and a corresponding increase in the amount of 2,3,6-tri-0-methyl-D-glucose in the hydrolysates after methylation (to an extent dependent upon the number of cellulose molecules released), together with an increase in the amount of 2,3,4,6-tetra-0-methyl-D-glucose (G_{2346}) associated with the production of any non-reducing chain ends.

Small reductions in the amounts of G_{26} were observed for all the samples treated with C_1 or C_x alone, but the level of significance of these changes is very uncertain.

Small amounts of G_{2346} would not normally be detected by the standard g.l.c. methods used for these analyses. Attempts have therefore been made to develop an improved method for quantitative estimation of this sugar at very low concentrations (Section 3.5) but so far it has not proved possible to detect with certainty any G_{2346} in any of the trimethylsilylated hydrolysates from extensively methylated samples. A problem with this analysis is the difficulty of separating tiny amounts of the G_{2346} component from the tail of the trimethylsilylating reagents when operating the g.c. detector under conditions of maximum sensitivity. Experiments with various amounts of G_{2346} added to the hydrolysates before trimethylsilylation has indicated that the limits of detection of this tetramethylglucose with the present g.l.c. set-up is around 0.2% of the total weight of glucose and partially methylated glucoses in the hydrolysate, corresponding to an overall average DP of only 500. But if only 30% of the cellulose chains are accessible to methylation, i.e. those situated on the surface of the elementary fibrils, this level of sensitivity limits the detection of chain ends to degraded samples where the average DP of their accessible cellulose molecules is less than about 150. Encouragingly, when G_{2346} was added to hydrolysates from extensively methylated samples of the four samples treated with the cellulase components by Dr Wood, somewhat higher recoveries of the tetramethyl glucose were observed with the C_{1+x} sample, indicating the presence of rather more accessible chain ends in this extensively degraded sample.

Much improved sensitivity in the detection of G_{2346} is required but this may be possible by developing capillary columns for the separation of these trimethylsilylated, partially methylated glucoses.

5.5 Scanning electron microscopical examination

The unground and Wiley-milled 'University' plugging cotton, variously treated with enzymes by Dr Reese, have been previously examined with the scanning electron microscope and representative scanning electron photomicrographs, showing enzymic removal of material from the collapsed, concave parts of the fibre cross-section and spiralling cracks between the lamellar sheets of secondary cell wall material, were

given in an earlier report [1]. This earlier SEM work showed that the enzymically degraded fibres are further fragmented during methylation with sodium hydroxide/dimethyl sulphate/toluene, probably associated with the preferential removal of the methylated material by the sodium hydroxide during each methylation cycle. It was concluded that this methylation technique for measuring accessible hydroxyl groups is also inappropriate for following the changes in the fine-structure of Wiley-milled cotton, brought about by the various enzyme treatments; internal disruption of the fibre structure during the grinding process would appear to be responsible for extensive fragmentation of the control fibre, which occurred during the methylation.

In the earlier part of the present work these various unground and Wiley-milled 'University' plugging cotton samples were methylated by the alternative diazomethane technique. Representative SEM photomicrographs (at the three magnifications used previously[1]) of the fibres after methylation with diazomethane for 28 days are shown in Figures 37 through to 46. None of these photomicrographs shows any evidence of additional damage to the fibres (i.e. other than that brought about by the enzyme treatments and shown[1] on the photomicrographs before methylation) which may be associated with the diazomethane treatment, confirming that this methylation technique does not cause any significant disruption of the fibre morphology.

All the Acala cotton samples, (i.e. samples A to H treated by Dr Reese and samples C_o, C_x, C_1, C_{1+x} treated by Dr Wood) were examined by SEM in the moist 'as-received' state. Specimens were prepared for vacuum coating by the Critical Point drying technique, as described in Section 3.6. Typical SEM photomicrographs of fibres taken from each sample, under three conditions of magnification are presented in Figures 47 through to 58.

The photomicrographs of the two control samples, A and C_o, (Figures 47 and 55 respectively) show the typical convoluted, ribbon-like morphological appearance of native cotton fibres. Examination of fibres treated with the C_x and C_1 components of T. viride by Dr Reese (respectively sample E, Fig. 51 and sample F, Fig. 52), and the separated components of T. koningii by Dr Wood (Figs. 56 and 57) by SEM revealed little evidence of significant damage. All of the other fibres however showed some change in their morphological appearance as the result of the various enzyme treatments. Samples B (Fig. 48) and H (Fig. 54) treated with the total cellulase complex from T. viride and sample C_{1+x} (Fig. 58) treated with the total enzyme

complex from T. koningii showed evidence of severe degradation involving extensive removal of parts of the collapsed, concave regions of the cross-section, which Kassenbeck[66] has shown to be by far the most accessible part of the fibre due to its more disorganised and 'open' structure. The two samples (H and C_{1+x}) degraded to the most advanced degree exhibited clear evidence of extensive erosion of the fibre structure.

About 35% of the fibres from the less extensively degraded sample, C, treated with a combined cellulase complex from T. viride of lower activity, showed (Fig. 49) only slight cracking of the dense helical fibrillar bands in the secondary wall oriented at 40 - 60° to the longitudinal fibre axis. Examination of fibres from the sample (D) treated with total T. viride complex with added Methocel indicated (Fig. 50) similar helical cracking in about 45% of the fibres, confirming earlier observations that the C_1 component in this cellulase complex is not completely inhibited by this material.

Sample G treated with the total cellulase from P. westerdijkii showed (Fig. 53) a surprisingly extensive degree of helical cracking in about 30% of the fibres and it is obvious from these SEM photomicrographs that the earlier conviction that this micro-organism does not elaborate a C_1 component cannot be correct, a conclusion borne out to some extent by the production of reducing sugars corresponding to an extent of hydrolysis of 5.4% (Table 1).

5.6 X-ray crystallography

Visual examination of the small angle X-ray photographs of the contrd Acala sample (C_o) and the sample (C_{1+x}) treated with the mixture of C_1 and C_x fractions from T. koningii showed that they were very similar with slight absorption edge effects superimposed. The wide angle photographs, recorded at the same time for calibration purposes, also showed little difference between the two specimens.

5.7 Density of enzyme treated cottons

In Table 13 are given the fibre densities of the twelve Acala cotton samples treated by Dr Reese and Dr Wood. It is clear from these results that the various enzyme treatments have no significant effect on the fibre density. No explanation can be given for the marginally higher densities of the four samples treated by Dr Wood.

6. Conclusions

6.1 Whilst it has been confirmed that the accessibility to water vapour of the moist, 'never-dried' fibres is significantly reduced by enzymic hydrolysis, the size of this reduction was nearly twice as large with the cellulase complex from T. viride acting at 50°C as that produced by the combined action at 37°C of the C_1 plus C_x components from T. koningii, for equivalent extents of degradation measured by production of soluble reducing sugars. These reductions in water vapour accessibility are considered to be associated with the preferential enzymic removal of the more accessible, collapsed, concave part of the fibre cross-section; in order to account for the different levels of reduction in accessibility, the slower attack by the T. koningii enzyme system at 37°C would appear to be relatively more uniformly distributed throughout the fibre structure, compared with a more rapid selective attack by the T. viride cellulase at 50°C.

6.2 Drying the fibres reduces their overall accessibility to water vapour. Enzyme treated fibres still show lower accessibilities than control fibres after drying but the magnitude of the drop in accessibility due to the enzyme treatment is now reversed for the T. viride and T. koningii systems, perhaps because of a greater collapse of the structure on drying the fibres treated at 37°C with the C_1 plus C_x enzymes from the latter organism, related perhaps to the more uniform enzyme action suggested above.

6.3 Enzymic degradation increases the water of imbibition of the cotton fibres; this is assumed to be due to the introduction of the micro-cracks and holes in the fibre surface, as revealed by SEM. Quantitative differences in the water of imbibition values of the fibres treated with the cellulase systems from the two Trichoderma sp. again suggests that the morphological distribution of enzymic attack may be different in these two cases.

6.4 Values for the percent total disordered cellulose in the various enzymic treated samples obtained by an improved infrared deuteration technique, indicated a reduction in accessibility to D_2O for the sample extensively degraded by the combined C_1 and C_x components from T. koningii, little change due to the action of the C_x fraction from the organism, but apparently a marked increase in accessibility following treatment with the C_1 fraction. However, absence of any

corresponding increase in water-vapour accessibility associated with the action of this C_1 component casts doubts on the reliability of this observation. The poor degree of precision of this infrared deuteration technique, despite repeated attempts at improvement, suggests that this approach is too unreliable to detect any small changes in accessibility of the cellulose hydroxyl groups following action by either C_1 or C_x components.

6.5 Diazomethane methylation in moist ethereal solution is largely confined to the accessible hydroxyl groups on the surface of the elementary fibrils and takes place with minimum disruption of the cotton morphology, as evidenced by SEM examination of methylated fibres. By measuring the distribution of the methyl substituents in the D-glucopyranosyl units at successive stages of methylation it is possible to determine any changes in accessibility of the cotton fibre as a result of treatment with the different enzyme components.

Results obtained for control samples indicate that the average cross-section of the elementary fibrils in the starting material has $10\bar{1}$ and 101 planar sides of 10 x 16 anhydroglucose units. Wiley-milling causes a partial disaggregation of the elementary fibrils and the ground fibres are more accessible to the diazomethane reagent, as indicated by a reduction in the size of the structural units to an average cross-section around 10 x 12 a.g.u.

The combined attack by the C_1 plus C_x components appears to be confined to the 'A' faces of the elementary fibril containing the 2,6 - and 2,3,6 - hydroxyl groups accessible on alternate anhydroglucose units, since the average length of this side of the cross-section remains unchanged at 10 a.g.u., whereas the average size of the 'B' side has been observed to increase up to 24 a.g.u. or decrease to 10 a.g.u. with different extents of enzymolysis, with the different enzyme systems at either 37° or 50°C, and depending on whether the samples are shaken or not during the enzyme treatment. Preferential removal of smaller, less aggregated elementary fibrils in the more accessible regions of the fibre, under certain conditions, is believed to be the explanation of the observed increases in the average size of the cross-section.

These results support the hypothesis that a component of the cellulase system has a specific affinity for the cellulose molecules situated in the 'A' faces of the elementary fibrils (associated with the unique configuration of their free hydroxyl groups at the 2,6 - and 2,3,6 - positions on adjacent anhydroglucose units along the cellulose chains), which leads to chain scission on this pair of surfaces, to produce free cellulose chain ends accessible to C_x endo- on exo-glucanases. The possibility must be considered that this unique, stereospecific endo-glucanase activity and a cellobiohydrolase activity resides in the same protein which we shall continue to call C_1.

6.6 Attempts to detect changes in the numbers of chain ends on the elementary fibrillar surfaces, by measuring 2,3,4,6 - tetra - O - methyl -D - glucose after acid hydrolysis of the diazomethane methylated materials, have so far proved unsuccessful because of insufficient sensitivity of the current g.l.c. technique developed for this purpose.

6.7 Small-angle X-ray photographs and fibre densities were not influenced by the combined action of the C_1 and C_x components.

6.8 No significant changes in any of the fibre properties examined to date have been observed with fibres treated with either C_1 or C_x components from T. viride or T. koningii acting alone. Whilst every effort should continue to be made to find suitable means of detecting even the smallest changes in the cotton fibre's fine-structure brought about by these individual enzyme components, it is possible that highly-ordered native cellulose is unaffected by C_1 or C_x acting alone; the possibility that the unique endo-glucanase activity referred to in 6.5 above is the result of a complex formed between cellobiohydrolase and C_x protein molecules merits consideration.

6.9 Appreciable helical cracking of the fibres treated with the total cellulase from P. westerdijkii indicates that, contrary to earlier beliefs, this organism probably produces some C_1 activity.

7. Recommendations for Further Work

In addition to seeking alternative and even more sensitive methods for following changes in accessibility, attention should be given to other aspects of the cellulase action, related to the scission of cellulose molecules on the elementary fibrillar surface. Measurement of DP changes and the detection of liberated chain ends merit particular attention. Assessment of changes in the distribution of molecular weights, by gel-filtration chromatography of the enzyme-treated cellulose dissolved in Cadoxen, should be rewarding.

The size, accessibility, and surface structure of the elementary fibrillar units are important factors determining the ease of enzymic hydrolysis and considerable information could be obtained, using the diazomethane methylation technique, by examining the influence of substrates such as mercerised cotton, viscose rayon, HWM rayon, ramie, and flax, all of which have microstructures very different from that of native cotton. This approach could be extended to include differential speed two roll mill pretreatments of cellulosic materials prior to enzymic hydrolysis, an area of current interest as a means of enhancing the susceptibility to enzymic hydrolysis.

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TABLE 1

ENZYME TREATMENT CONDITIONS FOR SAMPLES INVESTIGATED IN THIS REPORT

Series No.	Sample Designation	Source of Enzyme	Treatment Temp (°C)	Time (days)	Extent of Hydrolysis[a] (%)
1		Ground University Plugging Cotton Treated by Dr E.T.Reese, Natick Development Centre			
	2C	Control - no enzyme Treated with pH 4.5 citrate buffer	50	4	0.0
	2XP	C_x enzyme from Pestalotiopsis westerdijkii, QM 381-23; 75 C_x units/ml	50	5	1.2
	2E	Total cellulase complex from T.viride QM 9414; Batch 23 mm; FP 3.5 = 2.5 μl/ml	50	4	30.8
	2EM	As for 2E plus Methocel DS 1.89 (0.25 mg/ml)	50	5	14.0
		Unground University Plugging Cotton			
	2C-F	Control - no enzyme Treated with pH 4.5 citrate buffer	50	5	0.1
	2X-F	As for 2XP	50	5	1.5
	2E-F	As for 2E	50	5	24.1
	2EM-F	As for 2EM	50	5	5.0
2		Scoured Acala Cotton Samples Treated by Dr E.T.Reese, Natick Development Centre			
	A	Control - no enzyme; treated with pH 4.5 citrate buffer	50	2.9	-
	B	Total cellulase complex from T.viride, QM 9414. 50 C_x-Iu/ml	50	2.9	19.3
	C	Total cellulase complex from T.viride, QM 9414. 12.5 C_x-Iu/ml	50	2.9	5.0
	D	As for B plus Methocel (0.3mg/ml)	50	2.9	6.75
	E	C_x fraction from T.viride, QM 9414. 15 C_x-Iu/ml	50	4.9	0.3
	F	C_1 fraction from T.viride, QM 9414	50	4.9	2.0
	G	Total cellulase from Pestalotiopsis westerdijkii; QM 381. 84C_x-Iu/ml; no C_1	50	5.0	5.4
	H	As for B but sample unshaken during incubation	50	2.8	44.1
3		Scoured Acala Cotton Samples Treated by Dr T.M.Wood, The Rowett Research Institute			
	C_o	Control - no enzyme; treated with pH 4.8 sodium acetate/acetic acid buffer containing Na N_3 (0.005%).	37	11	-
	C_x	C_x fraction from T.koningii	37	11	1.7
	C_1	C_1 fraction from T.koningii	37	11	0.5
	C_{1+x}	Mixture of C_1 and C_x fractions from T.koningii	37	11	32.0

[a] Based on reducing sugar, as glucose, using air dry weight of original cotton sample.

TABLE 2

Effect of Enzyme Treatments on Accessibility to Water Vapour of 'Never-dried' Samples

Sample Desig-nation.[a]	Extent of enzymolysis [b] (%)	Water Retention (%)	Moisture Regain at 25° C.		Moles of water per D-glucopyranosyl unit		Proportion of accessible groups		Mean Accessibilit (%)
			65% r.h.	88% r.h.	65% r.h.	88% r.h.	65% r.h.	88% r.h.	
A	0	46.2 ± 3.3	8.6 ± 0.1	14.6 ± 0.7	0.774	1.314	0.506	0.505	50.6
B	19.3	56.6 ± 0.7	7.8 ± 0.2	13.4 ± 0.4	0.702	1.206	0.459	0.464	46.2
C	5.0	50.3 ± 0.6	8.3 ± 0.1	13.9 ± 0.2	0.747	1.251	0.488	0.481	48.5
D	6.75	52.9 ± 0.1	7.9 ± 0.6	13.4 ± 0.8	0.711	1.206	0.465	0.464	46.5
E	0.3	48.5 ± 0.4	8.5 ± 0.2	14.5 ± 1.1	0.765	1.305	0.500	0.502	50.1
F	2.0	48.9 ± 5.8	8.5 ± 0.3	14.3 ± 0.9	0.765	1.287	0.500	0.495	49.8
G	5.4	48.1 ± 1.4	8.4 ± 0.0	14.2 ± 0.4	0.756	1.278	0.494	0.492	49.3
H	44.1	54.3 ± 3.9	7.9 ± 0.3	13.5 ± 0.7	0.711	1.215	0.465	0.467	46.6
C_0	0	45.7 ± 4.1	8.7 ± 0.6	14.8 ± 0.0	0.783	1.332	0.512	0.512	51.2
C_1	0.5	45.9 ± 0.4	8.6 ± 0.0	14.9 ± 0.1	0.774	1.341	0.506	0.516	51.1
C_X	1.7	47.5 ± 4.7	8.6 ± 0.0	15.0 ± 0.1	0.774	1.350	0.506	0.519	51.3
$C_1 + C_X$	32.0	52.9 ± 0.6	8.4 ± 0.3	14.0 ± 0.0	0.756	1.260	0.494	0.485	49.0

[a] See Table 1.

[b] Based on reducing sugar as glucose, using air dry weight of original cotton sample.

TABLE 3

Effect of Enzyme Treatments on Accessibility to Water Vapour after Drying over P_2O_5

ample esig-ation.[a]	Extent of enzymolysis[b] (%)	Water Retention (%)	Moisture Regain at 25° C		Moles of water per D-glucopyranosyl unit		Proportion of accessible groups		Mean Accessibility (%)
			65% r.h.	88% r.h.	65% r.h.	88% r.h.	65% r.h.	88% r.h.	
A	0	37.9 ± 0.6	7.5 ± 0.2	12.2 ± 0.3	0.675	1.098	0.441	0.422	43.2
B	19.3	49.4 ± 7.3	6.9 ± 0.4	11.8 ± 1.4	0.621	1.062	0.406	0.408	40.7
C	5.0	40.2 ± 0.3	7.3 ± 0.1	11.8 ± 0.1	0.657	1.062	0.429	0.408	41.9
D	6.75	41.0 ± 0.3	7.0 ± 0.8	11.6 ± 1.1	0.630	1.044	0.412	0.402	40.7
E	0.3	41.1 ± 1.2	7.5 ± 0.1	12.1 ± 0.5	0.675	1.089	0.441	0.419	43.0
F	2.0	40.6 ± 1.8	7.6 ± 0.2	12.2 ± 1.0	0.684	1.098	0.447	0.422	43.5
G	5.4	38.9 ± 0.9	7.5 ± 0.1	12.0 ± 0.2	0.675	1.080	0.441	0.415	42.8
H	44.1	50.4 ± 1.2	7.1 ± 0.1	11.9 ± 0.9	0.639	1.071	0.418	0.412	41.5
C_0	0	40.4 ± 1.8	7.3 ± 0.5	11.9 ± 0.5	0.657	1.071	0.429	0.412	42.1
C_1	0.5	38.9 ± 1.7	6.9 ± 0.1	11.4 ± 0.2	0.621	1.026	0.406	0.395	40.1
C_X	1.7	39.4 ± 3.7	6.8 ± 0.5	11.3 ± 0.2	0.612	1.017	0.400	0.391	39.6
$_1+C_X$	32.0	45.5 ± 0.3	6.4 ± 0.7	10.8 ± 0.7	0.576	0.972	0.376	0.374	37.5

[a] See Table 1.

[b] Based on reducing sugar as glucose, using air dry weight of original cotton sample.

TABLE 4

Effect of Enzyme Treatment on Accessibility
Assessed by Infrared-Deuteration Studies

Sample Designation	Enzyme Treatment	Specimen Number	Total Disordered Cellulose Content (%)[a]	Rate of Deuteration of Crystalline O-H (increase in % disordered cellulose content per hour).
A	Control-no enzyme; Treated with pH 4.5 citrate buffer	1	35.4)	0.08)
		2	38.3) 36.9	0.07) 0.07$_5$
B	Total cellulase complex from *T.viride*, QM 9414; 50 C_xIu/ml	1	36.4)	0.10)
		2	45.1 40.7	0.02 0.06
C	- ditto - 12.5 C_xIu/ml	1	42.7)	0.08)
		2	38.4) 40.6	0.04) 0.06
D	As for B plus Methocel 0.3 mg/ml)	1	36.9)	0.05)
		2	37.9) 37.4	0.07) 0.06
E	C_x fraction from *T.viride*, QM 9414; 15 C_x Iu/ml	1	41.6)	0.08)
		2	47.8) 42.7	0.08) 0.09
		3	38.6)	0.12)
F	C_1 fraction from *T.viride*, QM 9414	1	40.2) 38.7	0.04) 0.05$_5$
		2	37.2)	0.07)
G	Total cellulase from *Pestalotiopsis westerdijkii*; QM 381; 84 C_xIu/ml; no C_1	1	46.6)	0.10)
		2	42.0) 44.3	0.13) 0.11$_5$
H	As for B but sample unshaken during incubation	1	41.5) 42.7	0.01) 0.02$_5$
		2	43.9)	0.04)
C_o	Control enzyme; treated with pH 4.8 acetate buffer	1	50.9)	0.09)
		2	43.2) 47.8	0.04) 0.08
		3	49.3)	0.11)
C_1	C_1 fraction from *T.koningii*	1	53.3)	0.15)
		2	54.9) 55.0	0.15) 0.16
		3	56.9)	0.19)
C_x	C_x fraction from *T. koningii*	1	45.5)	0.03)
		2	51.1)	0.08)
		3	45.4) 47.0	0.08) 0.09
		4	45.8)	0.17)
C_1+C_x	Mixture of C_1 and C_x Fractions from *T. koningii*	1	43.0) 42.7	0.04) 0.04
		2	42.4)	0.04)

[a]Extrapolated to zero time of deuteration.

Table 5

Analysis of Hydrolysates from Original Air-Dry University Plugging Cotton and from a Scoured Cotton Yarn Methylated with Diazomethane

Sample	Reaction Time (days)	Composition of hydrolysate (mole-%)[a]								Hydroxyl groups (%) methylated			Methoxyl D.S. (calc.[d])
		G	G_2	G_3	G_6	G_{23}	$G_{2,6}$	G_{36}	G_{236}	at C-2	at C-3	at C-6	
Scoured Cotton Yarn	1	88.7	4.0	0.7	4.2	0.8	1.1	0.5	0.2	6.1	2.2	6.0	0.14
	2	80.0	6.3	0.8	5.3	2.4	2.1	1.2	1.6	12.4	6.0	10.2	0.29
	4	77.6	5.7	0.9	7.3	2.1	3.9	1.2	2.3	14.0	6.5	14.7	0.35
	7	75.4	5.5	0.5	9.7	2.6	3.8	0.5	3.6	15.5	7.2	17.6	0.40
	10	70.2	7.0	1.0	8.4	2.9	4.1	1.9	5.4	19.4	11.2	19.8	0.50
	15	68.2	7.4	1.2	8.4	2.2	4.9	1.8	5.7	20.2	10.9	20.8	0.52
	21	64.7	7.9	1.0	7.6	4.2	5.9	1.5	7.1	25.1	13.8	22.1	0.61
	28	67.2	6.8	0.9	7.3	3.1	5.4	0.9	8.7	24.0	13.6	22.3	0.60
Original University Plugging Cotton	1	80.0	6.9	-	5.4	2.0	2.7	0.8	1.1	12.7	3.9	10.0	0.26
	2	77.8	8.7	-	6.0	2.0	3.1	1.9	1.7	15.5	5.6	12.7	0.34
	4	71.6	9.1	0.9	8.1	2.6	4.5	1.2	2.7	18.9	7.4	16.5	0.43
	7	70.3	9.0	0.8	8.4	2.8	4.7	0.8	3.5	20.0	7.9	17.4	0.45
	10	69.0	7.6	0.9	8.2	3.1	6.4	1.2	4.5	21.6	9.7	20.3	0.52
	15	64.9	8.9	0.8	10.1	3.3	4.9	1.1	6.3	23.4	11.5	22.4	0.57
	21	64.8	8.5	1.7	9.7	2.7	4.8	1.3	7.1	23.1	12.8	22.9	0.59
	28	64.5	8.3	1.1	9.8	3.0	4.7	1.1	7.4	23.4	12.6	23.0	0.59

[a] G = D-glucose, G_2 = 2-O-methyl-D-glucose, etc.

Table 6

Analysis of Hydrolysates from Unground Control and Enzyme Treated University Plugging Cotton Methylated with Diazomethane

Sample[a]	Reaction Time (days)	Composition of hydrolysate (mole-%)[b]								Hydroxyl groups (%) methylated			Methoxyl D.S. (calc.[d])
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
2 CF	1	88.9	4.7	0.4	3.0	0.9	1.0	-	0.3	6.9	1.6	4.3	0.13
	2	79.9	6.7	1.0	6.6	1.8	2.6	1.0	0.9	12.0	4.7	11.1	0.17
	4	73.8	8.0	0.8	7.9	2.4	4.0	0.8	2.4	16.8	6.4	15.1	0.38
	7	72.2	7.8	1.2	8.5	2.7	4.0	0.9	3.1	17.6	7.9	16.5	0.42
	10	72.3	7.4	0.9	7.9	2.9	3.4	0.9	4.4	18.1	9.1	16.6	0.44
	15	67.4	7.3	0.8	9.6	2.9	4.4	1.1	6.6	21.2	11.4	21.7	0.54
	21	63.5	9.4	(2.5)	9.0	3.7	3.5	(2.1)	6.9	23.5	(15.5)	21.5	(0.60)
	28	67.7	7.5	1.3	9.2	2.6	4.5	1.0	6.8	21.4	11.7	21.5	0.55
2 XF	1	91.8	2.4	0.4	2.8	0.9	1.3	0.4	0.3	4.9	2.0	4.8	0.12
	2	81.0	5.3	0.6	6.5	2.2	2.8	0.6	1.1	11.4	4.5	11.0	0.27
	4	73.0	8.7	0.4	8.5	2.4	3.6	0.8	2.3	17.0	5.9	15.2	0.38
	7	73.7	7.8	0.3	8.5	2.4	3.4	0.7	3.0	16.6	6.4	15.6	0.39
	10	70.9	7.6	0.6	9.6	2.8	4.1	0.8	3.7	18.2	7.9	18.2	0.44
	15	69.0	6.9	0.8	9.9	2.8	4.5	1.0	5.5	19.7	10.1	20.9	0.51
	21	67.8	8.6	1.1	9.7	2.2	4.0	0.8	5.6	20.4	9.7	20.1	0.50
	28	67.8	7.1	1.4	9.9	2.7	4.6	1.9	5.7	20.1	11.7	22.1	0.54
2 EMF	1	87.8	4.4	0.5	4.0	0.6	1.5	0.3	0.7	7.2	2.1	6.5	0.16
	2	78.5	7.7	0.9	6.4	2.0	2.6	0.6	1.4	13.7	4.9	11.0	0.30
	4	75.1	7.0	0.9	8.3	2.4	3.7	0.5	1.9	15.0	5.7	14.4	0.35
	7	72.2	7.1	1.1	8.9	2.8	3.6	1.1	3.4	16.9	8.4	17.0	0.42
	10	70.8	6.6	0.7	9.5	2.8	4.2	1.3	4.6	18.2	9.4	19.6	0.47
	15	65.1	6.9	0.9	8.8	3.4	5.1	1.0	8.9	24.3	14.2	23.8	0.62
	21	65.8	8.4	1.8	9.8	3.1	3.6	1.3	6.6	21.7	12.8	21.3	0.56
	28	66.5	7.3	0.9	10.0	2.6	4.6	0.6	7.3	21.8	11.4	22.5	0.56
2 EF	1	84.3	5.4	0.5	6.4	1.2	2.0	0.2	0.3	8.9	2.2	8.9	0.20
	2	80.3	5.5	0.8	7.3	1.8	2.0	0.8	1.4	10.7	4.8	11.5	0.27
	4	73.4	7.5	0.4	8.5	3.3	3.3	0.9	2.5	16.6	7.1	15.2	0.39
	7	71.7	7.2	1.0	9.5	2.6	4.0	1.2	3.3	17.1	8.1	18.0	0.43
	10	68.8	6.5	1.4	9.3	3.0	6.0	1.4	4.7	20.2	10.5	21.4	0.52
	15	68.6	6.9	0.8	9.3	2.7	4.1	1.3	5.9	19.6	10.7	20.6	0.51
	21	68.4	7.9	0.6	9.8	2.7	3.9	0.9	5.6	20.1	9.8	20.2	0.50
	28	67.5	7.3	0.9	9.8	2.6	4.0	0.9	7.0	20.9	11.4	21.7	0.54

[a] See Table 1,

[b] G = D-glucose, G_2 = 2-0- methyl-D-glucose, etc.

Table 7

Analysis of Hydrolysates from Wiley-milled Control and Enzyme Treated University Plugging Cotton Methylated with Diazomethane

Sample[a]	Reaction Time (days)	Composition of hydrolysate (mole-%)[b]								Hydroxyl groups (%) methylated			Methoxyl D.S. (calc.[d])
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
2 C	1	87.0	5.1	0.4	4.2	1.0	1.5	0.3	0.3	7.9	2.0	6.3	0.16
	2	73.3	7.8	0.8	7.9	2.7	4.5	0.8	2.6	17.6	6.9	15.8	0.40
	4	71.3	7.9	0.6	8.2	3.3	4.4	1.3	3.2	18.8	8.4	17.1	0.44
	7	67.9	8.2	0.8	8.3	3.1	5.0	0.8	5.4	21.7	10.1	19.5	0.51
	10	67.9	6.9	0.6	7.6	3.2	5.5	1.3	7.9	23.5	13.0	21.4	0.58
	15	62.6	6.9	0.9	10.3	3.7	6.0	1.1	8.8	25.4	14.5	26.2	0.66
	21	64.1	7.7	1.5	7.4	2.8	5.0	2.0	10.6	26.1	16.9	25.0	0.68
	28	62.5	6.8	1.5	8.0	3.5	5.6	1.5	11.0	26.9	17.5	26.1	0.70
2 XP	1	86.9	3.4	0.9	5.2	1.2	1.6	0.6	0.6	6.8	3.3	8.0	0.18
	2	72.8	8.4	1.1	8.1	2.9	3.4	0.7	2.1	16.8	6.8	14.3	0.38
	4	72.0	6.6	0.7	7.7	3.3	5.3	1.0	3.3	18.5	8.3	17.3	0.44
	7	69.1	7.2	1.0	8.0	3.3	4.5	1.0	5.4	20.4	10.7	18.9	0.50
	10	66.8	7.2	0.7	9.3	3.2	5.1	1.5	7.0	22.5	12.4	22.9	0.58
	15	64.5	7.1	0.7	8.1	4.0	5.6	1.5	8.7	25.4	14.9	23.9	0.64
	21	64.7	7.8	1.4	7.9	2.8	5.0	1.1	9.5	25.1	14.8	23.5	0.63
	28	62.8	7.6	1.1	7.7	3.0	5.9	1.1	10.8	27.3	16.0	25.5	0.69
2 EM	1	92.5	2.6	0.3	1.8	1.2	0.5	0.3	0.5	4.8	2.3	3.1	0.10
	2	86.0	4.8	0.9	4.9	1.1	0.5	9.3	0.6	7.0	2.9	6.3	0.16
	4	76.4	6.6	0.5	7.5	2.6	3.3	1.0	1.8	14.3	5.8	13.6	0.34
	7	69.9	7.2	1.5	8.6	3.2	4.5	1.0	4.3	19.2	10.0	18.4	0.48
	10	71.0	6.2	1.1	7.7	3.0	4.8	1.6	5.3	19.3	11.0	19.4	0.50
	15	67.4	6.4	0.7	8.9	3.2	5.7	0.7	7.1	22.4	11.7	22.4	0.57
	21	67.0	6.8	1.4	8.2	2.8	4.5	1.4	8.3	22.4	13.9	22.4	0.59
	28	65.7	7.0	0.7	8.5	3.2	4.8	1.0	8.9	23.9	13.8	23.2	0.61
2 E	1	81.6	5.5	1.4	5.7	1.6	3.4	0.7	0.7	11.2	4.4	10.5	0.26
	2	75.9	6.3	0.7	8.5	2.2	3.4	1.4	2.0	13.9	6.3	15.3	0.36
	4	72.1	7.5	1.0	7.9	2.8	4.0	1.3	3.6	17.9	8.7	16.8	0.43
	7	66.5	7.1	1.1	8.7	3.7	4.9	0.8	6.9	22.6	12.5	21.3	0.56
	10	68.7	6.8	0.6	8.6	2.8	4.5	0.6	7.3	21.4	11.3	21.0	0.54
	15	69.6	6.8	0.6	9.3	2.9	4.5	0.8	5.5	19.7	9.8	20.1	0.50
	21	66.9	5.8	1.4	7.2	3.1	5.2	0.9	9.5	23.6	14.9	22.8	0.61
	28	65.9	5.7	0.8	8.1	3.0	5.6	1.4	9.8	24.1	15.0	24.9	0.64

[a] See Table 1,

[b] G = D-glucose, G_2 = 2-O-Methyl-D-glucose, etc.

TABLE 8

G.L.C. Analys s of Hydrolysates from Scoured Acala Cotton Fibres Treated with Cellulase Fractions from T. viride (Dr E.T.Reese) and Subsequently Methylated with Diazomethane

SAMPLE [a]	Time of Reaction with Diazomethane (DAYS)	COMPOSITION OF HYDROLYSATE (mole-%)[b]								HYDROXYL GROUPS (%) METHYLATED			METHOXYL D.S. (Galc)
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
A	1	85.5	5.5	0.2	4.9	1.3	2.1	0.3	0.3	9.2	2.1	7.6	0.19
Control - no	2	75.9	7.5	0.6	8.0	2.1	3.0	0.6	2.7	15.2	6.0	13.3	0.35
enzyme; treated	4	75.2	7.5	0.6	6.9	2.3	3.8	0.7	2.9	16.4	6.4	14.2	0.37
with pH 4.5	7	65.0	11.2	0.8	6.2	4.1	6.3	0.9	5.9	27.5	11.8	19.3	0.59
citrate buffer	11	72.5	8.3	0.4	7.6	2.1	4.4	0.9	4.3	19.0	7.7	17.1	0.44
	16	66.5	9.2	1.0	6.7	2.9	6.6	0.3	7.1	25.7	11.3	20.7	0.58
	21	66.9	8.7	1.2	7.2	3.8	5.1	0.7	6.8	24.3	12.5	19.8	0.57
	28	66.5	7.4	1.5	6.1	2.9	7.2	0.8	7.9	25.4	13.1	22.0	0.61
	35	65.6	8.3	0.9	6.6	2.8	7.7	0.7	8.3	27.1	12.7	23.3	0.63
	42	65.2	8.8	0.3	8.1	3.0	6.4	0.7	9.1	27.3	13.1	24.3	0.65
B	1	86.1	4.5	0.3	5.3	1.1	2.1	0.3	0.5	8.2	2.2	8.2	0.19
Total cellulase	2	76.6	7.2	0.6	7.9	2.1	3.4	0.6	2.0	14.6	5.3	13.8	0.34
complex from	4	74.8	7.4	0.7	8.1	2.3	3.4	0.7	3.1	16.0	6.7	15.2	0.39
T.viride,	7	73.6	7.2	0.9	7.7	2.5	3.7	0.9	3.7	17.1	8.0	16.0	0.42
QM 9414.	11	75.2	6.7	0.6	8.0	2.0	3.6	0.9	3.5	15.7	6.9	15.4	0.38
50 C_x-Iu/ml	16	69.8	6.5	1.2	8.5	2.2	4.0	1.0	5.7	19.4	10.1	19.2	0.49
	21	71.1	6.6	0.6	9.0	3.0	4.0	0.3	4.8	18.3	8.7	18.1	0.45
	28	67.6	6.0	0.8	9.6	2.4	4.7	0.4	8.2	21.2	11.8	24.8	0.56
	35	63.5	8.1	0.8	8.6	3.0	5.6	0.8	9.2	26.4	14.3	24.1	0.65
	42	61.8	7.8	1.8	10.0	3.6	6.1	0.9	8.8	20.4	15.1	25.8	0.67
C	1	79.0	6.6	0.8	7.2	1.8	2.4	1.2	1.4	11.4	5.2	12.0	0.29
Total cellulase	2	76.3	7.0	0.5	7.8	2.4	3.3	0.8	2.3	14.9	5.9	14.1	0.35
complex from	4	73.2	7.6	0.6	8.3	2.2	4.1	0.6	3.8	17.6	7.2	16.7	0.42
T.viride,	7	70.4	7.4	0.8	8.6	2.0	4.2	0.8	4.3	17.8	7.8	17.8	0.44
QM 9414.	11	70.1	6.8	0.6	8.5	2.6	5.1	0.6	5.4	19.9	9.2	19.6	0.49
12.5 C_x-Iu/ml	16	71.1	7.1	0.6	9.2	2.5	3.8	0.8	5.5	18.8	9.3	19.2	0.48
	21	70.9	6.8	0.3	8.8	2.4	4.7	0.7	5.4	19.3	8.8	19.6	0.48
	28	71.7	7.1	0.9	9.5	2.2	3.3	0.8	5.1	17.6	9.0	18.6	0.46
	35	67.7	7.6	0.8	9.2	2.3	4.7	1.0	7.1	21.6	11.1	21.9	0.55
	42	69.2	7.7	0.8	9.0	2.2	4.4	0.8	5.8	20.0	9.6	19.9	0.50

[a] See Table 1 [b] G = D-glucose, G_2 = 2-O- methyl-D-glucose, etc

TABLE 8 contd

SAMPLE[a]	Time of Reaction with Diazomethane (DAYS)	COMPOSITION OF HYDROLYSATE (mole-%)[b]								HYDROXYL GROUPS (%) METHYLATED			METHOXYL D.S.(Calc)
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
D	1	81.7	5.9	0.7	7.0	1.3	2.5	0.5	0.6	10.3	3.1	10.5	0.24
As for B plus	2	71.9	6.9	0.9	7.4	2.2	2.8	0.9	2.4	14.3	6.4	13.5	0.34
Methocel	4	68.0	8.9	1.4	8.6	3.2	4.9	1.4	4.6	21.5	10.6	19.4	0.52
(0.3mg/ml)	7	67.9	7.9	0.6	8.8	2.9	4.7	0.6	7.0	22.4	11.2	21.0	0.55
	11	69.8	8.3	0.8	7.9	2.4	5.2	1.3	5.9	20.4	10.4	19.0	0.49
	16	71.8	6.6	0.8	7.5	2.5	3.9	0.8	6.3	19.3	10.4	18.5	0.48
	21	72.4	6.8	0.8	6.8	2.4	3.7	0.8	6.6	19.4	10.6	17.9	0.48
	28	71.2	7.4	0.5	8.2	2.6	4.3	0.7	5.4	19.5	9.1	18.5	0.48
	35	67.0	8.2	1.0	8.4	2.6	4.7	1.0	7.4	23.0	12.1	21.6	0.57
	42	66.4	8.1	1.0	9.4	2.3	4.6	0.5	8.0	23.0	11.8	22.4	0.58
E	1	80.2	6.2	0.4	7.2	2.0	2.9	0.4	1.0	12.0	3.8	11.5	0.27
C_x fraction from	2	77.7	6.5	0.5	7.9	1.9	2.9	0.6	2.2	13.8	5.2	13.5	0.32
T.viride,	4	72.6	7.5	0.3	8.9	2.6	3.8	0.7	4.0	17.8	7.5	17.3	0.43
QM 9414.	7	71.8	7.5	1.3	8.9	2.6	3.6	1.3	3.5	17.1	8.7	17.3	0.43
15 C_x-Iu/ml	11	70.8	6.8	0.8	7.8	2.5	4.4	0.8	5.8	17.0	10.1	19.0	0.46
	16	72.7	6.8	0.5	8.9	2.3	4.1	1.2	4.2	15.9	8.0	18.1	0.42
	21	69.4	5.1	1.1	8.2	2.6	4.6	0.7	6.3	19.1	10.7	19.7	0.49
	28	62.2	6.9	1.3	8.9	3.0	3.5	1.3	7.5	20.4	13.0	21.1	0.55
	35	66.4	7.7	1.3	8.1	2.8	3.4	1.3	8.4	21.9	13.8	22.2	0.58
	42	67.3	6.7	1.1	8.4	2.8	4.9	1.0	8.2	20.6	13.1	22.5	0.56
F	1	81.5	6.3	0.6	7.0	1.3	2.2	0.6	1.1	10.1	3.6	10.9	0.25
C_1 fraction	2	68.1	7.3	0.8	11.0	2.0	4.2	1.0	3.9	16.8	7.7	20.0	0.45
from *T.viride*,	4	75.8	7.0	0.5	8.5	1.6	3.3	0.5	3.2	13.5	5.8	14.9	0.35
QM 9414	7	71.2	7.6	0.5	9.5	2.7	4.1	0.5	4.2	16.7	7.9	18.3	0.43
	11	72.4	6.5	0.5	7.8	2.6	4.9	0.8	4.9	16.5	8.7	18.3	0.44
	16	70.0	7.6	0.3	8.8	2.3	4.0	0.6	6.6	18.7	9.8	20.0	0.49
	21	65.3	8.3	0.6	9.0	2.9	4.5	0.7	8.8	22.6	13.0	23.0	0.59
	28	64.1	8.3	1.2	8.7	3.3	4.3	1.2	9.4	24.2	15.1	23.6	0.63
	35	66.4	8.4	0.6	9.5	2.6	5.3	0.8	6.8	21.0	10.8	22.3	0.54
	42	68.6	7.4	0.9	8.7	2.1	4.4	0.9	7.3	19.7	11.2	21.2	0.52

[a] See Table 1 [b] G = D-glucose, G_2 = 2-O- methyl-D-glucose, etc

TABLE 8 contd

SAMPLE [a]	Time of Reaction with Diazomethane (DAYS)	COMPOSITION OF HYDROLYSATE (mole-%) [b]								HYDROXYL GROUPS (%) METHYLATED			METHOXYL D.S.(Calc)
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
G. Total cellulase from Pestalotiopsis westerdijkii; QM 381. $84C_x$-Iu/ml; no C_1	1	81.6	5.0	0.4	7.5	1.5	2.1	1.6	0.7	9.4	4.2	11.9	0.26
	2	76.2	7.0	0.9	7.6	2.2	2.7	1.1	2.4	14.1	6.6	13.9	0.35
	4	71.7	7.6	0.8	8.3	2.6	4.5	0.8	4.2	16.5	8.8	17.7	0.43
	7	67.3	7.6	1.3	9.1	2.9	4.2	1.3	7.0	19.8	12.6	21.7	0.54
	11	67.2	6.5	1.9	8.6	2.8	4.5	1.3	6.8	20.1	12.7	21.2	0.54
	16	69.4	7.6	0.5	8.5	1.8	4.2	1.0	6.8	19.2	10.1	20.5	0.50
	21	68.5	8.3	0.9	9.2	3.0	4.3	1.2	6.3	19.4	11.3	21.0	0.52
	28	67.8	8.3	0.8	9.2	2.1	3.6	1.1	7.6	20.2	11.6	21.4	0.53
	35	69.7	8.4	0.7	9.1	2.8	4.4	1.2	6.5	18.4	11.2	21.1	0.51
	42	66.4	7.4	0.8	9.3	2.0	5.3	0.8	7.6	20.4	12.2	22.9	0.56
H. As for B but sample unshaken during incubation	1	77.0	5.0	0.7	7.7	2.2	3.1	0.7	2.0	12.8	5.6	13.5	0.32
	2	79.2	7.4	0.3	7.7	1.6	2.8	0.2	1.9	17.6	4.0	12.6	0.35
	4	70.5	8.2	1.0	8.6	2.5	3.9	1.0	4.8	17.9	9.2	18.2	0.45
	7	73.6	7.5	0.5	7.6	1.7	4.0	1.0	4.1	15.5	7.2	16.6	0.40
	11	70.4	7.7	0.8	9.2	1.7	3.6	0.8	5.8	17.4	9.0	19.3	0.46
	16	66.3	8.0	0.7	9.0	2.8	4.9	0.7	8.0	21.6	12.2	22.5	0.56
	21	61.8	8.9	1.0	9.7	2.9	4.7	1.0	10.2	24.6	15.1	19.7	0.65
	28	66.2	7.9	0.4	10.0	2.7	5.6	0.4	6.6	22.8	10.1	22.6	0.56
	35	61.7	8.1	1.2	9.4	3.1	4.7	1.2	11.0	25.5	16.5	26.3	0.69
	42	62.1	8.0	1.1	9.8	3.0	4.5	1.1	10.7	24.8	16.0	26.2	0.67

[a] See Table 1

[b] G = D-glucose, G_2 = 2-O methyl-D-glucose, etc.

TABLE 9 contd

SAMPLE [a]	Time of Reaction with Diazomethane (DAYS)	COMPOSITION OF HYDROLYSATE (mole-%) [b]								HYDROXYL GROUPS (%) METHYLATED			METHOXYL D.S.(Calc)
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
C_x fraction from T.koningii	1	86.5	5.0	0.4	4.7	1.1	1.7	0.2	0.2	8.0	1.9	6.8	0.17
	2	82.0	5.2	0.7	5.7	1.3	2.9	0.5	1.9	11.2	4.4	11.0	0.27
	4	79.4	5.3	0.5	5.6	1.9	3.7	0.7	2.6	13.5	5.7	12.7	0.32
	7	71.1	8.3	0.5	8.2	1.9	4.3	1.0	4.6	19.0	8.0	18.1	0.45
	11	71.4	7.5	0.8	8.7	1.9	3.6	1.1	5.4	18.3	9.1	18.7	0.46
	16	71.5	7.9	0.9	7.6	2.8	2.9	0.2	5.3	17.6	8.1	14.8	0.41
	21	69.0	6.8	1.1	8.8	2.6	4.3	0.7	8.0	21.7	12.4	21.7	0.56
	28	65.7	8.6	1.3	8.5	2.0	4.0	0.7	9.2	23.7	13.2	22.4	0.60
	35	68.4	8.0	0.9	7.6	2.4	3.7	0.9	8.3	22.4	12.5	20.5	0.56
	42	69.5	7.0	0.7	7.8	2.4	4.0	0.7	8.2	21.6	12.0	20.7	0.55
C_{1+x} Mixture of C_1 and C_x fractions from T.koningii	1	87.0	4.7	0.5	4.0	1.0	2.1	0.1	0.8	8.6	2.4	6.9	0.18
	2	81.1	5.8	0.6	4.8	2.1	4.0	0.4	2.0	13.8	5.1	10.7	0.30
	4	72.9	8.3	0.9	4.4	2.3	6.3	0.3	5.0	20.3	8.3	14.4	0.43
	11	72.4	5.9	0.9	6.9	2.7	5.4	0.4	5.6	19.2	9.2	18.0	0.47
	16	63.6	8.5	1.0	7.4	2.9	7.0	0.3	10.0	28.3	14.2	24.6	0.60
	21	58.9	8.2	1.0	6.0	3.7	8.4	0.6	13.2	33.0	17.8	28.0	0.79
	28	62.3	7.0	1.2	6.3	3.3	9.0	0.5	10.8	29.8	16.1	26.4	0.72
	35	61.1	7.0	1.3	7.5	2.9	7.9	0.5	13.1	30.4	17.8	29.0	0.78

[a] See Table 1 [b] G = D-glucose, G_2 = 2-O - methyl-D-glucose, etc

TABLE 9

G.L.C. Analyses of Hydrolysates from Scoured Acala Cotton Fibres Treated with Cellulase Enzyme Fractions from T.Koningii (Dr T.M.Wood) and Subsequently Methylated with Diazomethane

SAMPLE [a]	Time of Reaction with Diazomethane (DAYS)	COMPOSITION OF HYDROLYSATE (mole-%) [b]								HYDROXYL GROUPS (%) METHYLATED			METHOXYL D.S.(Calc)
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	at C-2	at C-3	at C-6	
C_o Control - no enzyme Treated with pH 4.8 sodium acetate acetic acid buffer containing NaN_3(0.005%)	1	86.3	5.2	0.3	5.2	0.8	2.1	0.3	0.4	8.4	1.8	8.0	0.18
	2	77.2	7.5	0.6	6.8	2.2	3.2	0.8	2.1	14.9	5.7	12.9	0.34
	4	70.6	8.5	0.9	7.1	3.0	4.9	0.5	4.7	21.0	9.0	17.1	0.48
	7	73.8	6.5	1.0	8.4	2.1	4.3	0.5	3.8	16.6	7.4	17.0	0.41
	11	72.9	5.8	0.6	8.0	2.4	4.3	0.9	5.4	17.9	9.0	18.8	0.46
	16	70.3	6.1	0.5	8.8	2.7	5.2	1.3	6.7	20.4	11.1	22.2	0.54
	28	69.0	5.6	0.8	6.7	2.3	6.2	0.2	9.4	22.2	11.5	21.5	0.56
	35	68.7	6.3	0.9	7.0	2.4	5.1	0.6	8.9	22.6	12.7	21.5	0.57
	42	66.5	7.6	0.7	8.2	2.8	4.4	1.0	9.1	23.9	13.3	22.6	0.60
C_1 fraction from T.koningii	1	88.7	3.7	0.3	4.3	0.9	1.8	0.2	0.6	6.8	1.9	6.8	0.16
	2	79.1	6.8	0.7	6.0	2.3	2.8	0.7	1.7	13.7	5.5	11.3	0.31
	4	79.6	5.2	0.6	6.7	1.8	2.5	0.7	2.3	11.7	5.3	12.1	0.29
	7	75.5	6.0	0.4	7.5	2.2	3.9	0.9	4.0	16.0	7.5	16.1	0.40
	11	72.9	7.2	1.7	8.6	1.6	3.3	0.4	4.6	16.7	8.3	16.9	0.42
	16	67.9	8.6	0.8	8.8	2.8	4.1	0.8	6.7	22.1	11.1	20.3	0.54
	21	67.6	8.2	0.6	8.8	2.6	4.6	1.0	7.1	22.4	11.2	21.4	0.55
	28	70.0	7.1	0.5	7.6	2.3	4.8	0.5	7.5	20.8	9.8	19.8	0.51
	35	68.5	7.6	1.0	8.7	2.1	3.8	1.0	7.8	20.8	11.4	21.4	0.54
	42	68.9	7.2	0.7	7.2	2.1	5.1	0.7	7.9	22.2	11.4	20.8	0.54

[a] See Table 1

[b] G = D-glucose, G_2 = 2-O- methyl-D-glucose, etc.

Table 10

Diazomethane Methylation - Distribution of Methoxyl Groups on completion of the Initial Rapid Reaction

i.e. Extrapolated to Zero Reaction Time

Sugar[a]	Sugar Content (Moles %)											
	Scoured Cotton Yarn	Original University Plugging Cotton	Unground Samples[b]				Wiley-milled Samples[b]				Calculated[c] for 10x20 a.g.u.	Calculated[c] for 10x12 a.g.u.
			2C-F	2X-F	2EM-F	2E-F	2C	2XP	2EM	2E		
G	70.0	69.5	70.5	70.0	69.5	70.0	67.5	68.5	70.0	69.0	72.0	66.7
G_2	7.9	9.2	8.1	8.3	7.9	7.8	8.4	7.8	7.0	7.8	9.0	8.3
G_3	1.0	0.9	1.0	0.6	1.0	1.0	1.2	1.0	1.0	1.0	0.0	0.0
G_6	9.5	9.8	9.9	10.0	9.7	9.7	8.6	8.7	9.2	9.0	9.0	8.3
G_{23}	3.0	3.0	3.3	3.0	4.1	3.1	3.7	3.6	3.3	3.0	0.0	0.0
G_{26}	5.2	5.2	4.4	4.5	5.0	4.0	5.8	5.4	5.5	5.1	5.0	8.3
G_{36}	1.4	0.9	1.0	1.0	1.7	1.5	1.6	1.4	1.5	1.0	0.0	0.0
G_{236}	5.0	5.3	5.0	4.7	4.7	4.8	8.2	8.0	6.5	8.3	5.0	8.3
D.S.	0.53	0.54	0.51	0.50	0.54	0.50	0.65	0.62	0.57	0.61	0.43	0.58

[a] G = D-glucose; G_2 = 2-O-methyl-D-glucose; etc.

[b] See Table 1

[c] Sides A and B of average rectangular cross-section of crystalline elementary fibrils; side A is assumed to contain the corner units and to give rise to G_{26} and G_{236}, and side B to give G_2 and G_6. For the assumed model $G = 100(AB-2A-2B+4)/AB$; $G_2 = G_6 = 100(B-2)/AB$; $G_{26} = G_{236} = 100/B$; $DS = (5A + 2B - 4)/AB$

[d] D.S. = Degree of substitution.

TABLE 11

Diazomethane Methylation of Scoured Acala Cotton Treated with Cellulase Fractions from T.viride and P.westerdijkii (Dr E.T.Reese). - Distribution of Methoxyl Groups on Completion of the Initial Rapid Reaction, i.e. Partially Methylated Glucose Contents Extrapolated to Zero Reaction Time.

gar [a]	Sugar Content (mole - %)											
	Sample Designation [b]				Calc. for 10 x 16 [c] a.g.u.	Sample		Calc. for 10 x 18 a.g.u.	Sample	Calc. for 10 x 24 a.g.u.	Sample	Calc. for 10 x 12 a.g.u.
	A	E	F	G		C	D		B		H	
G	70	70	70	70	70	71	71	71	73	73	67	67
G_2	8.9	7.7	8.8	8.6	8.8	7.7	9.0	8.9	7.5	9.1	7.8	8.3
G_3	0.9	1.0	0.6	1.0	0	0.7	0.8	0	0.9	0	0.7	0
G_6	8.0	9.0	9.2	9.0	8.8	9.4	8.6	8.9	9.2	9.1	9.3	8.3
G_{23}	3.0	2.6	2.8	2.7	0	2.6	2.6	0	2.6	0	2.4	0
G_{26}	6.8	5.2	5.2	4.9	6.2	5.0	5.0	5.6	4.0	4.2	6.0	8.3
G_{36}	0.9	1.0	0.7	1.0	0	0.9	0.9	0	0.9	0	0.6	0
G_{236}	6.0	6.2	6.0	6.4	6.2	5.6	6.0	5.6	4.4	4.2	9.9	8.3
D.S.[d]	0.50	0.49	0.50	0.50	0.49	0.46	0.47	0.46	0.39	0.39	0.58	0.58

[a] G = D-glucose, G_2 = 2-0-methyl-D-glucose .

[b] See Table 1.

[c] Sides A and B of average rectangular cross-section of crystalline elementary fibrils; side A is assumed to contain the corner units and to give rise to G_{26} and G_{236}, and side B to give G_2 and G_6. For the assumed model $G = 100(AB-2A-2B+4)/AB$; $G_2 = G_6 = 100(B-2)/AB$; $G_{26} = G_{236} = 100/B$; $DS = (5A+2B-4)/AB$

[d] D.S. = Degree of substitution

TABLE 12

Diazomethane Methylation of Scoured Acala Cotton Treated with Cellulase Fractions from T.koningii (Dr T.M.Wood). - Distribution of Methoxyl Groups on Completion of the initial Rapid Reaction, i.e. Partially Methylated Glucose Contents Extrapolated to Zero Reaction Time.

Sugar [a]	Sugar Content (mole-%)					
	Sample [b]			Calc. for 10 x 16 [c] a. g. u.	Sample	Calc. for 10 x 10 a. g. u.
	C_0	C_1	C_X		$C_1 + X$	
G	70	70	70	70	64	64
G_2	8.7	8.8	8.6	8.8	8.2	8.0
G_3	1.0	0.8	1.0	0	0.9	0
G_6	8.9	9.0	9.0	8.8	8.0	8.0
G_{23}	2.8	2.5	2.4	0	3.2	0
G_{26}	6.0	5.6	5.2	6.2	9.8	10.0
G_{36}	0.9	1.0	1.0	0	0.6	0
G_{236}	6.8	6.4	7.2	6.2	9.7	10.0
D.S. [d]	0.49	0.49	0.49	0.49	0.66	0.66

See Table11for footnotes.

TABLE 13

DENSITY OF ENZYME - TREATED ACALA COTTON

Sample Designation [a]	Fibre Density (g/cm^3)	
	Knotted fibres	Fibre pellet
A	1.544	1.543
B		1.543
C	1.546	1.543
D	1.546	1.541
E	1.546	1.543
F	1.547	1.545
G	1.545	1.548
H		1.545
C_o		1.549
C_x		1.549
C_1		1.549
C_{1+x}		1.550

[a] See Table 1

Fig. 1

Influence of Enzyme Treatments on Accessibilities to Water Vapour of 'Never-Dried' Samples and after Drying over P_2O_5.

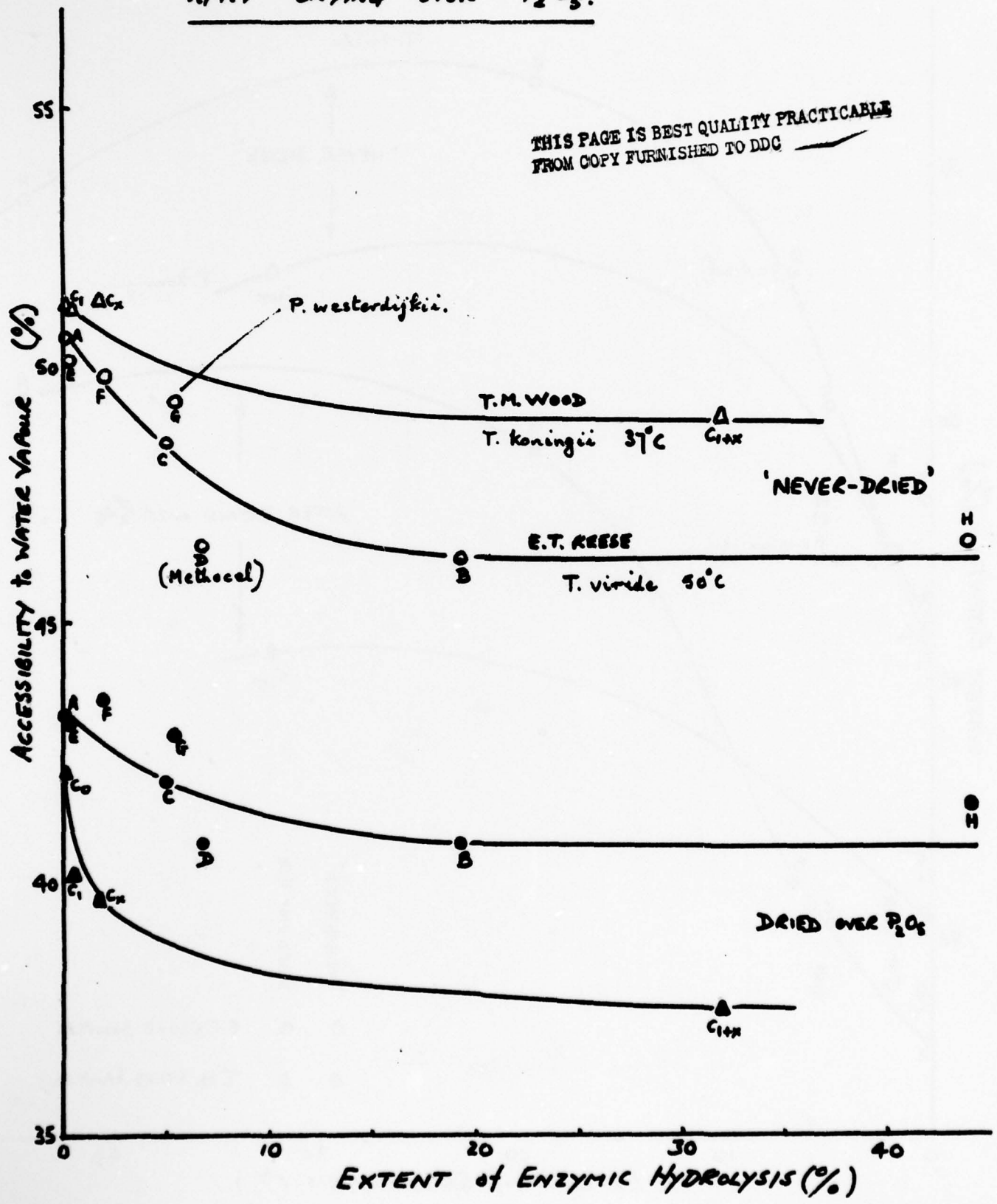
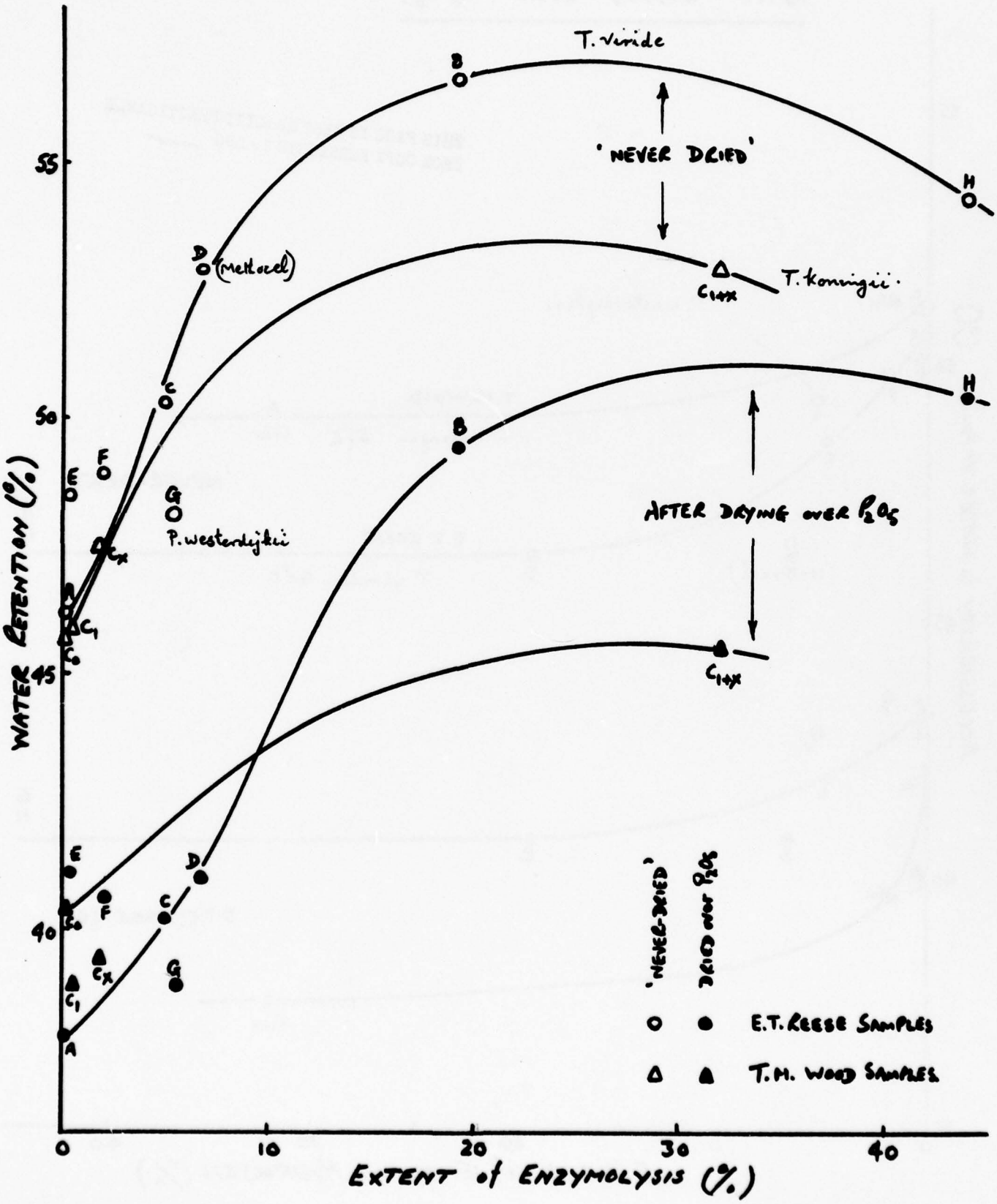
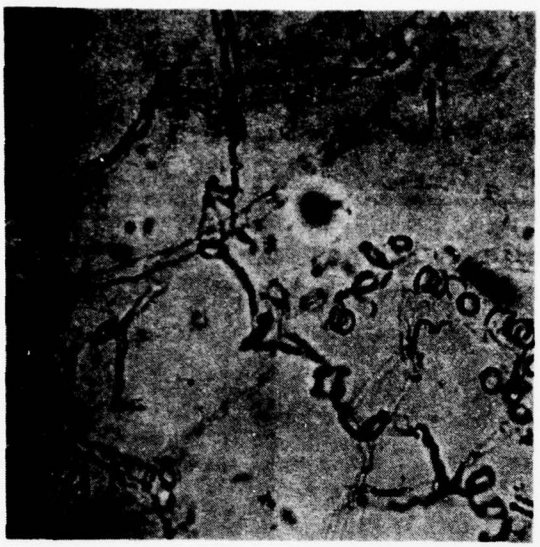


Fig 2

INFLUENCE of ENZYME TREATMENTS on WATER RETENTION



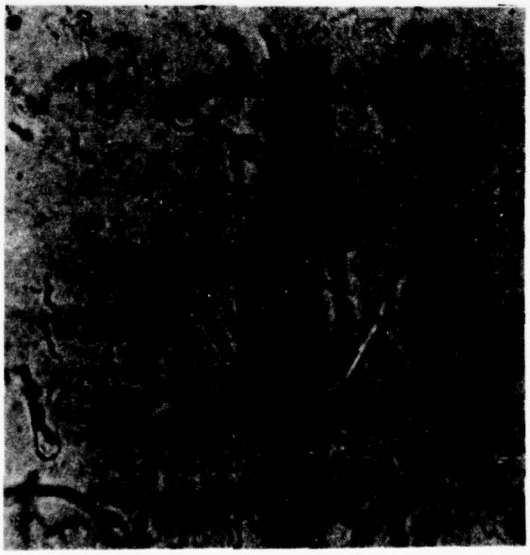


Original 'Unversity' plugging cotton

Sample 2C-F

Sample 2X-F

Sample 2EM-F

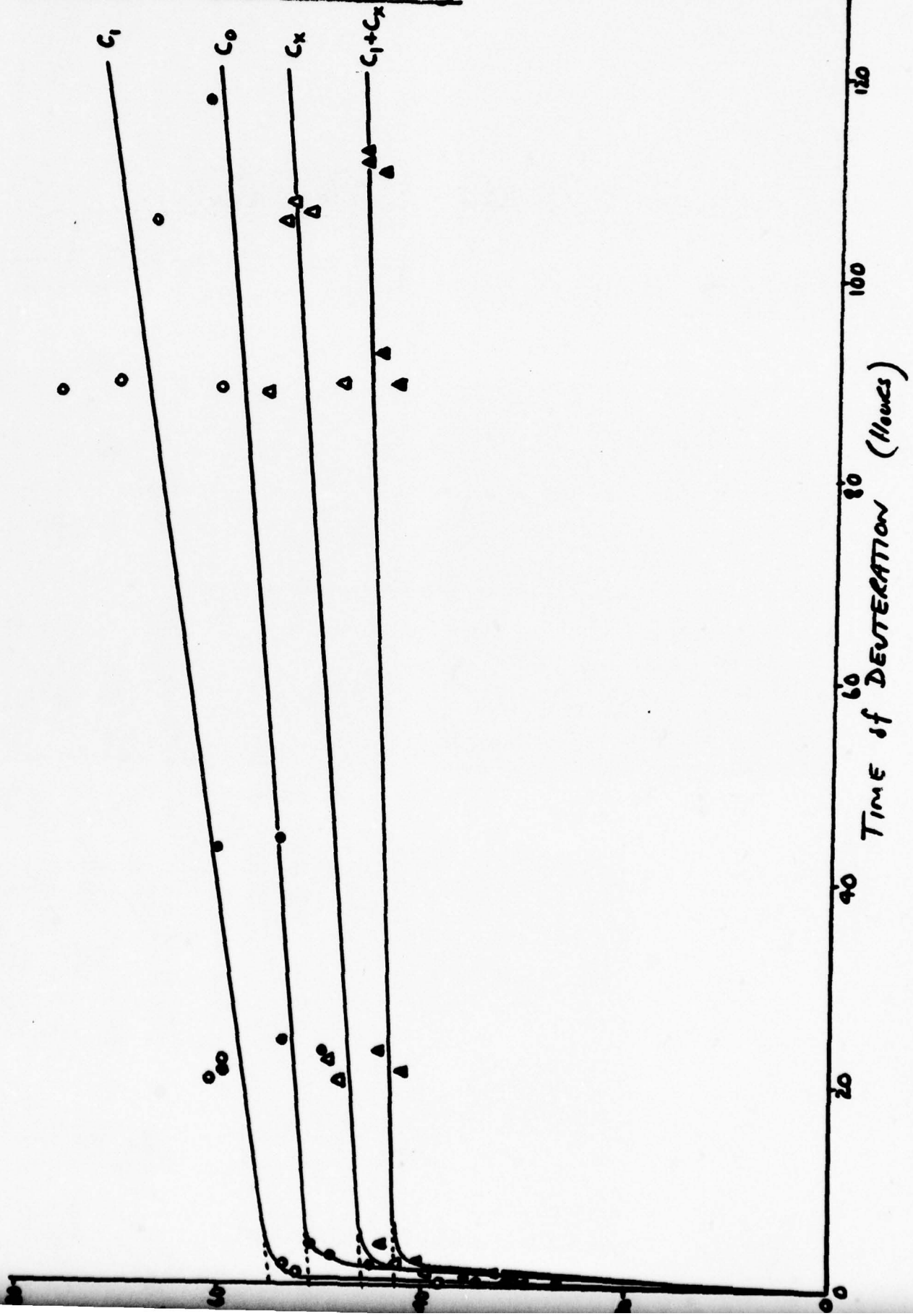


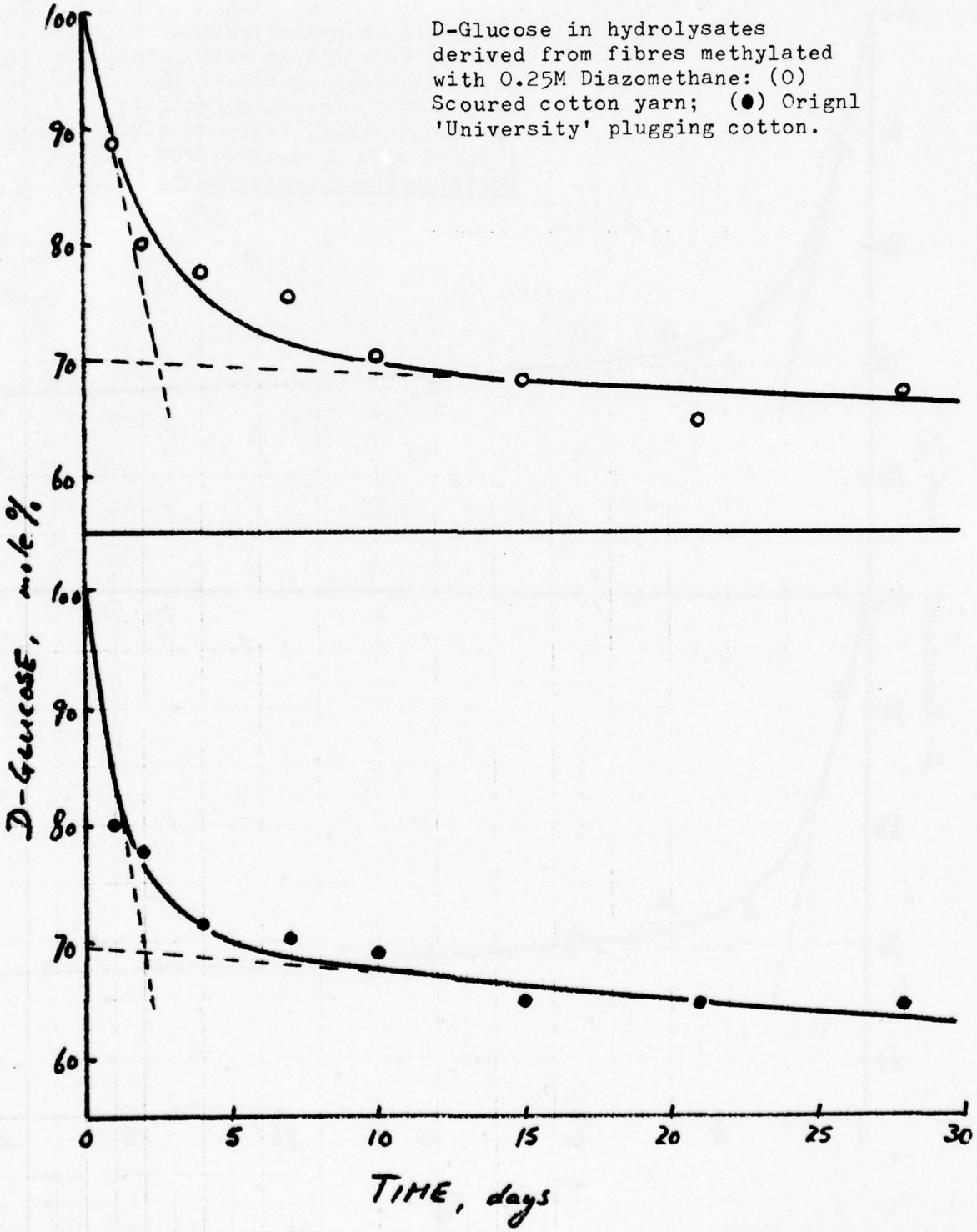
Sample 2E-F

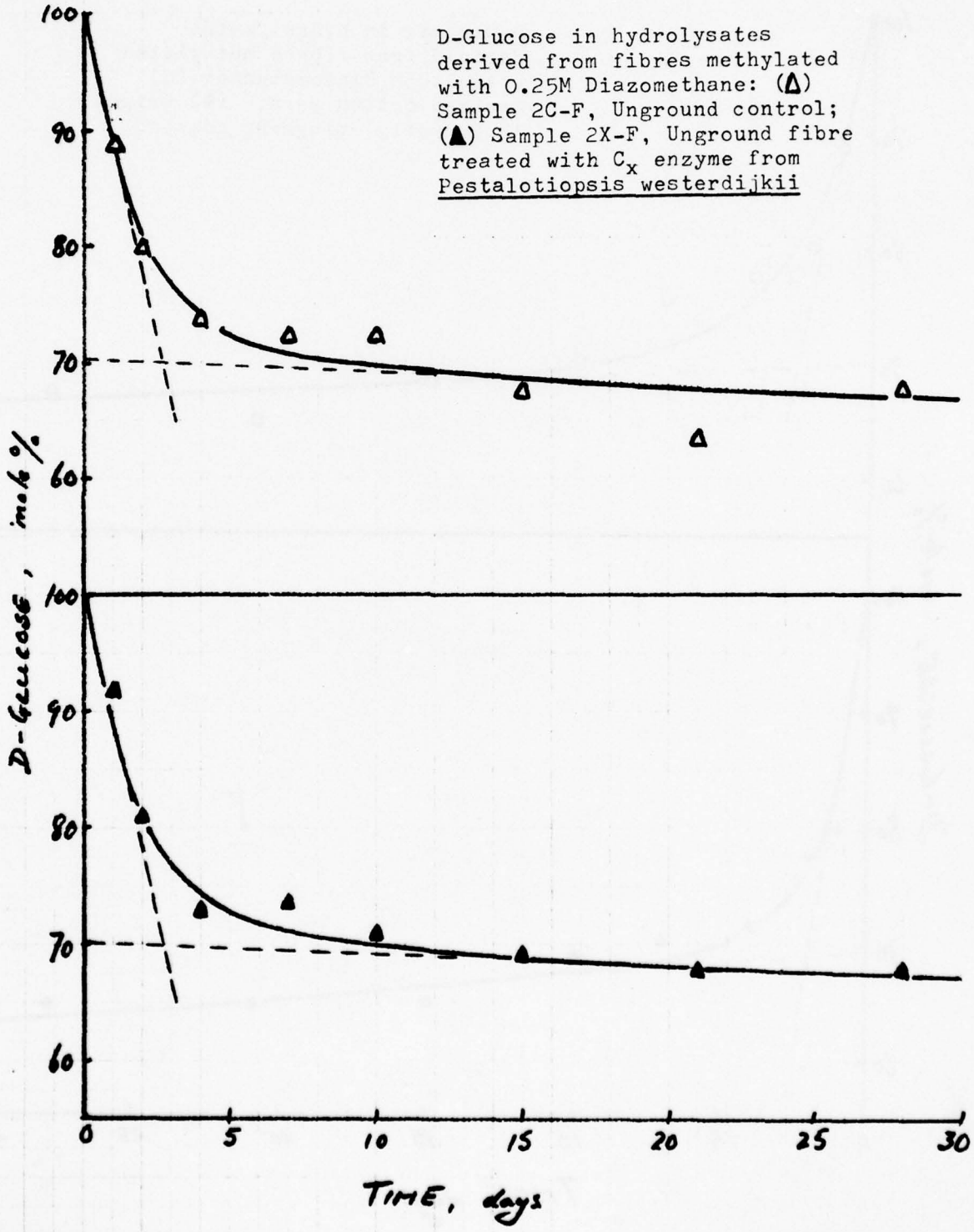
Photomicrographs of the residual material (cuticle) from the control and enzyme treated 'University' plugged cotton fibres after dissolving the cellulose in cuprammonium hydroxide.

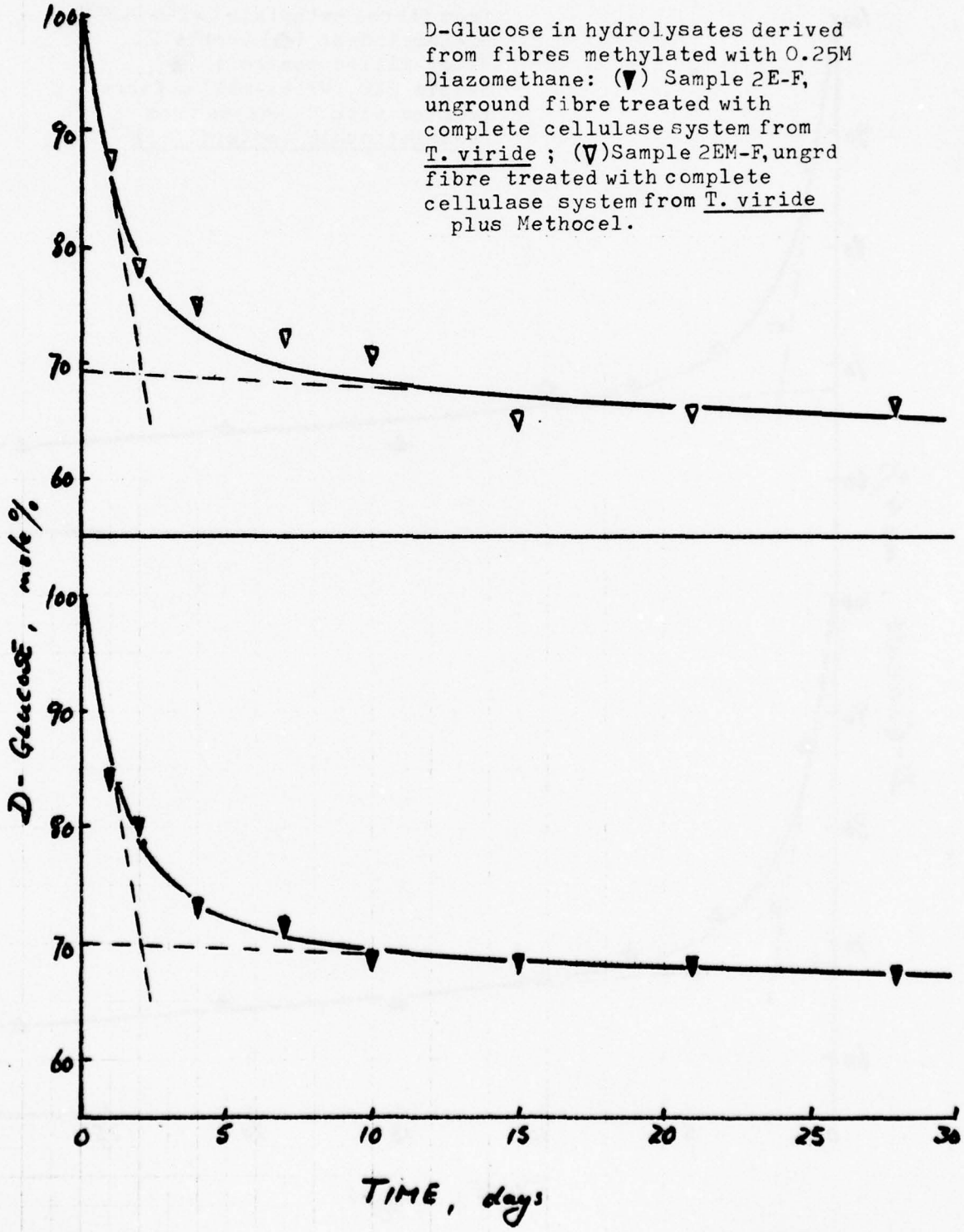
Fig. 4

INFRARED - DEUTERATION of COTTON SAMPLES TREATED with CELLULASE FRACTIONS from T. KONINGII (DR. T.M. WOOD)



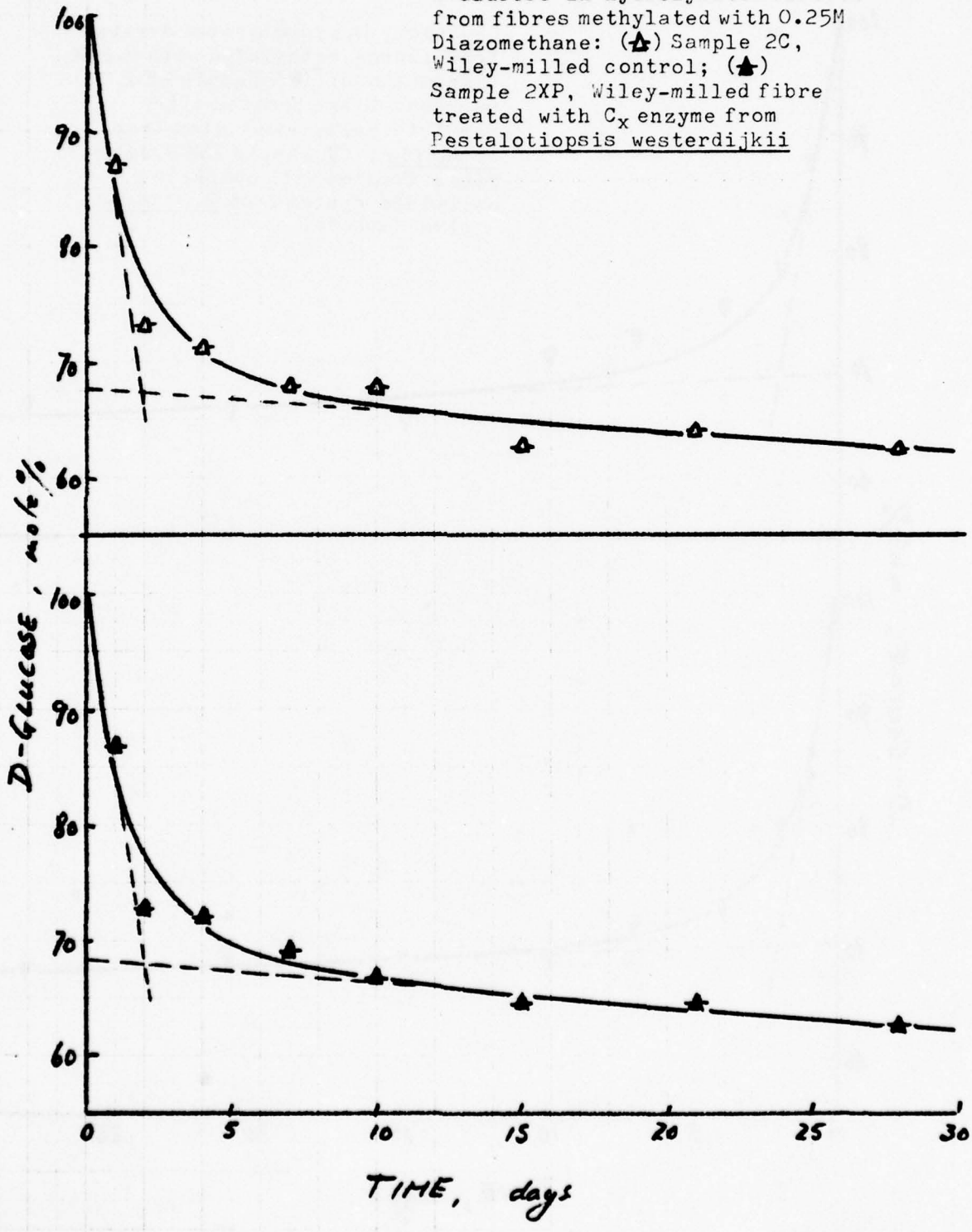

D-Glucose in hydrolysates derived from fibres methylated with 0.25M Diazomethane: (O) Scoured cotton yarn; (●) Orignl 'University' plugging cotton.
D-Glucose, mole %
100
90
80
70
60
0
5
10
15
20
25
30
TIME, days


D-Glucose in hydrolysates derived from fibres methylated with 0.25M Diazomethane: (Δ) Sample 2C-F, Unground control; (▲) Sample 2X-F, Unground fibre treated with C_x enzyme from Pestalotiopsis westerdijkii
100
90
80
70
60
D-Glucose, mol %
100
90
80
70
60
0
5
10
15
20
25
30
TIME, days

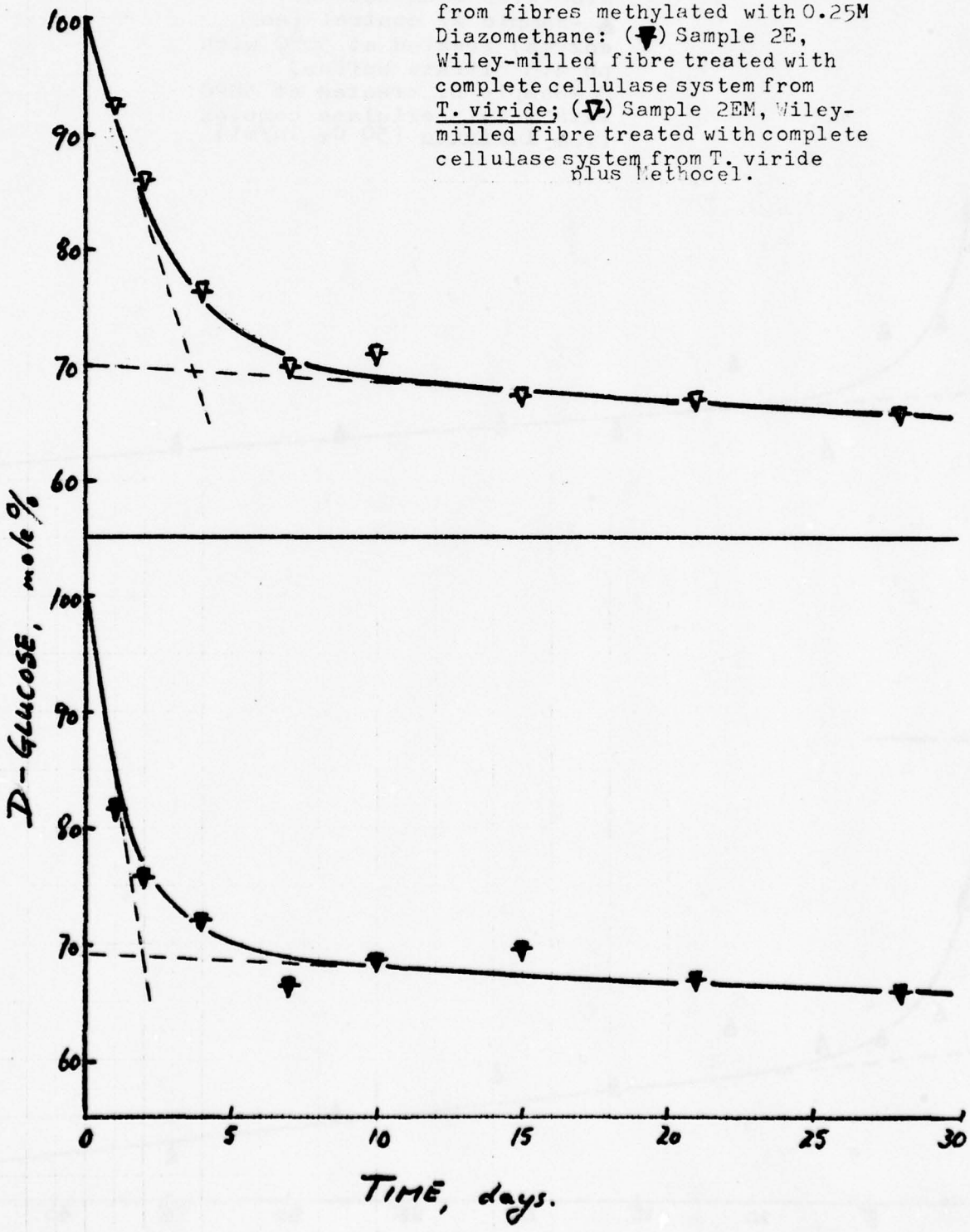


D-Glucose in hydrolysates derived from fibres methylated with 0.25M Diazomethane: (▼) Sample 2E-F, unground fibre treated with complete cellulase system from T. viride ; (▽)Sample 2EM-F, ungrd fibre treated with complete cellulase system from T. viride plus Methocel.

D-Glucose in hydrolysates derived from fibres methylated with 0.25M Diazomethane: (△) Sample 2C, Wiley-milled control; (▲) Sample 2XP, Wiley-milled fibre treated with C_x enzyme from Pestalotiopsis westerdijkii



D-Glucose in hydrolysates derived from fibres methylated with 0.25M Diazomethane: (▼) Sample 2E, Wiley-milled fibre treated with complete cellulase system from T. viride; (▽) Sample 2EM, Wiley-milled fibre treated with complete cellulase system from T. viride plus Methocel.



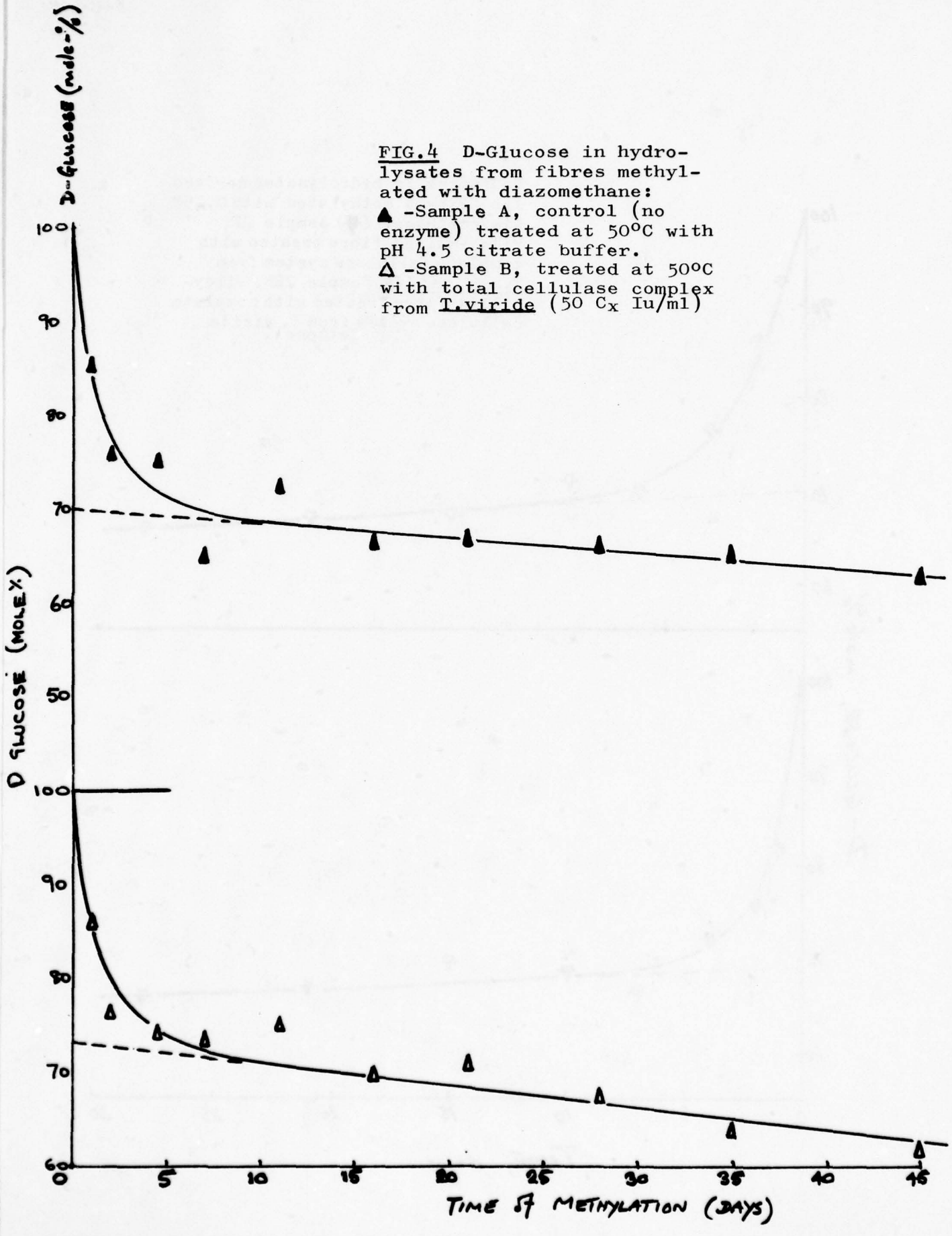


FIG.4 D-Glucose in hydrolysates from fibres methylated with diazomethane:
▲ -Sample A, control (no enzyme) treated at 50°C with pH 4.5 citrate buffer.
Δ -Sample B, treated at 50°C with total cellulase complex from T.viride (50 C_x Iu/ml)

FIG. 5 D-Glucose in hydrolysates from fibres methylated with diazomethane:-
★-Sample C, treated at 50°C with total cellulase complex from T.viride (12.5 C_x Iu/ml)
▲-Sample D, treated at 50°C with total cellulase complex from T.viride (50 C_x Iu/ml) plus Methocel (0.3 mg/ml)

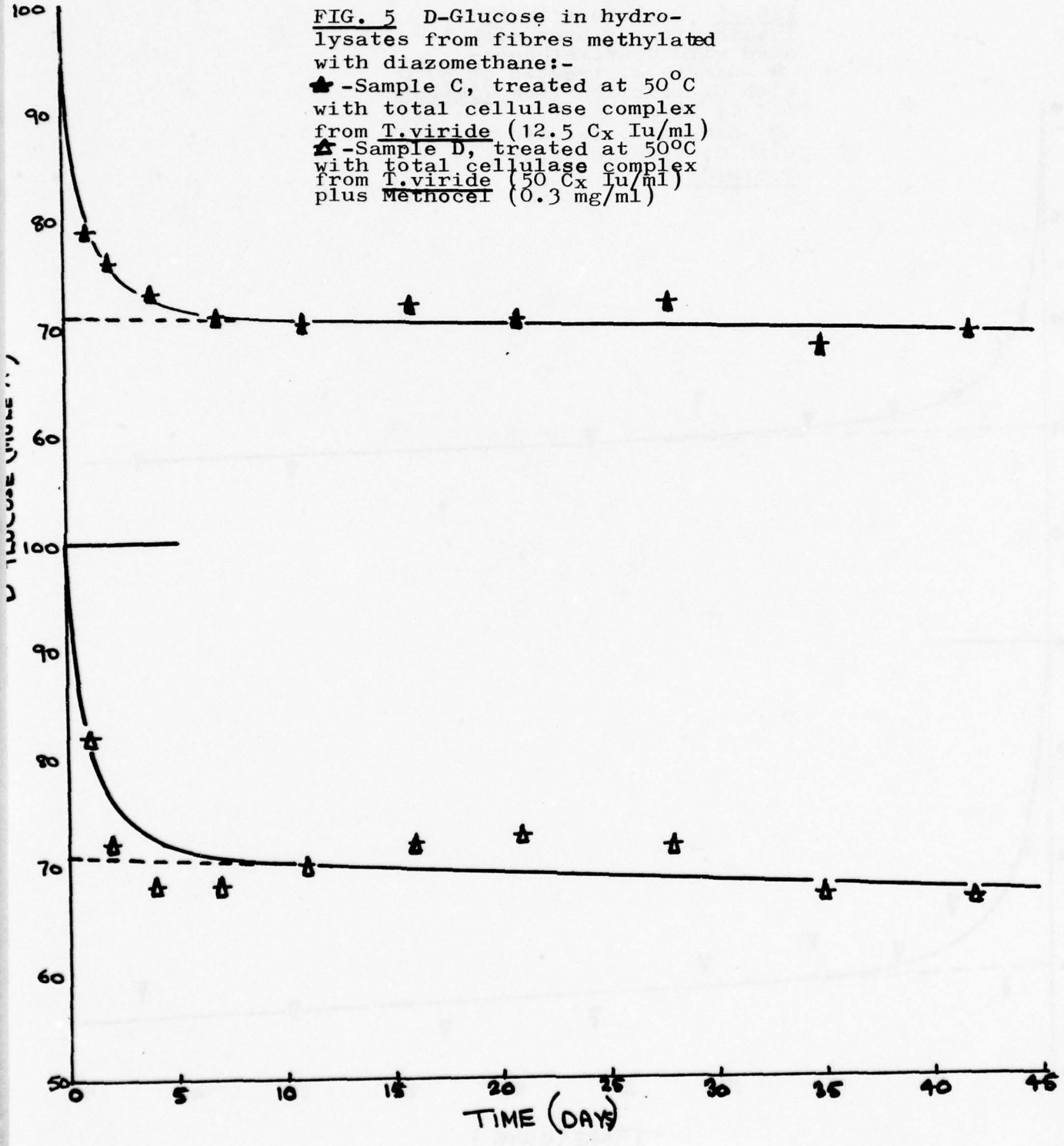


FIG. 6 D-glucose in hydrolysates from fibres methylated with diazomethane:-
▼ -Sample E, treated at 50°C with C_x fraction from T.viride (15 C_x Iu/ml)
▽ -Sample F, treated at 50°C with C_1 fraction from T.viride

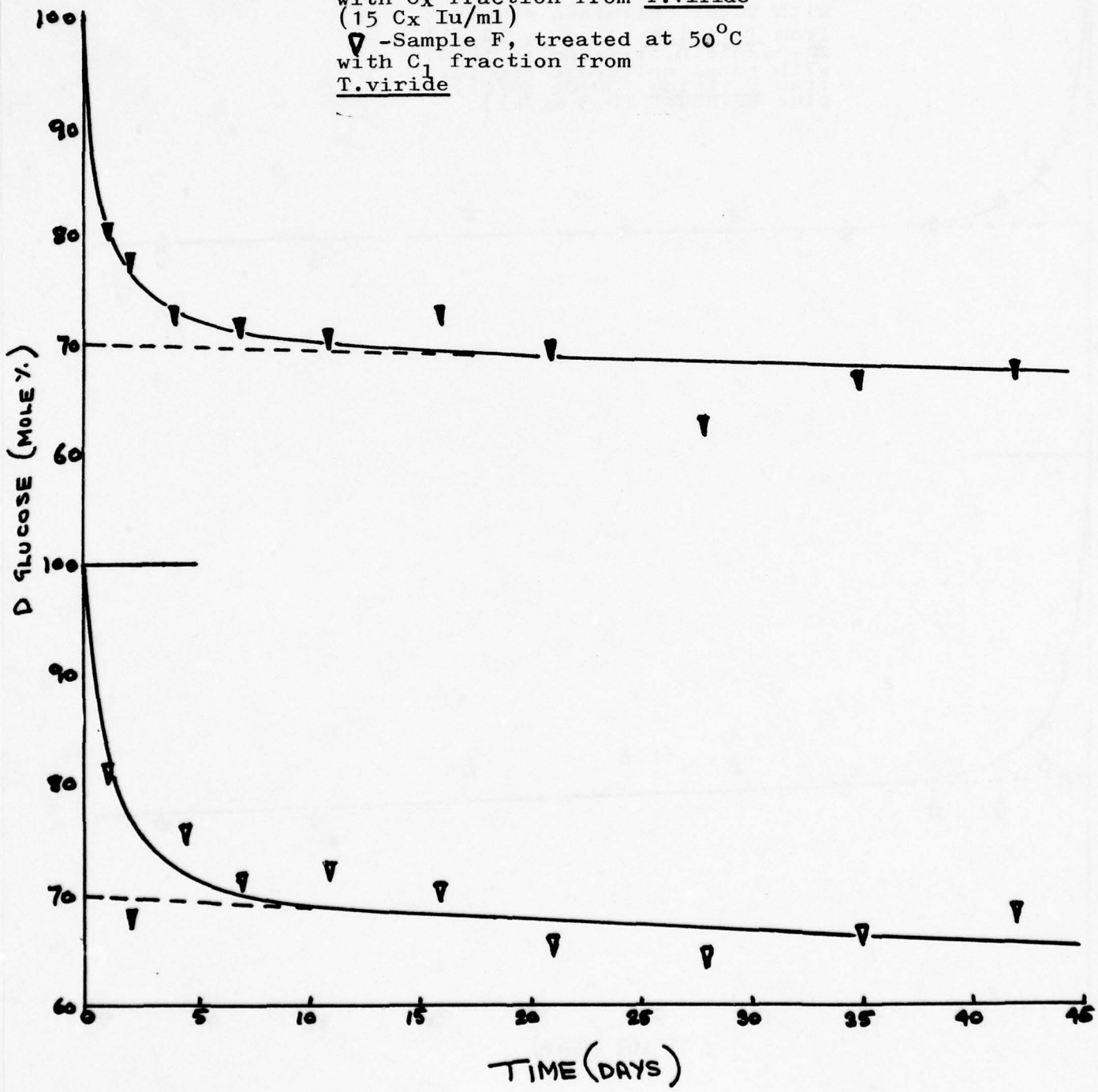


FIG. 7 D-glucose in hydrolysates from fibres methylated with diazomethane:-
▼ -Sample G, treated at 50°C with total cellulase complex from P.westerdijkii (84 C_x Iu/ml, No. C_1)
▽ -Sample H, treated as for sample B but unshaken during incubation.

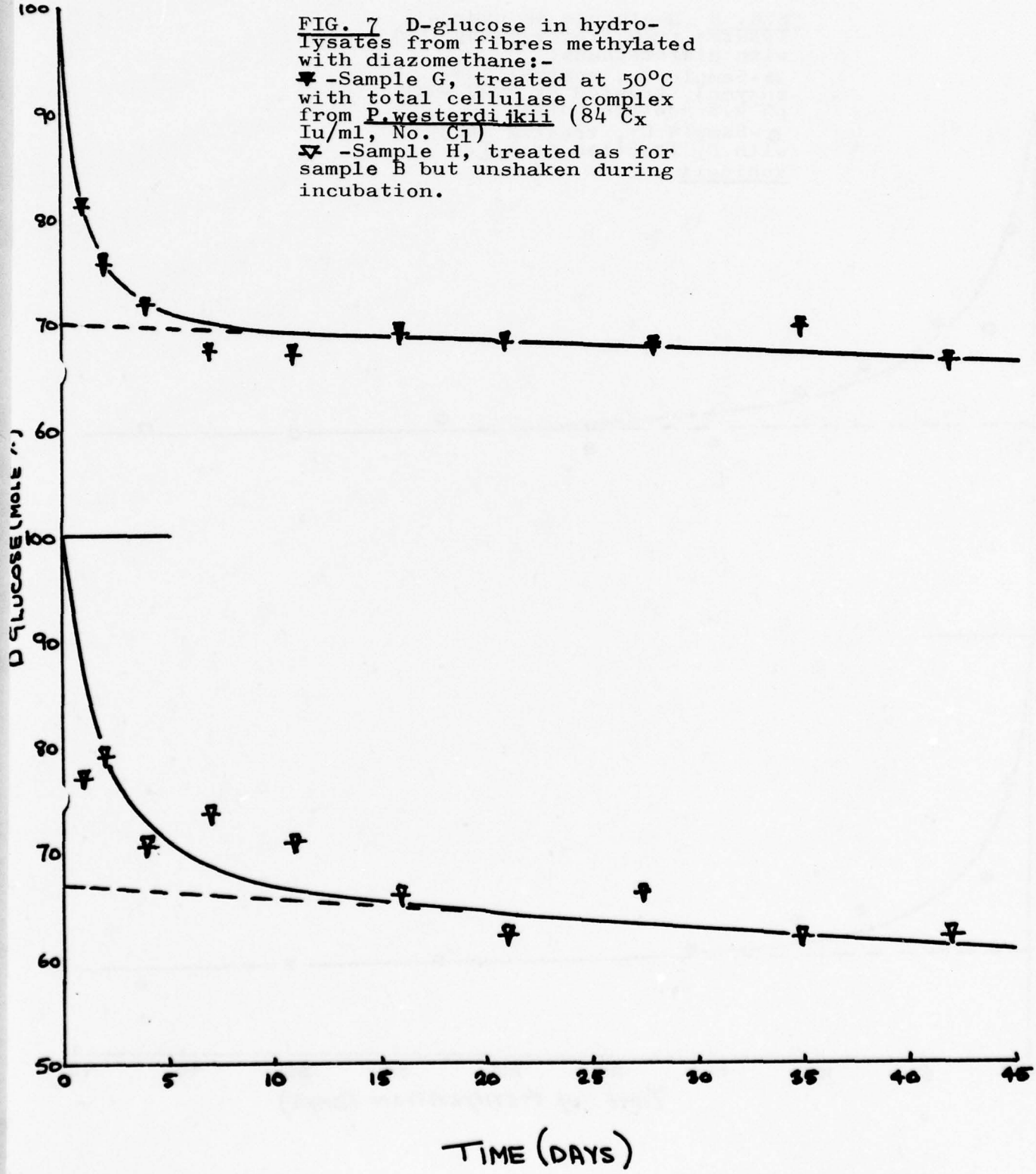


FIG. 8 D-glucose in hydrolysates from fibres methylated with diazomethane:-

●-Sample C_0, control (no enzyme), treated at 37°C with pH 4.8 acetate buffer

⊙-Sample C_1, treated at 37°C with C_1 fraction from *T. koningii*

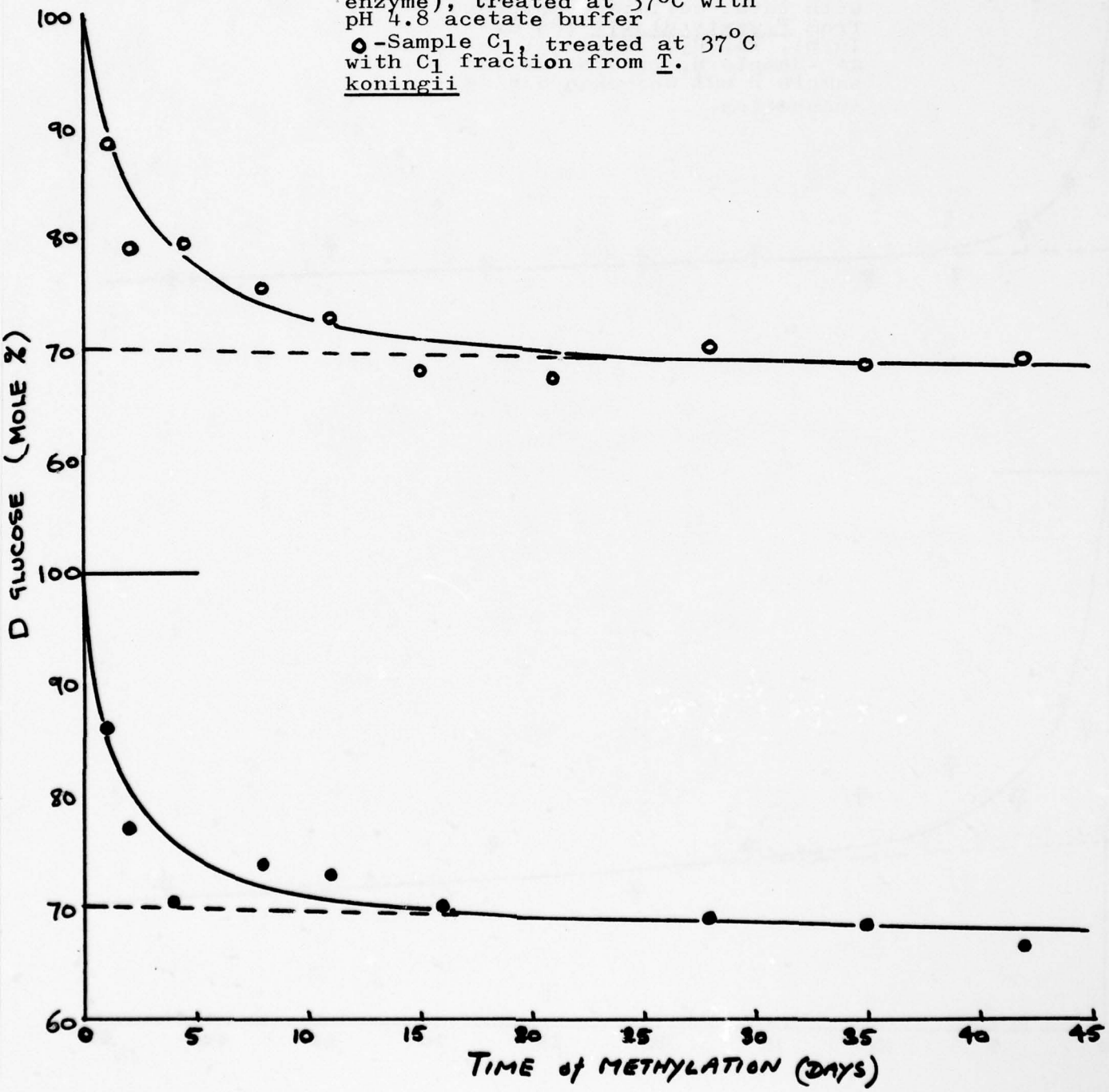


FIG. 9 D-glucose in hydrolysates from fibres methylated with diazomethane:-
-o- -Sample C_x, treated at 37°C with C_x fraction from T.koningii
-●- -Sample C_{1+x}, treated at 37°C with mixture of C1 and C_x fractions from T.koningii

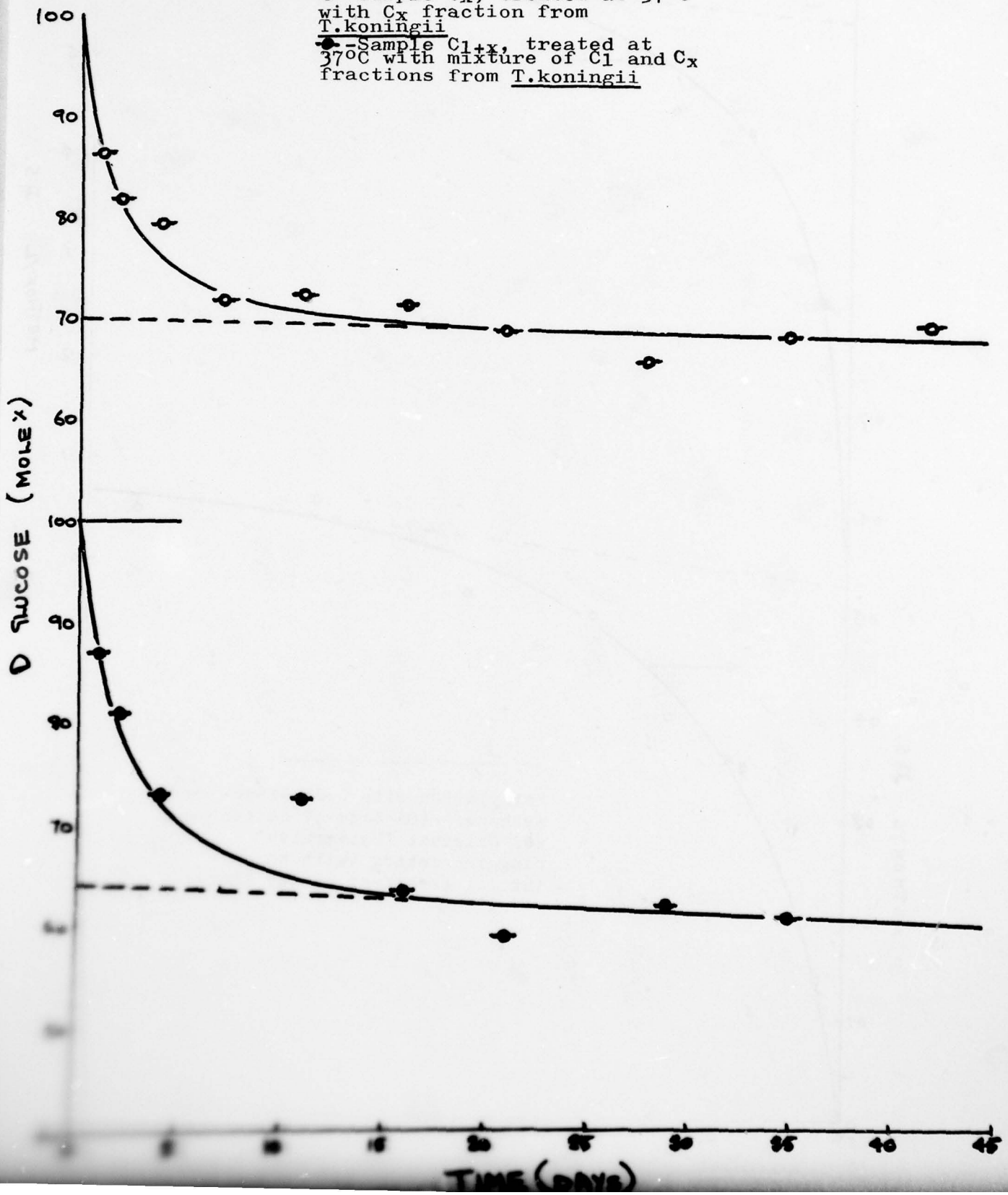
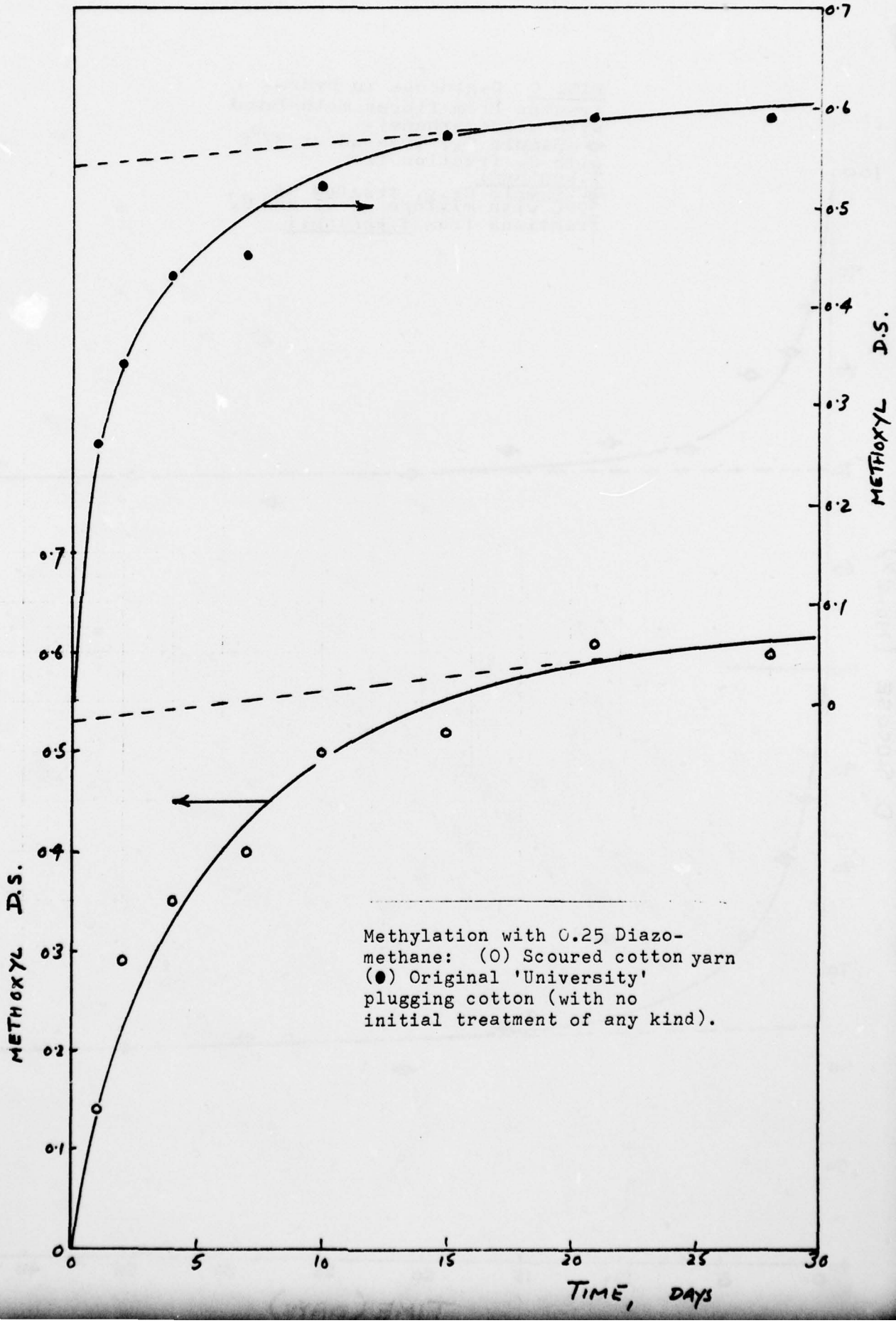
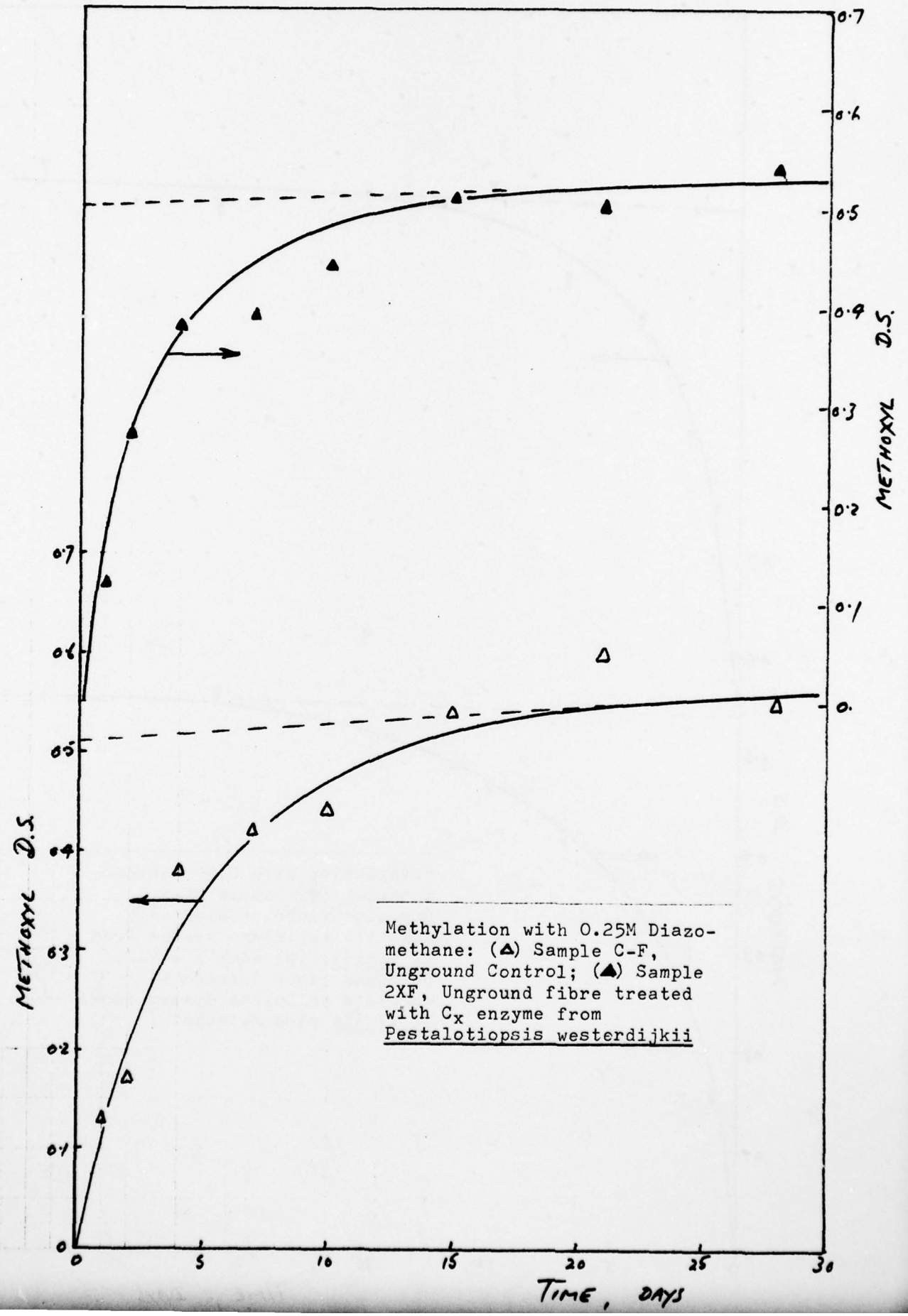


Fig. 16



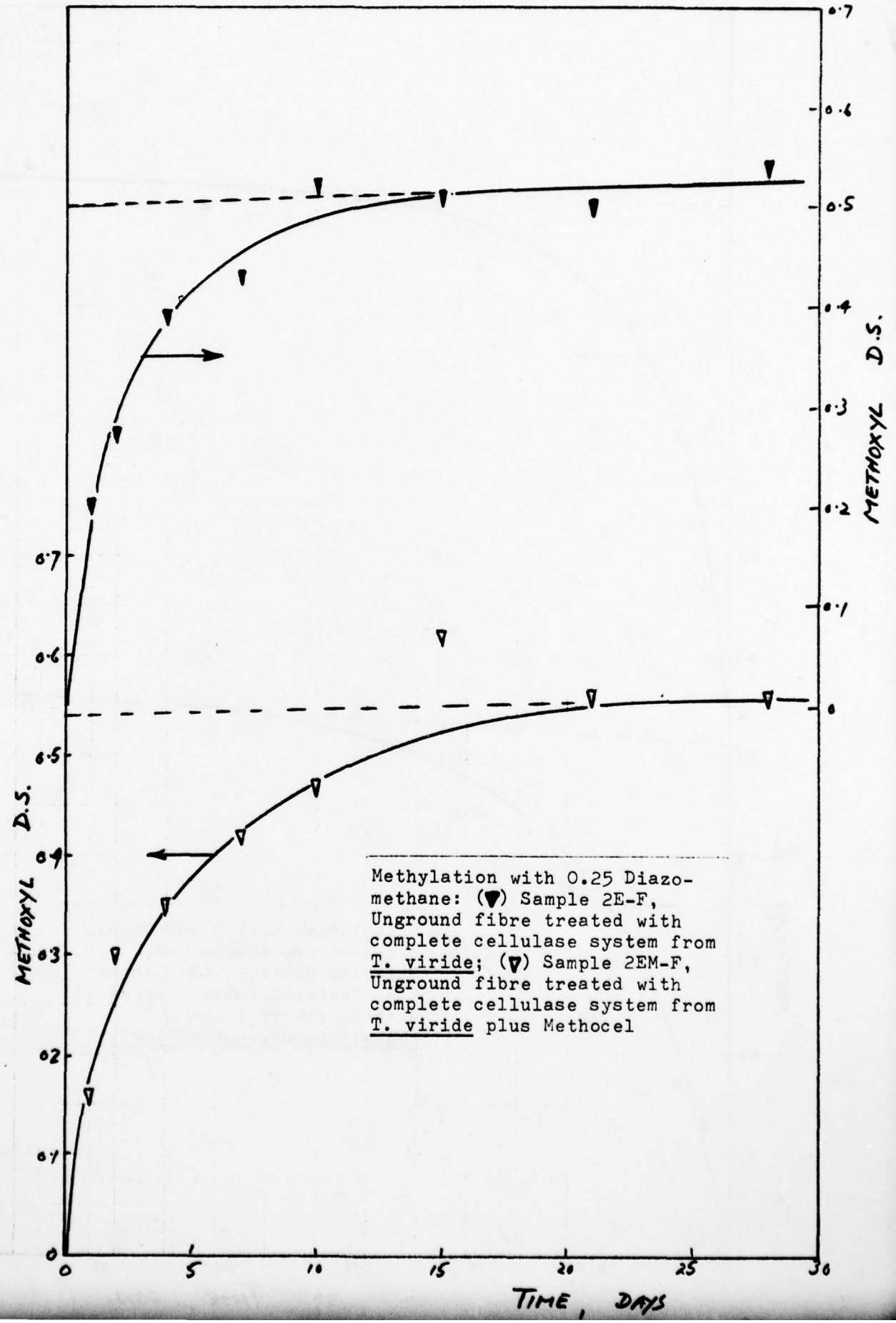
Methylation with 0.25 Diazomethane: (O) Scoured cotton yarn (●) Original 'University' plugging cotton (with no initial treatment of any kind).

Fig. 17



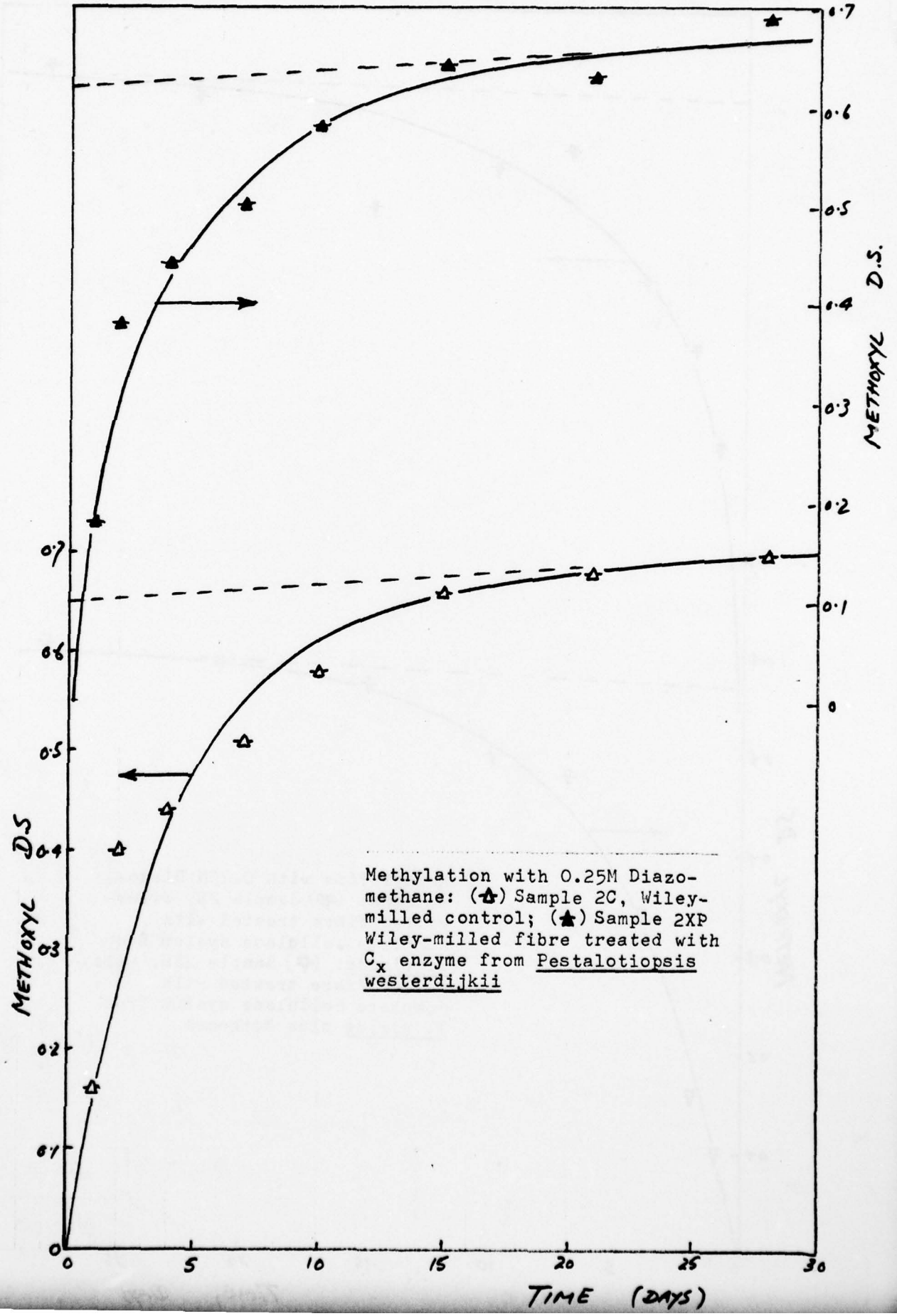
Methylation with 0.25M Diazomethane: (△) Sample C-F, Unground Control; (▲) Sample 2XF, Unground fibre treated with C_x enzyme from Pestalotiopsis westerdijkii

Fig. 18



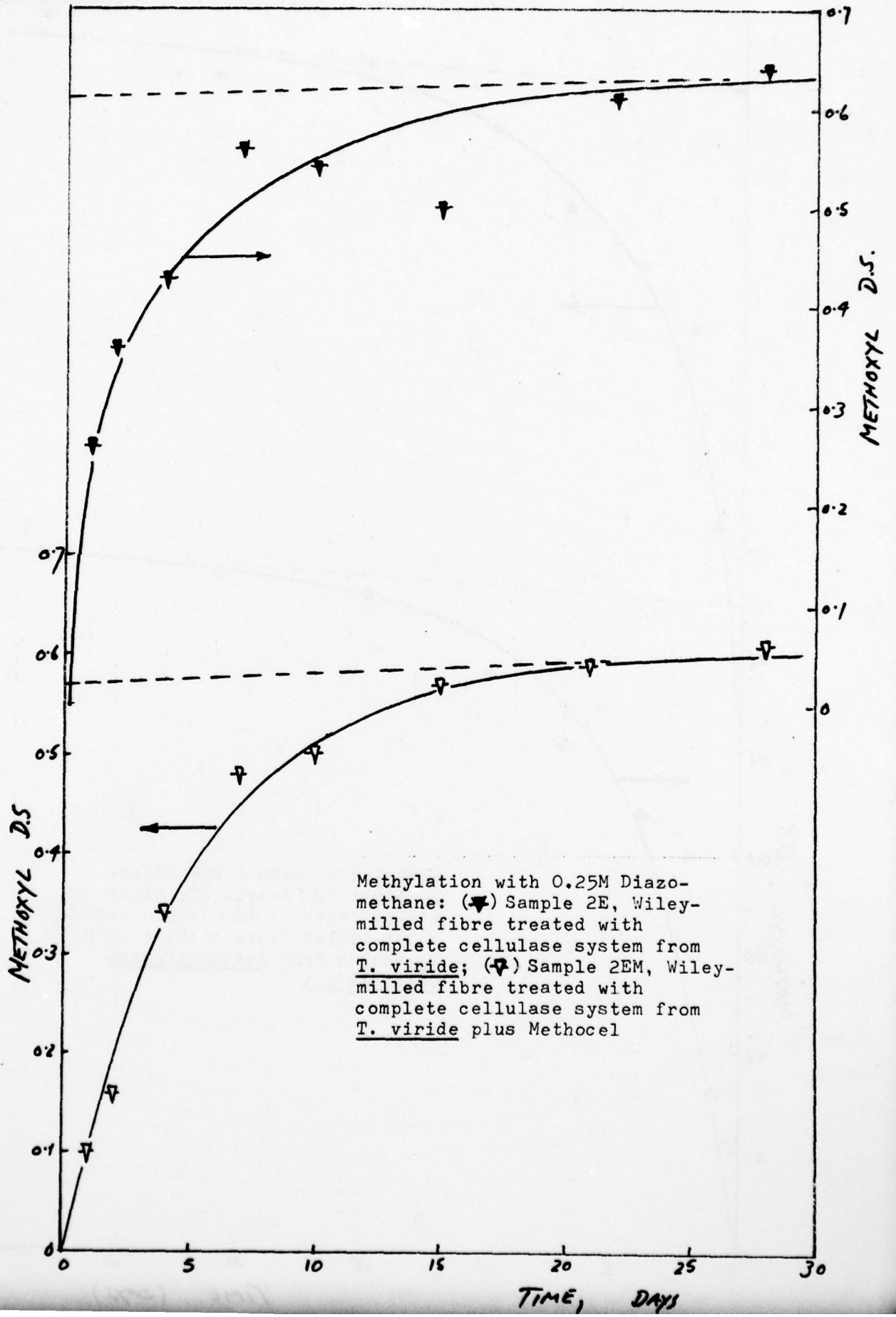
Methylation with 0.25 Diazomethane: (▼) Sample 2E-F, Unground fibre treated with complete cellulase system from T. viride; (▽) Sample 2EM-F, Unground fibre treated with complete cellulase system from T. viride plus Methocel

Fig. 19



Methylation with 0.25M Diazomethane: (☆) Sample 2C, Wiley-milled control; (★) Sample 2XP Wiley-milled fibre treated with C_x enzyme from Pestalotiopsis westerdijkii

Fig. 20



Methylation with 0.25M Diazomethane: (▼) Sample 2E, Wiley-milled fibre treated with complete cellulase system from T. viride; (▽) Sample 2EM, Wiley-milled fibre treated with complete cellulase system from T. viride plus Methocel

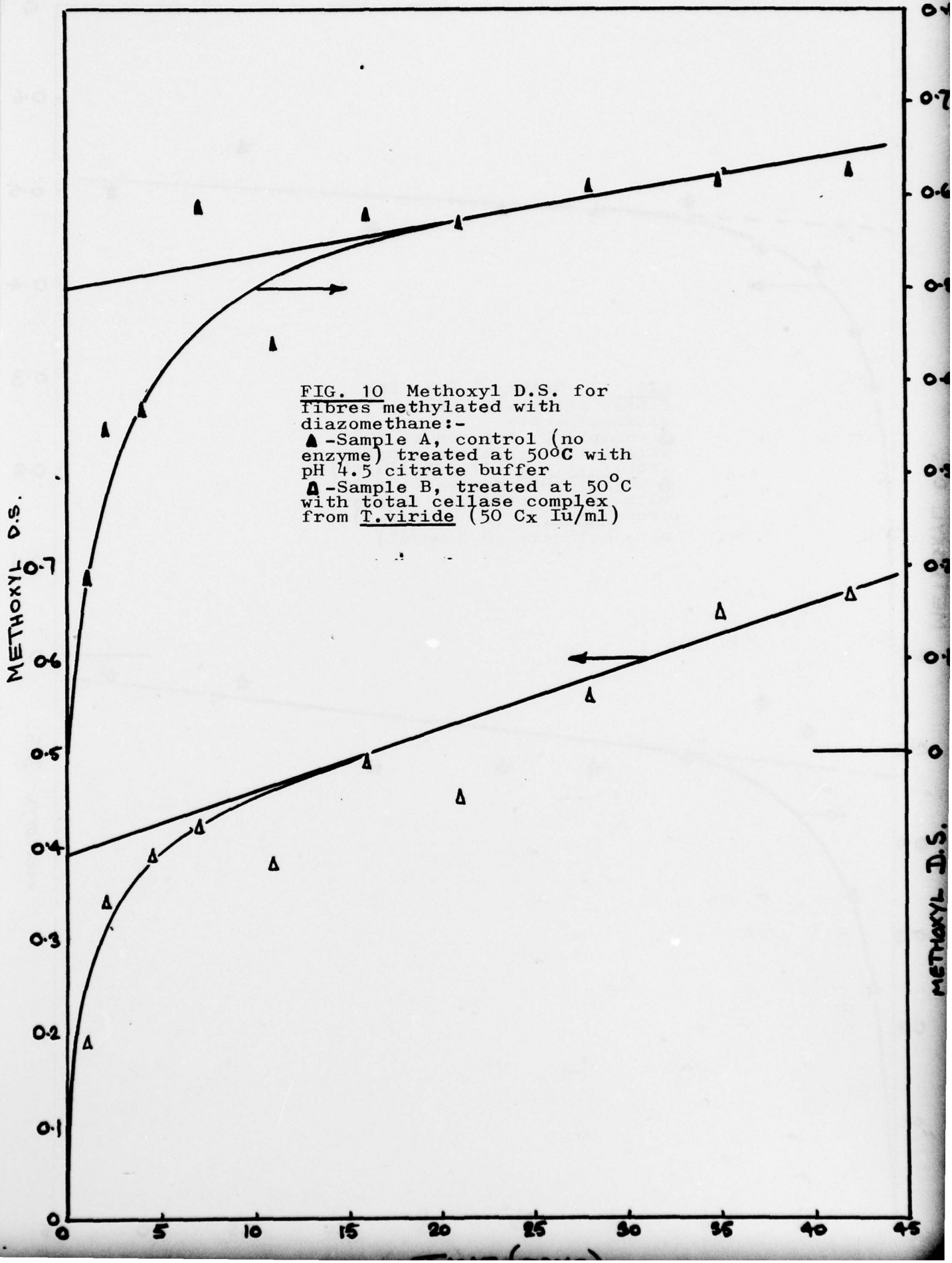


FIG. 10 Methoxyl D.S. for fibres methylated with diazomethane:-
▲-Sample A, control (no enzyme) treated at 50°C with pH 4.5 citrate buffer
Δ-Sample B, treated at 50°C with total cellase complex from T.viride (50 Cx Iu/ml)

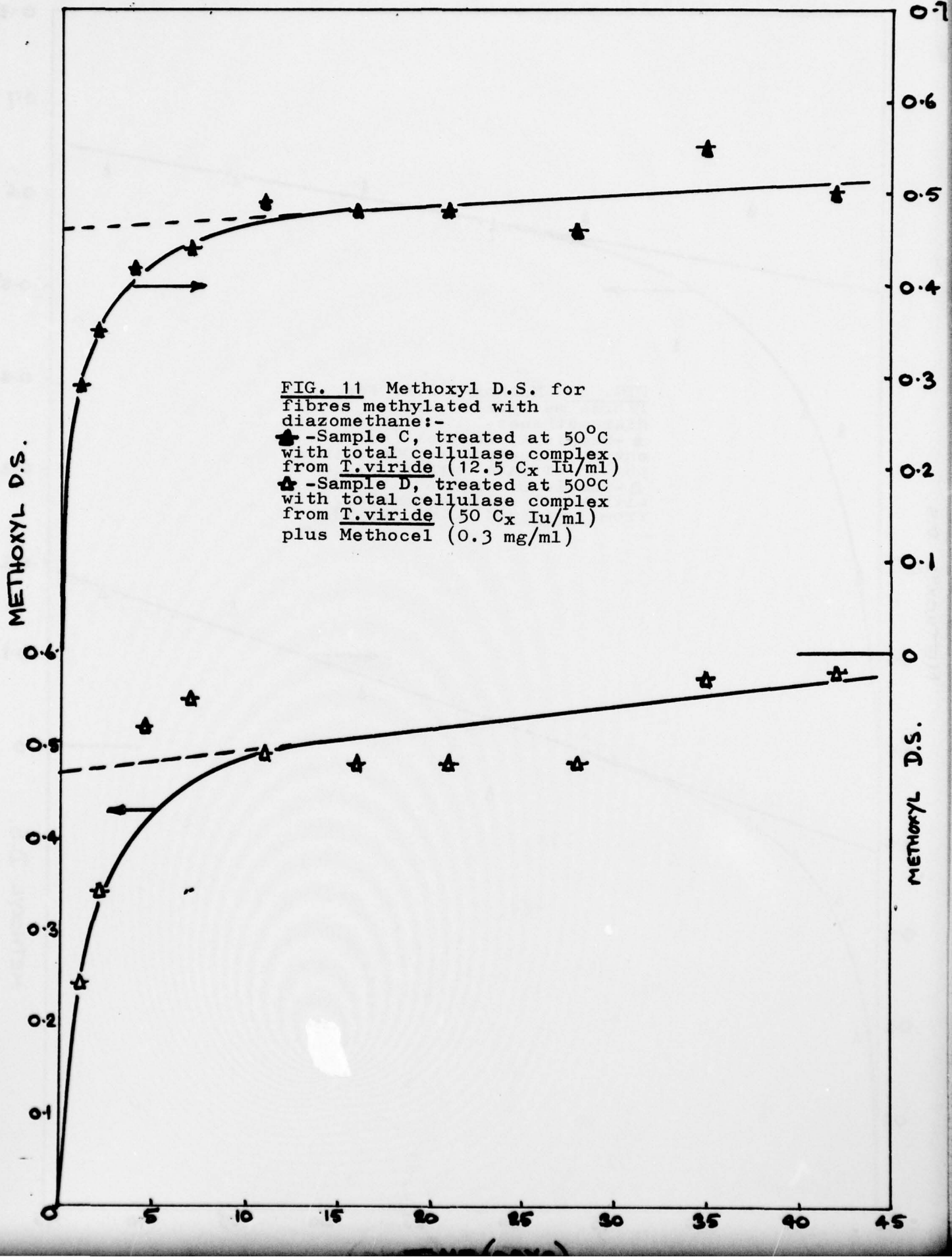


FIG. 11 Methoxyl D.S. for fibres methylated with diazomethane:-
-Sample C, treated at 50°C with total cellulase complex from T.viride (12.5 C_x Iu/ml)
-Sample D, treated at 50°C with total cellulase complex from T.viride (50 C_x Iu/ml) plus Methocel (0.3 mg/ml)

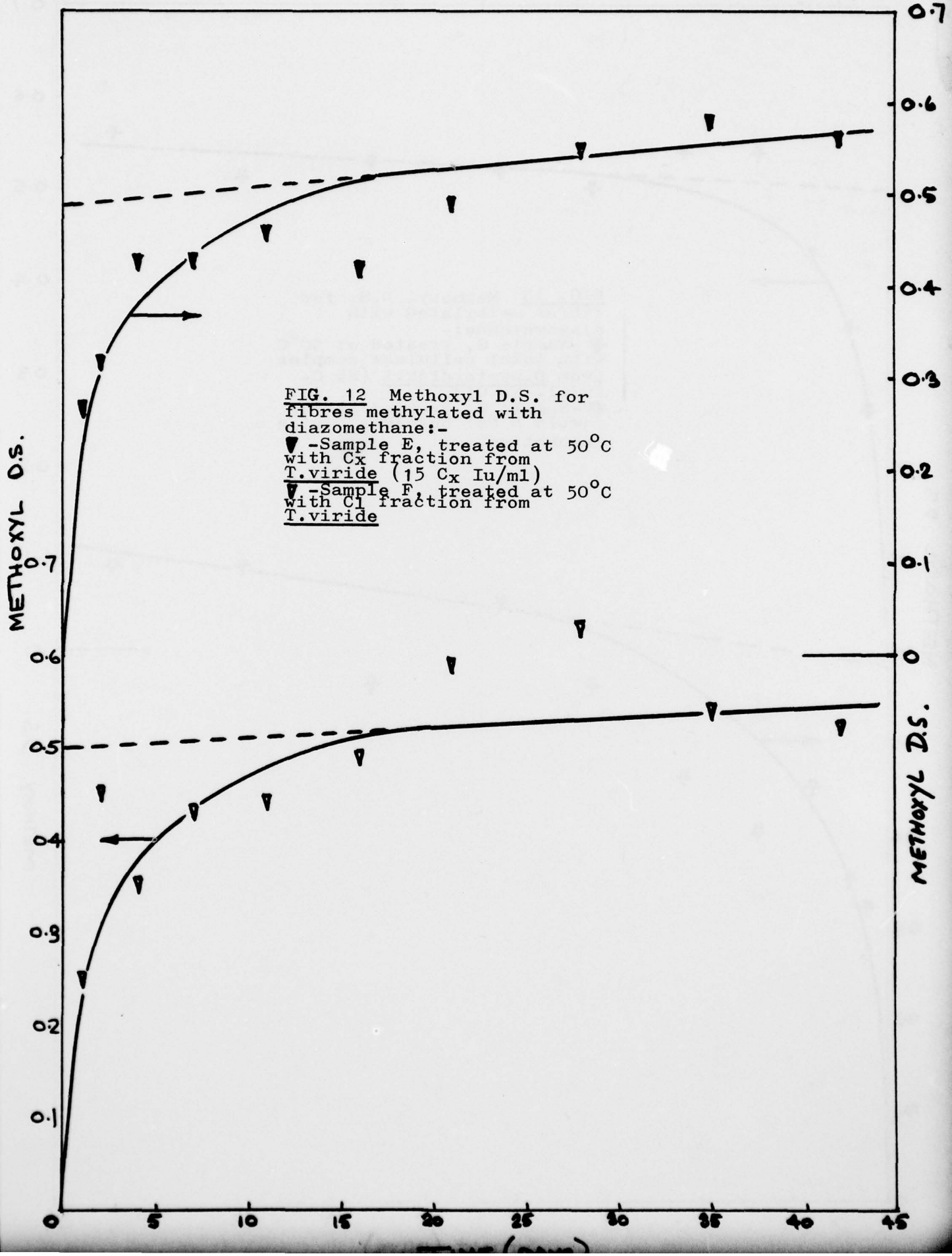


FIG. 12 Methoxyl D.S. for fibres methylated with diazomethane:-
▼-Sample E, treated at 50°C with Cx fraction from T.viride (15 Cx Iu/ml)
▼-Sample F, treated at 50°C with C1 fraction from T.viride

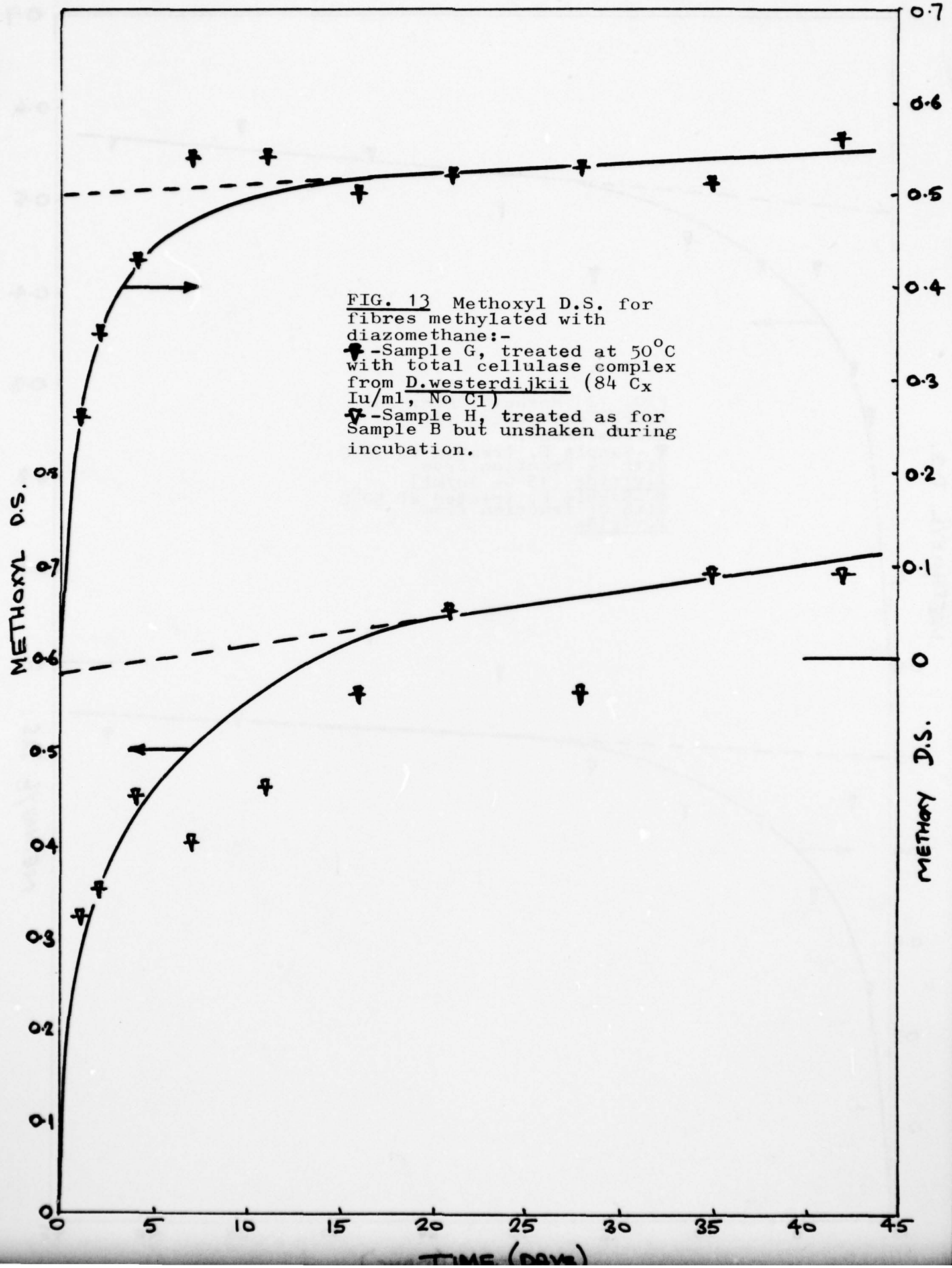


FIG. 13 Methoxyl D.S. for fibres methylated with diazomethane:-
-Sample G, treated at 50°C with total cellulase complex from D.westerdijkii (84 C_x Iu/ml, No C1)
-Sample H, treated as for Sample B but unshaken during incubation.

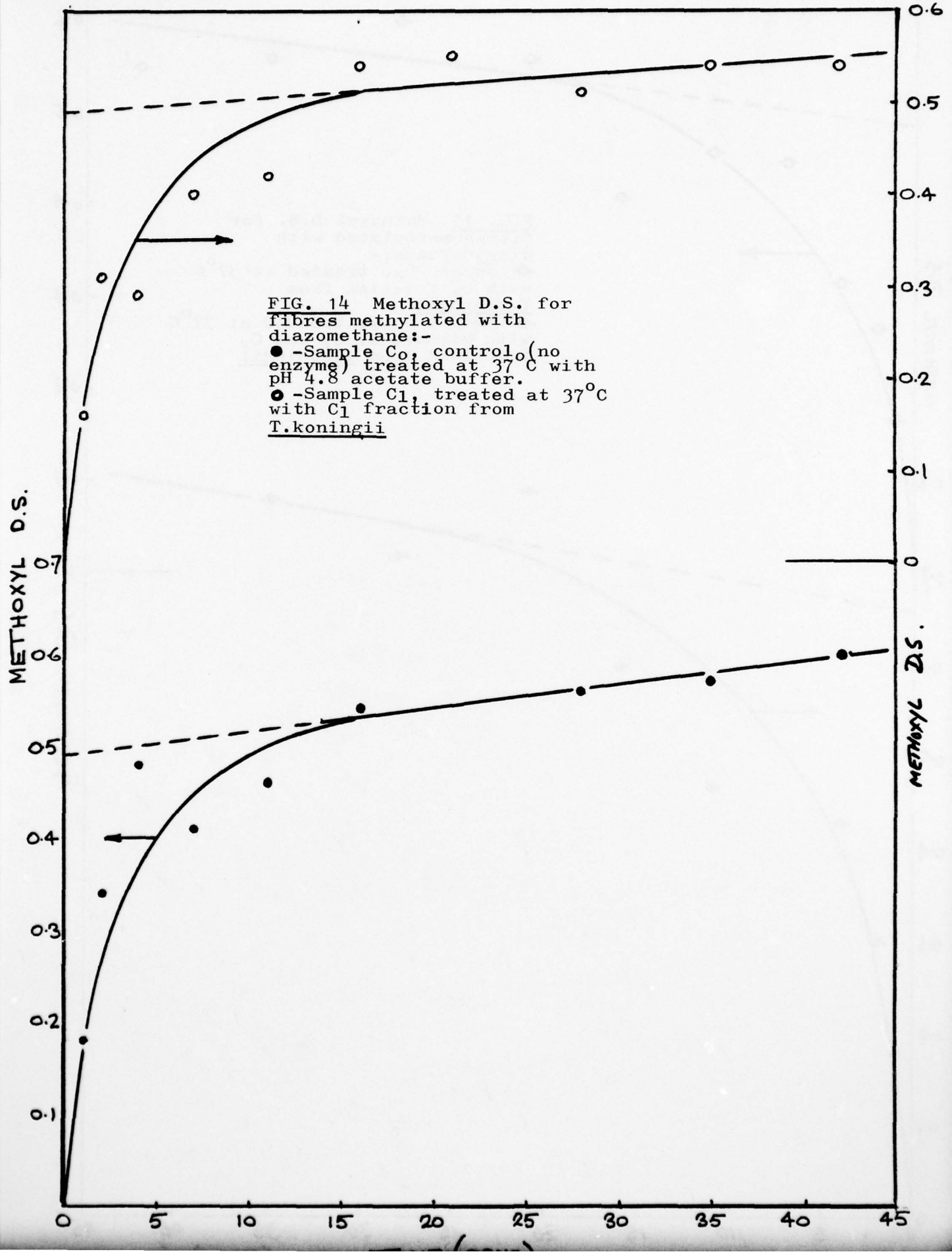


FIG. 14 Methoxyl D.S. for fibres methylated with diazomethane:-
● -Sample C_o, control$_o$ (no enzyme) treated at 37°C with pH 4.8 acetate buffer.
⊙ -Sample C_1, treated at 37°C with C_1 fraction from T.koningii

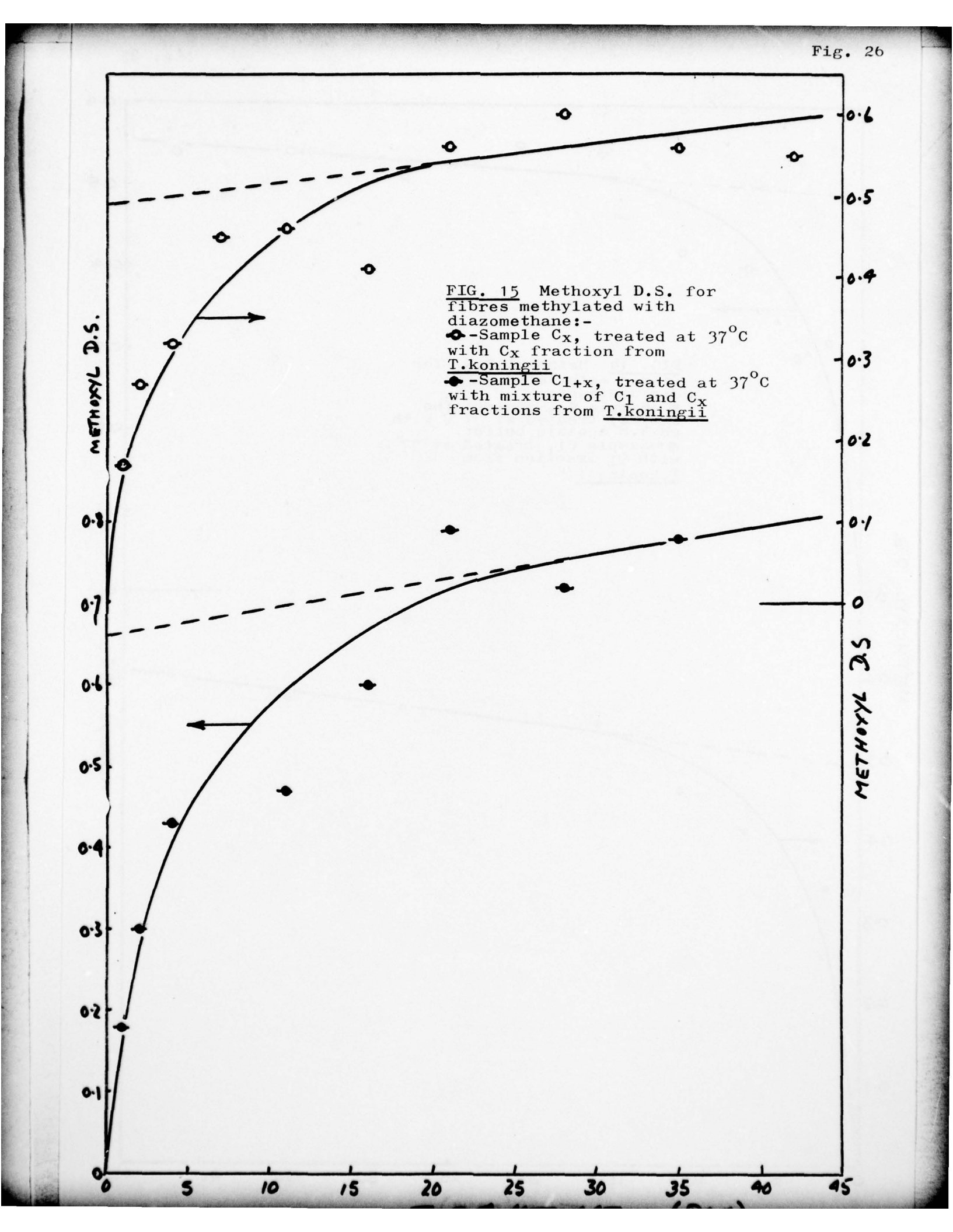
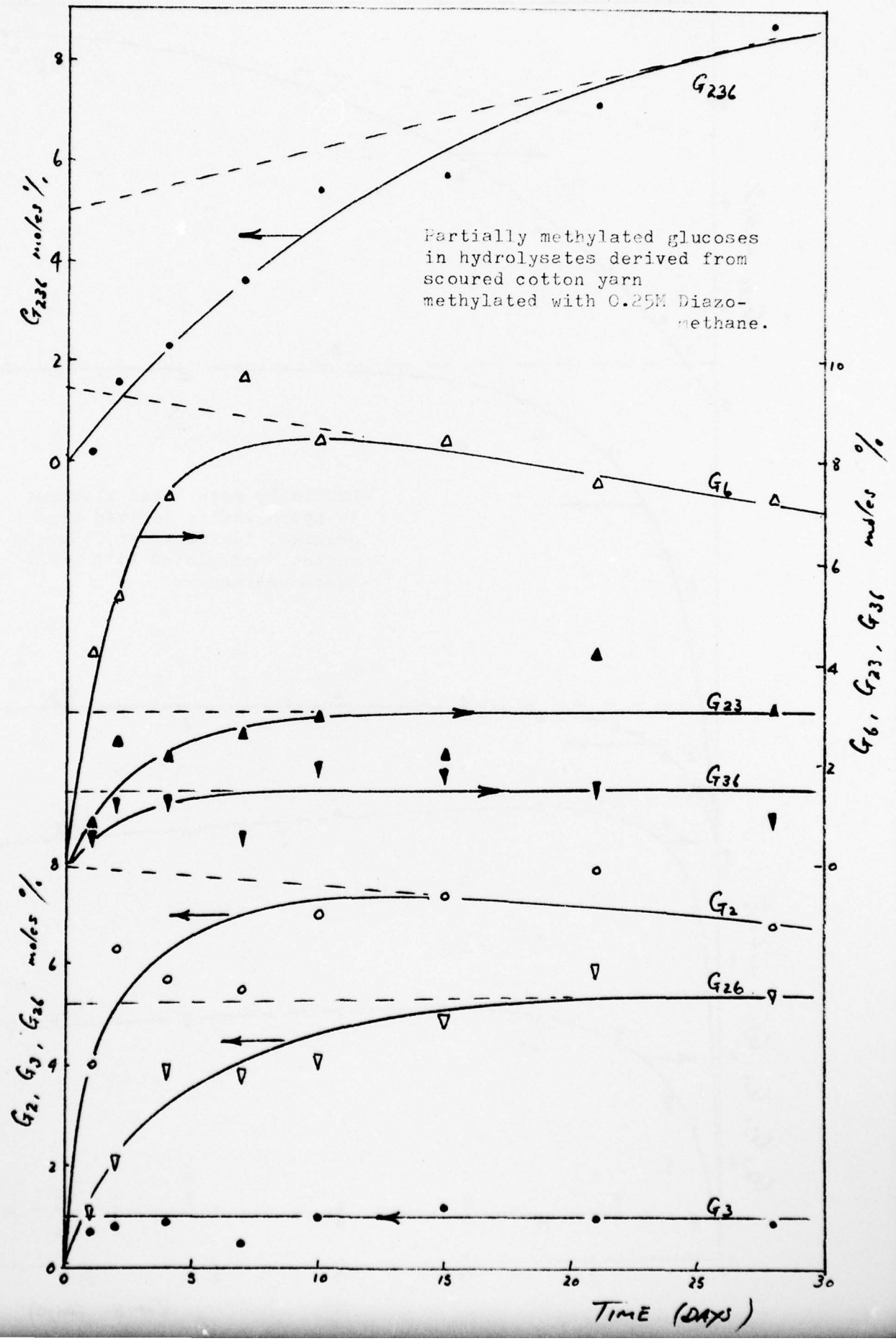
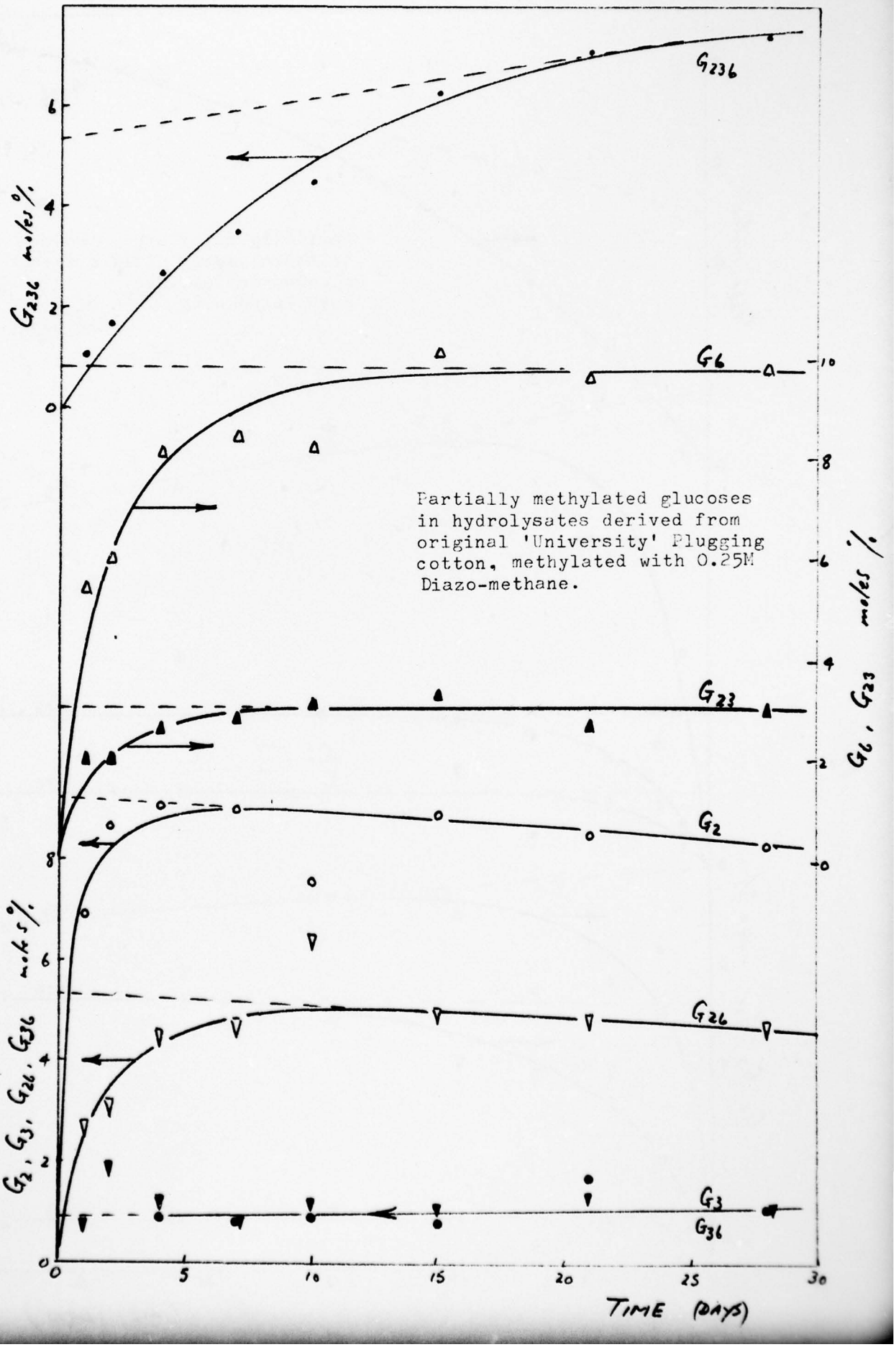


FIG. 15 Methoxyl D.S. for fibres methylated with diazomethane:-
-o-Sample C_x, treated at 37°C with C_x fraction from T.koningii
-●-Sample C_{1+x}, treated at 37°C with mixture of C_1 and C_x fractions from T.koningii

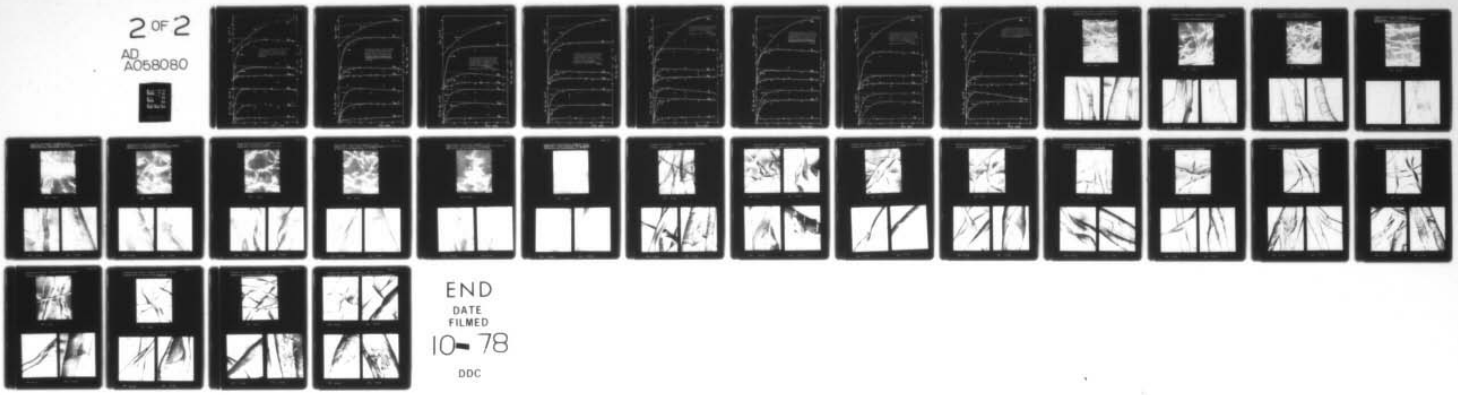
Fig. 27


Partially methylated glucoses in hydrolysates derived from scoured cotton yarn methylated with 0.25M Diazomethane.
G236
G6
G23
G36
G2
G26
G3
G2, G3, G26 moles %
G6, G23, G36 moles %
TIME (DAYS)


Partially methylated glucoses in hydrolysates derived from original 'University' Plugging cotton, methylated with 0.25M Diazo-methane.
G236
G6
G23
G2
G26
G3
G36
G236 moles %
G2, G3, G26, G36 moles %
G6, G23 moles %
TIME (DAYS)
0
5
10
15
20
25
30
2
4
6
8
10

AD-A058 080 SHIRLEY INST MANCHESTER (ENGLAND) F/G 6/1
INVESTIGATION OF ACCESSIBILITY CHANCES IN CRYSTALLINE CELLULOSE--ETC(U)
MAY 78 B F SAGAR DAERO-75-G-047
UNCLASSIFIED SI-REF-21/13/522 NL

2 OF 2
AD A058080



END
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Fig. 29

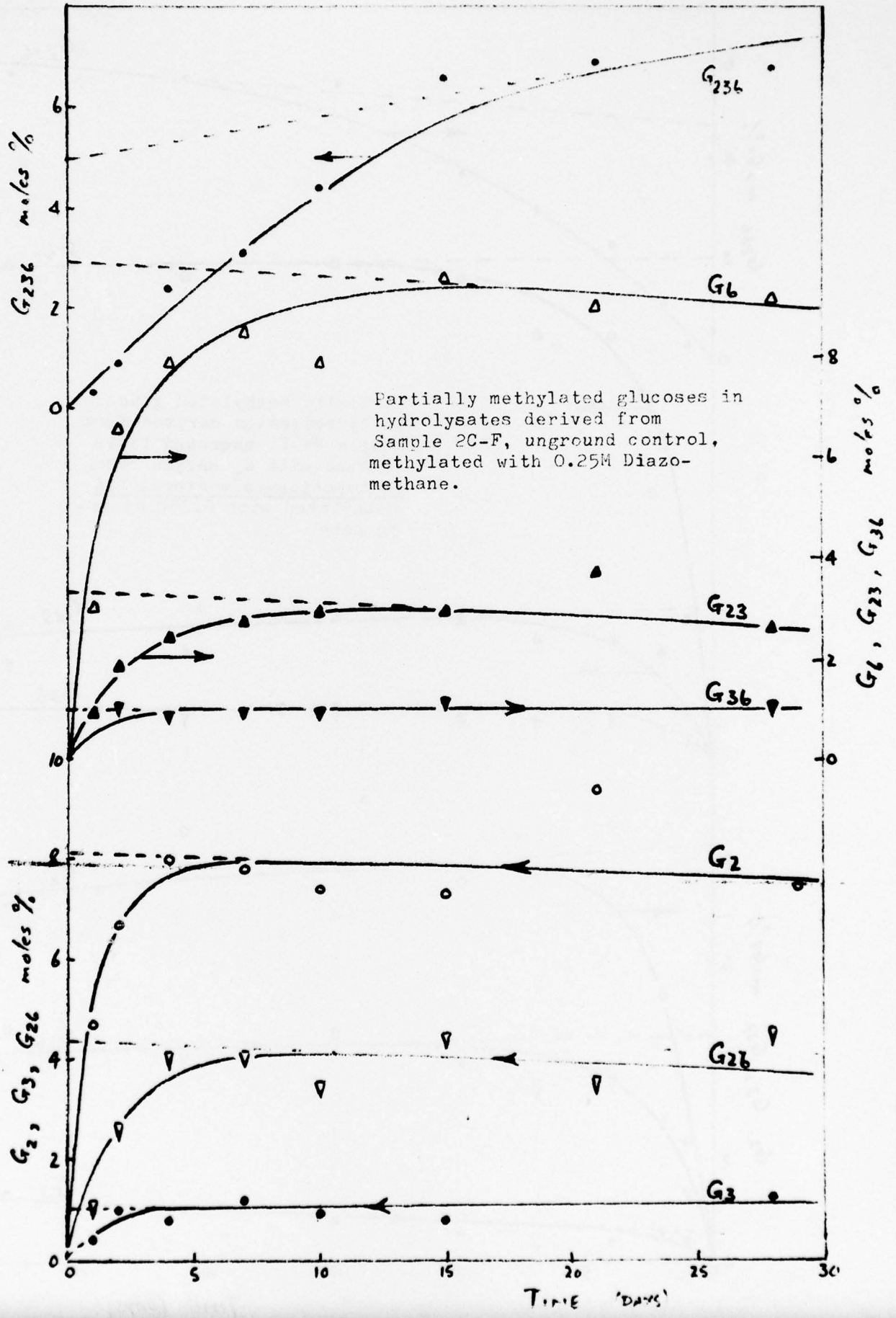
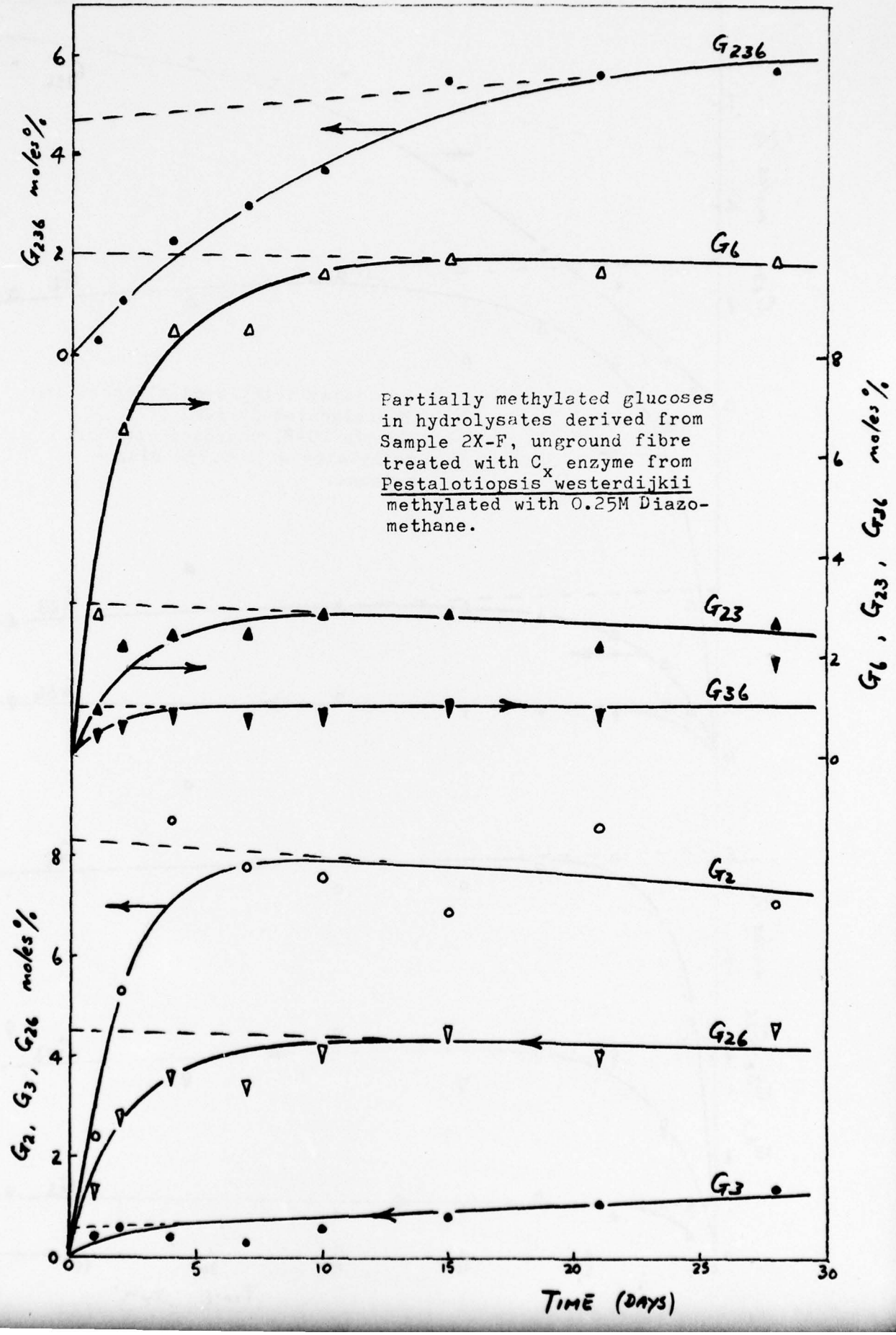
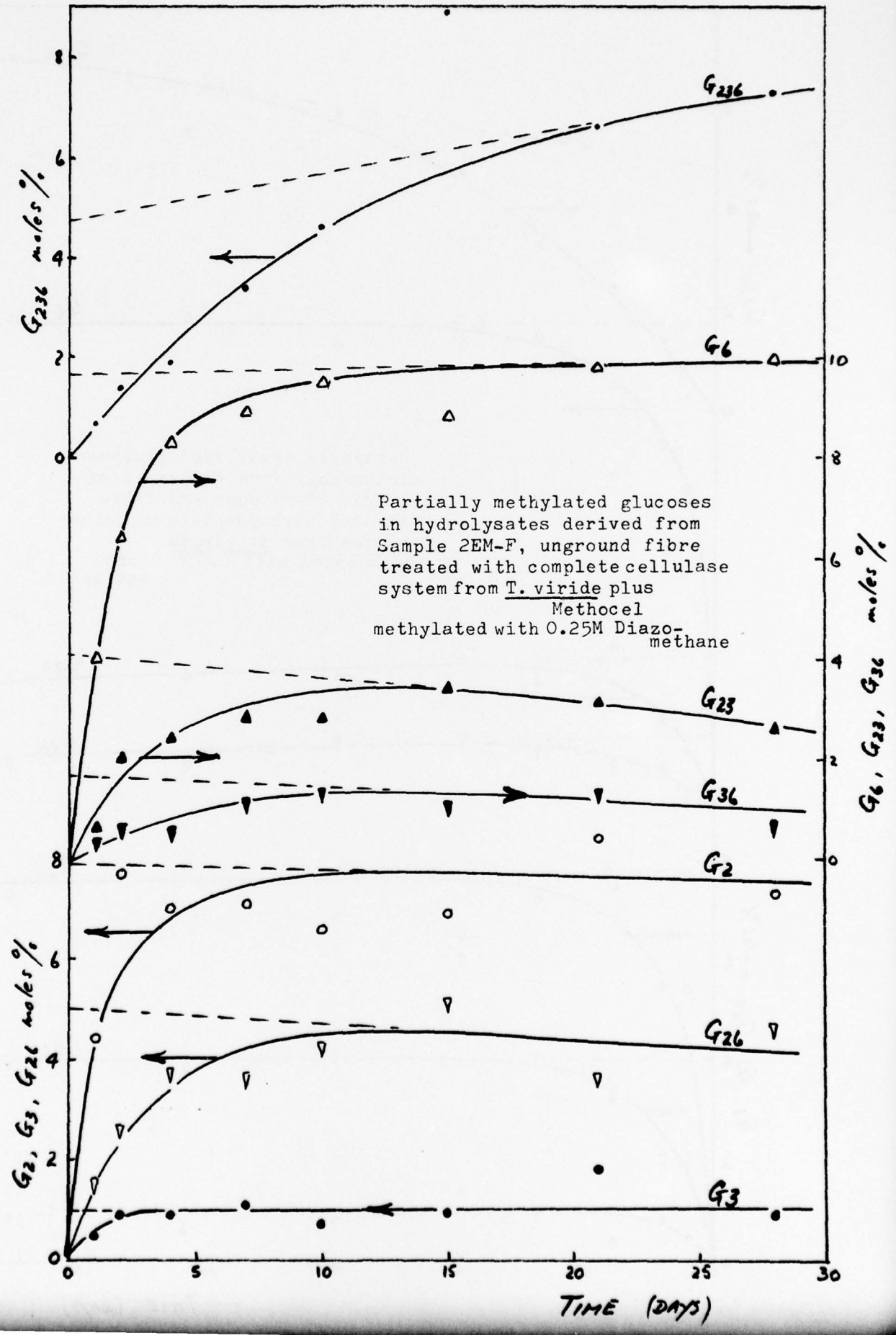

G234
G6
G23
G36
G2
G26
G3
Partially methylated glucoses in hydrolysates derived from Sample 2C-F, unground control, methylated with 0.25M Diazo-methane.
G234 moles %
G6, G23, G36 moles %
G2, G3, G26 moles %
TIME 'DAYS'
0
5
10
15
20
25
30

Fig. 30



Partially methylated glucoses in hydrolysates derived from Sample 2X-F, unground fibre treated with C_x enzyme from Pestalotiopsis westerdijkii methylated with 0.25M Diazomethane.

Fig. 31


G_{236}
G_6
G_{23}
G_{36}
G_2
G_{26}
G_3
G_{236} moles %
G_2, G_3, G_{26} moles %
G_6, G_{23}, G_{36} moles %
TIME (DAYS)
0
5
10
15
20
25
30
2
4
6
8
10
Partially methylated glucoses in hydrolysates derived from Sample 2EM-F, unground fibre treated with complete cellulase system from T. viride plus Methocel methylated with 0.25M Diazomethane

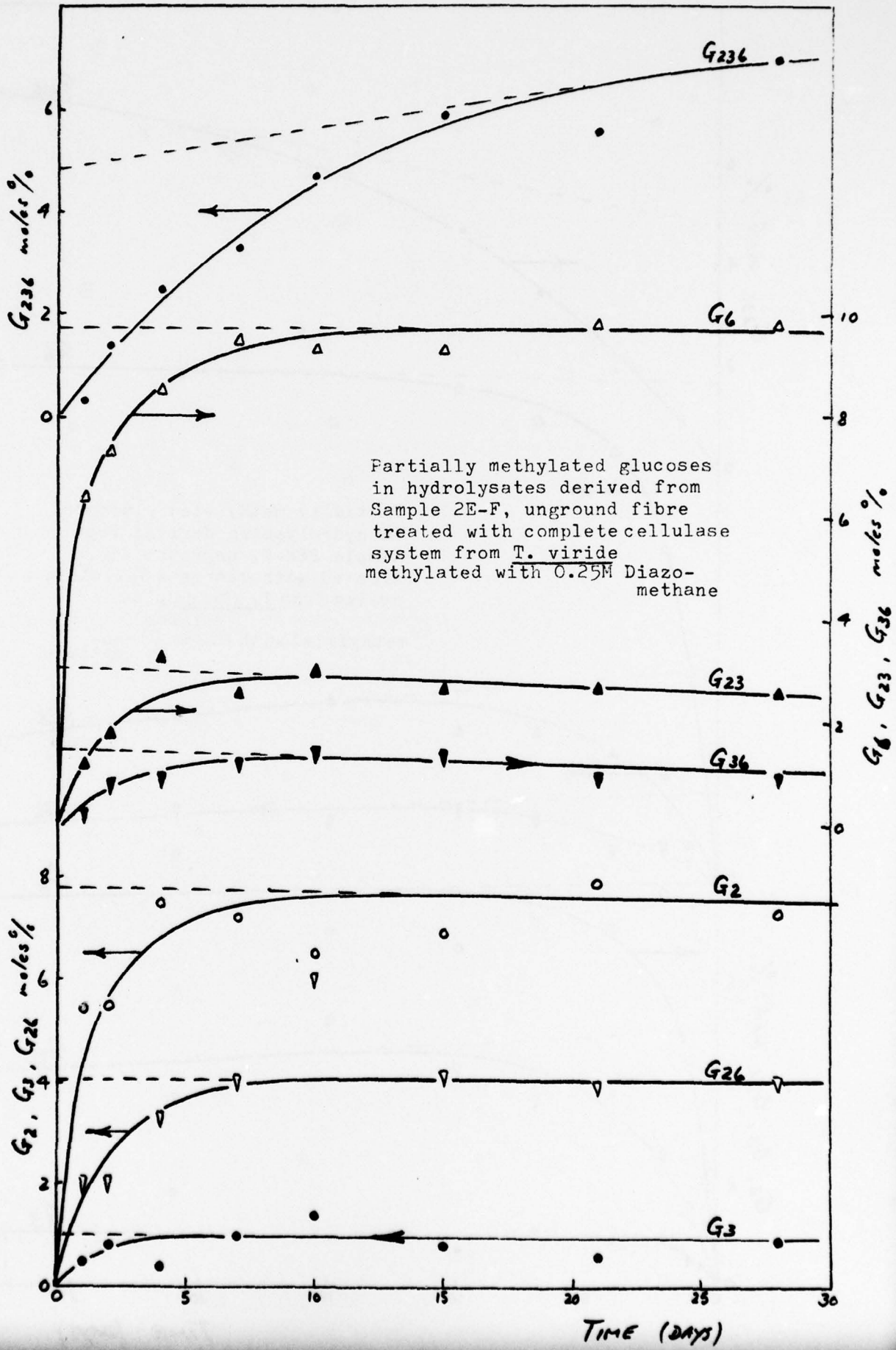

G236
G6
G23
G36
G2
G26
G3
Partially methylated glucoses in hydrolysates derived from Sample 2E-F, unground fibre treated with complete cellulase system from T. viride methylated with 0.25M Diazo-methane
G236 moles %
G6, G23, G36 moles %
G2, G3, G26 moles %
TIME (DAYS)
0
5
10
15
20
25
30
2
4
6
8
10

Fig. 33

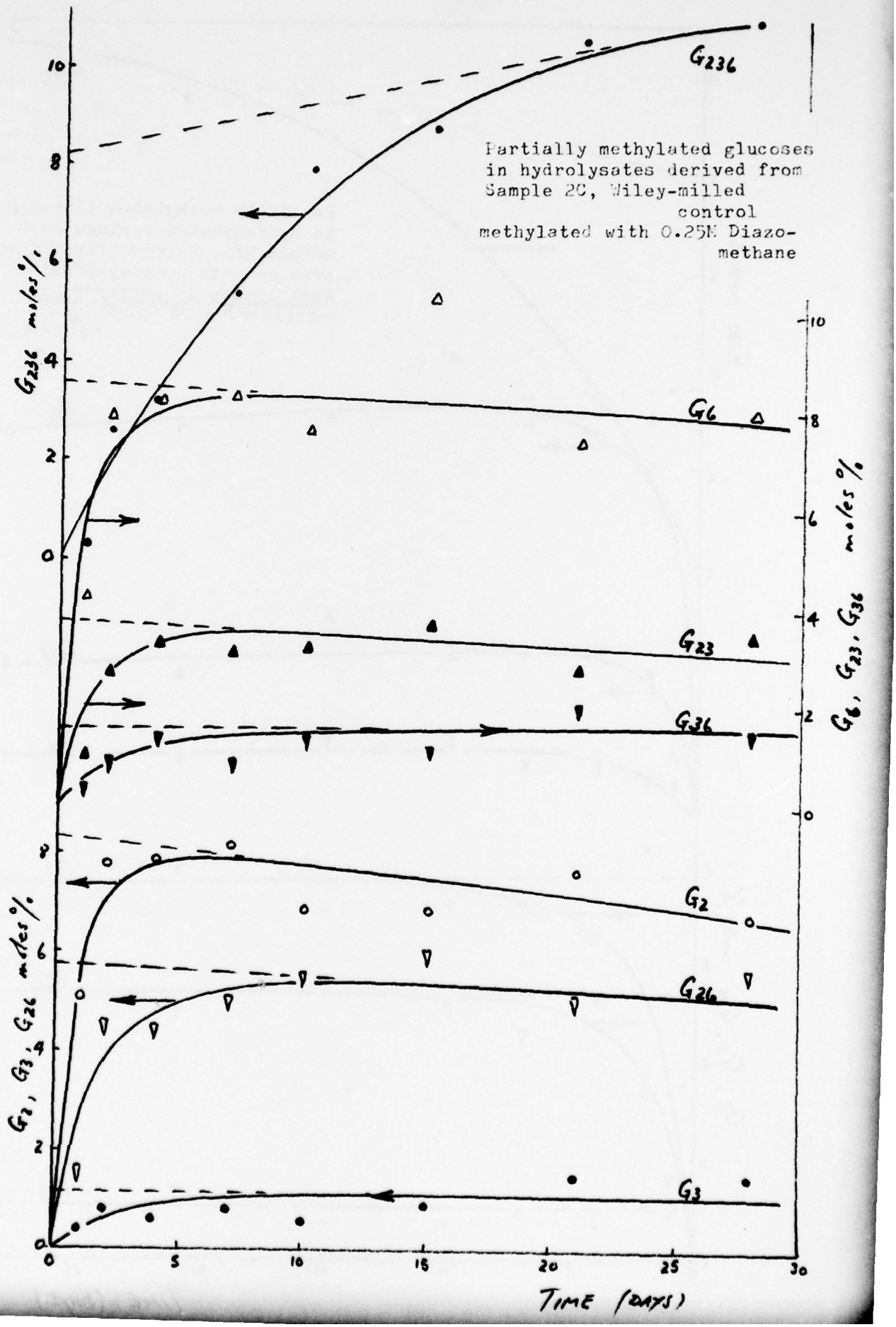

Partially methylated glucoses in hydrolysates derived from Sample 20, Wiley-milled control methylated with 0.25M Diazo-methane
G236
G6
G23
G36
G2
G26
G3
G236 moles %
G6, G23, G36 moles %
G2, G3, G26 moles %
TIME (DAYS)
0
5
10
15
20
25
30
2
4
6
8
10

Fig. 34

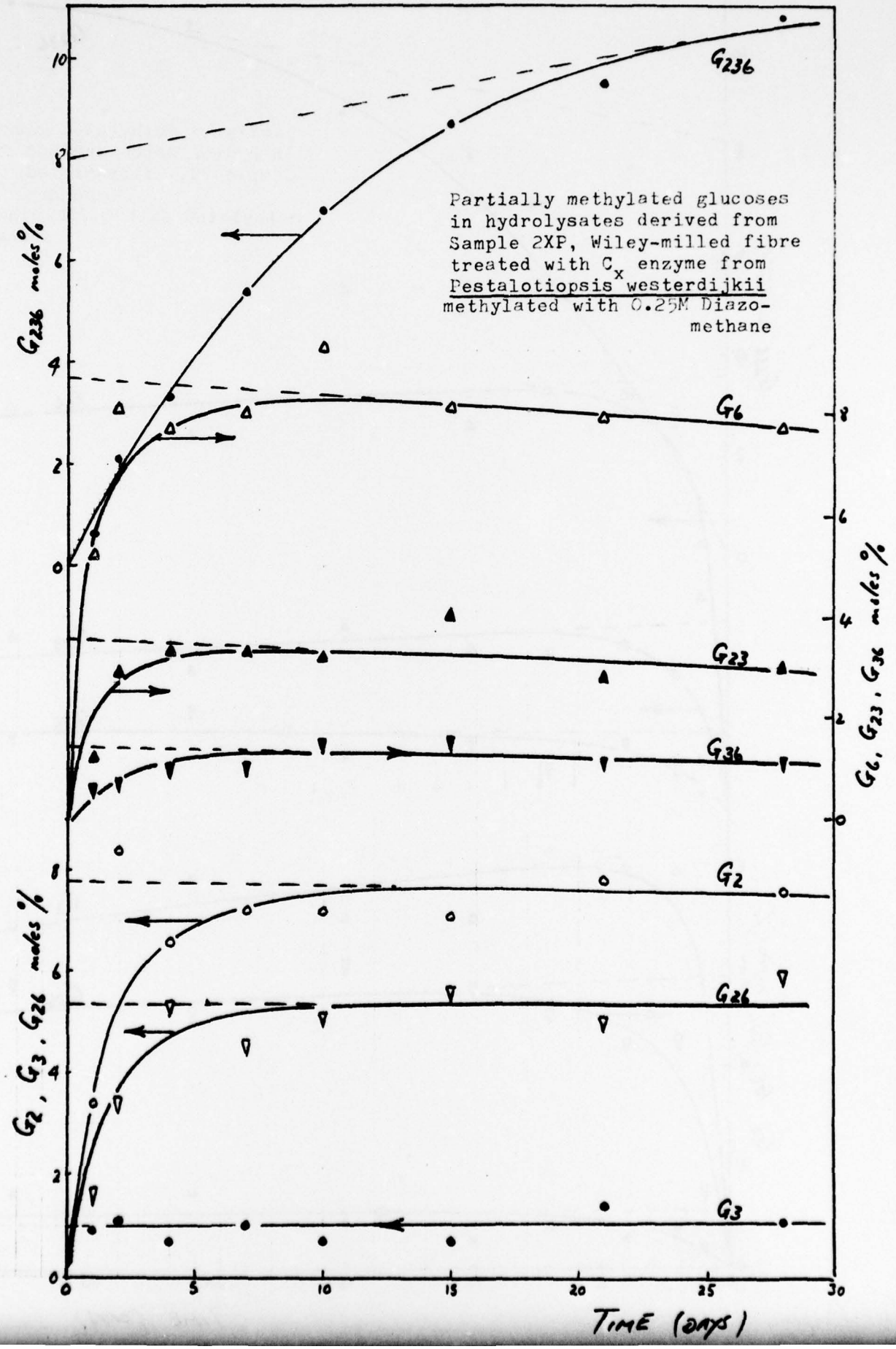
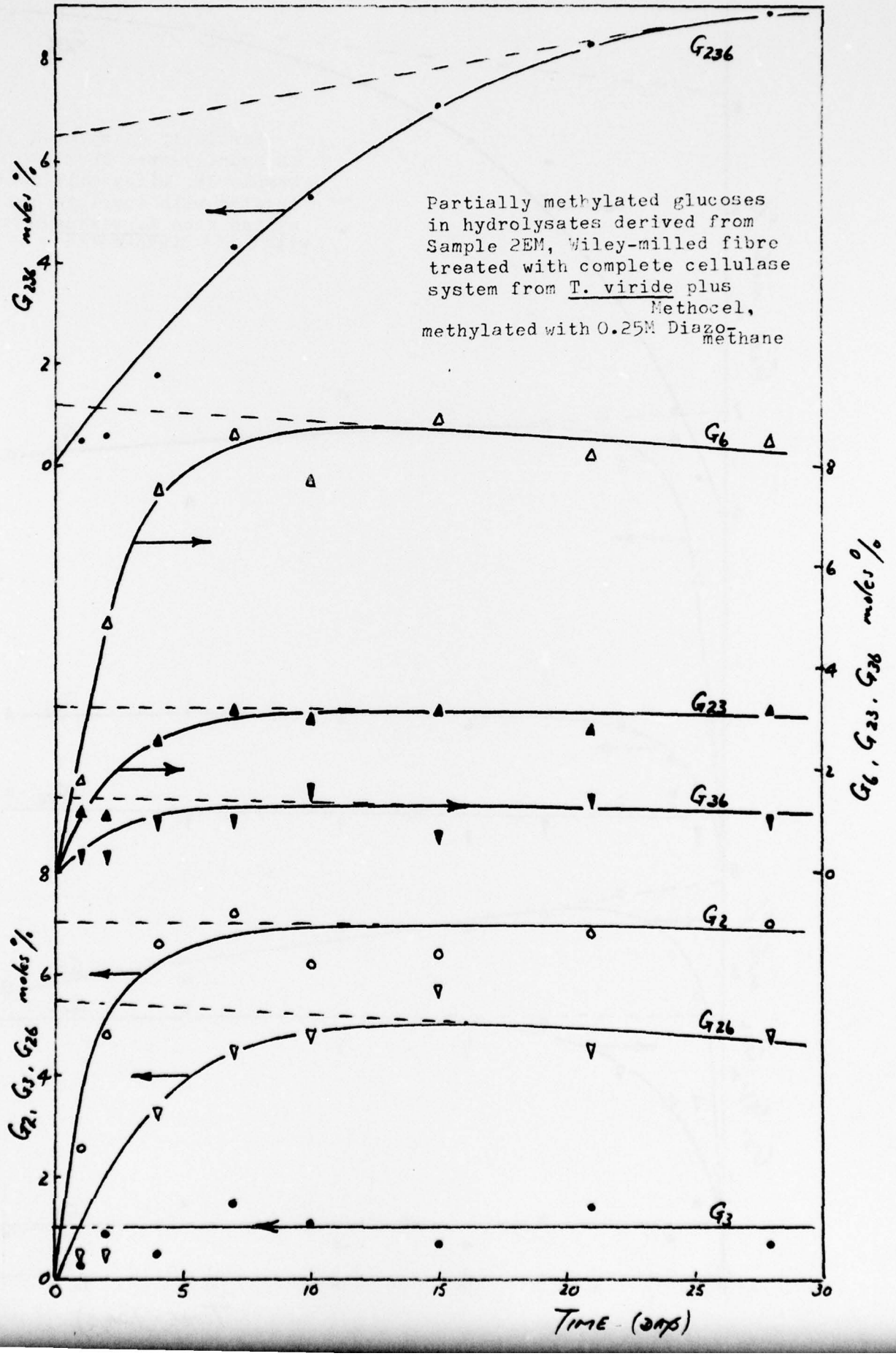

Partially methylated glucoses in hydrolysates derived from Sample 2XP, Wiley-milled fibre treated with C_x enzyme from Pestalotiopsis westerdijkii methylated with 0.25M Diazomethane
G236
G6
G23
G36
G2
G26
G3
G236 moles %
G2, G3, G26 moles %
G6, G23, G36 moles %
TIME (DAYS)

Fig. 35


Partially methylated glucoses in hydrolysates derived from Sample 2EM, Wiley-milled fibre treated with complete cellulase system from T. viride plus Methocel, methylated with 0.25M Diazomethane
G236
G6
G23
G36
G2
G26
G3
G2, G3, G26 moles %
G6, G23, G36 moles %
TIME (DAYS)

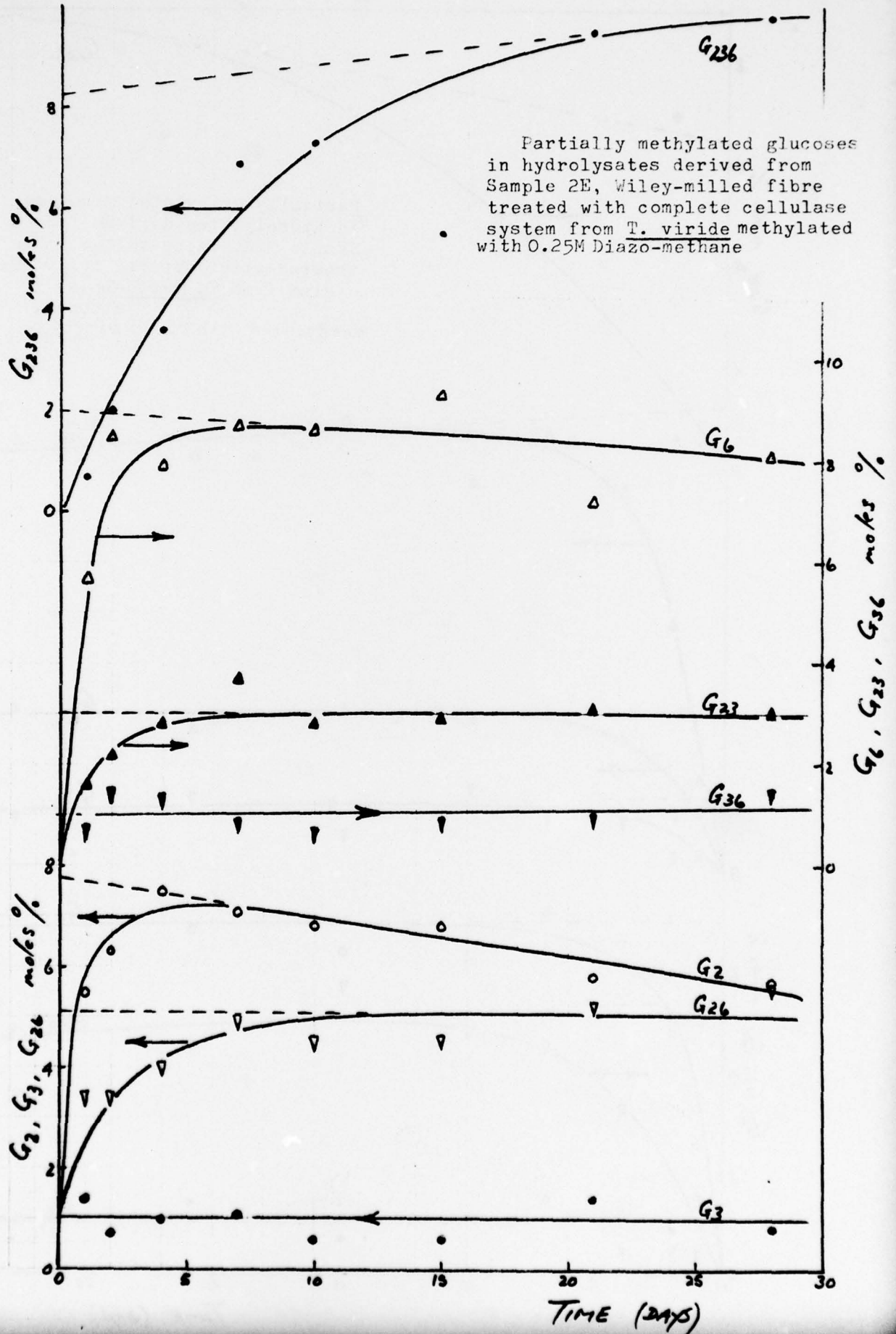
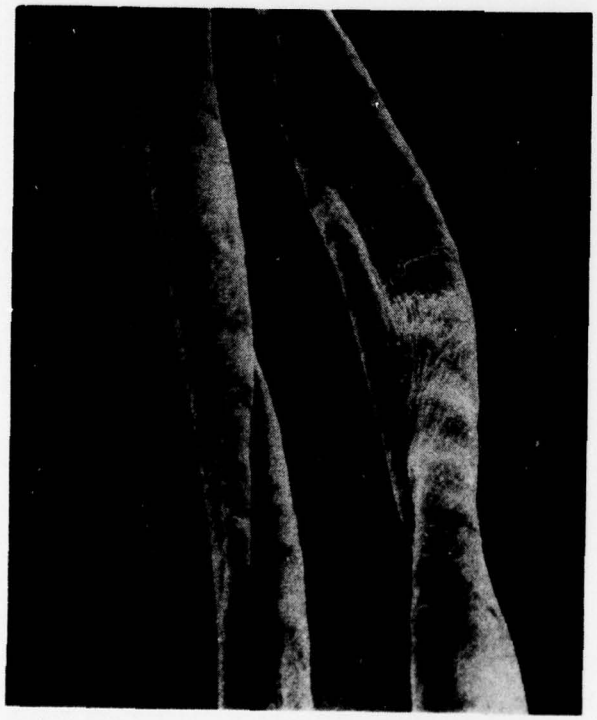

Partially methylated glucoses in hydrolysates derived from Sample 2E, Wiley-milled fibre treated with complete cellulase system from T. viride methylated with 0.25M Diazo-methane
G236
G6
G23
G36
G2
G26
G3
G236 moles %
G2, G3, G26 moles %
G6, G23, G36 moles %
TIME (DAYS)
0
5
10
15
20
25
30
2
4
6
8
10

Fig. 37

Scoured Cotton Yarn - No enzyme treatment
Methylated with Diazomethane for 28 days.

56 μm



5.6 μm

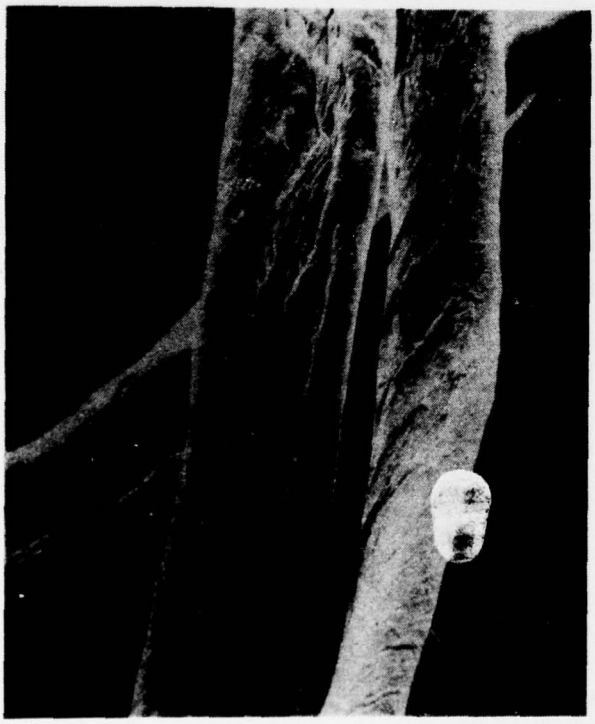
2.9 μm

Fig. 38

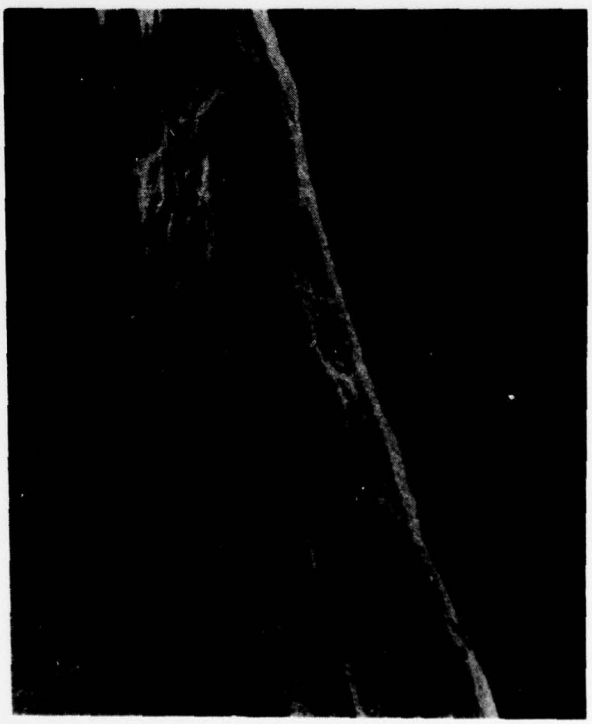
Original 'University' Plugging Cotton - No enzyme treatment.

Methylated with Diazomethane for 28 days.

56 μm

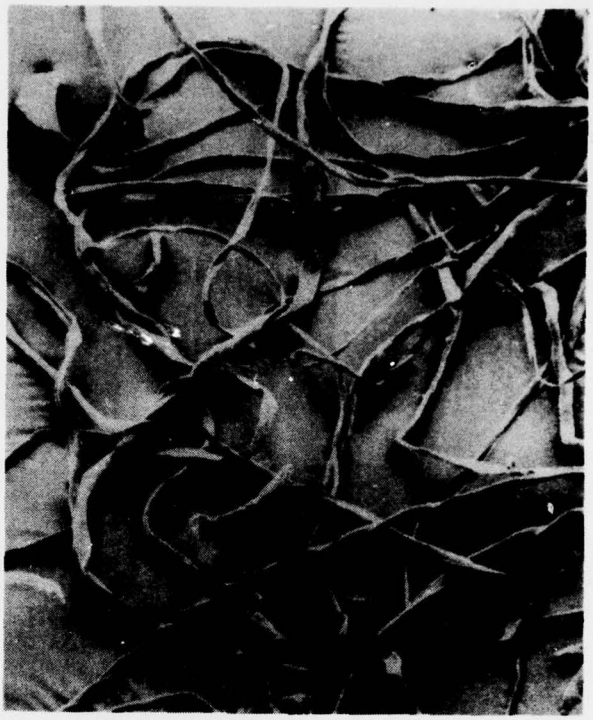


5.6 μm

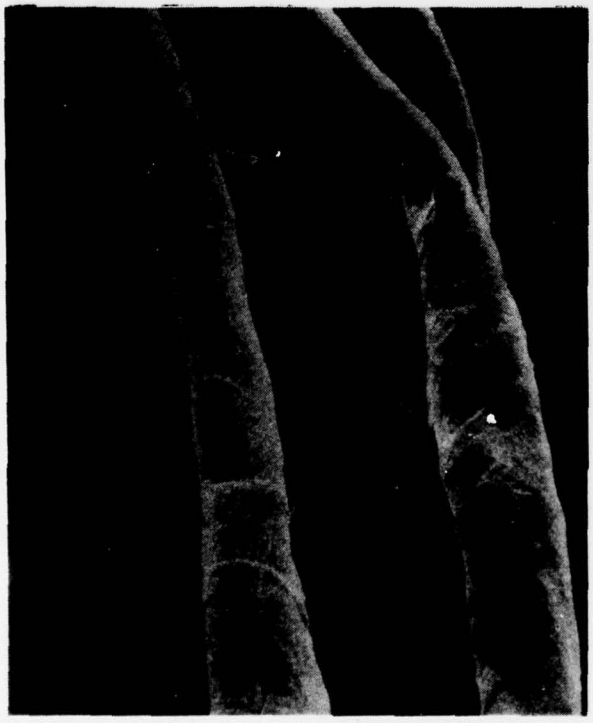


2.9 μm

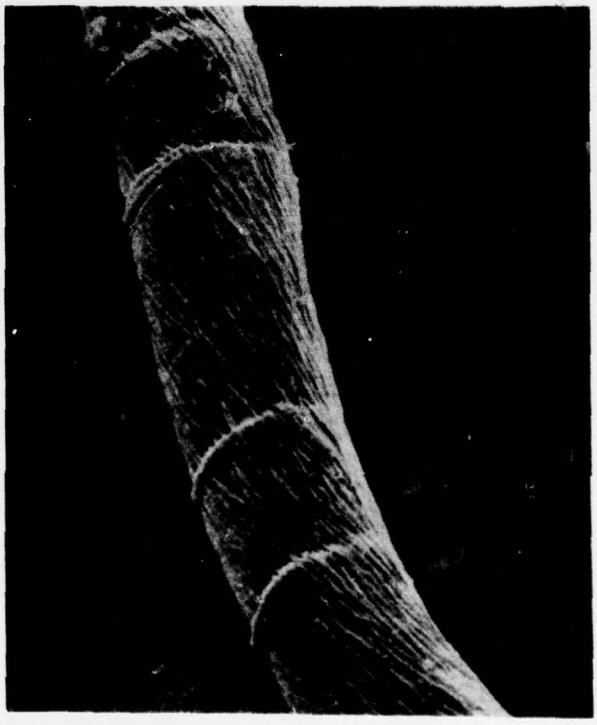
Unground 'University' plugging cotton
Sample 2C-F - Control (no enzyme)
Methylated with Diazomethane for 28 days



56 μm

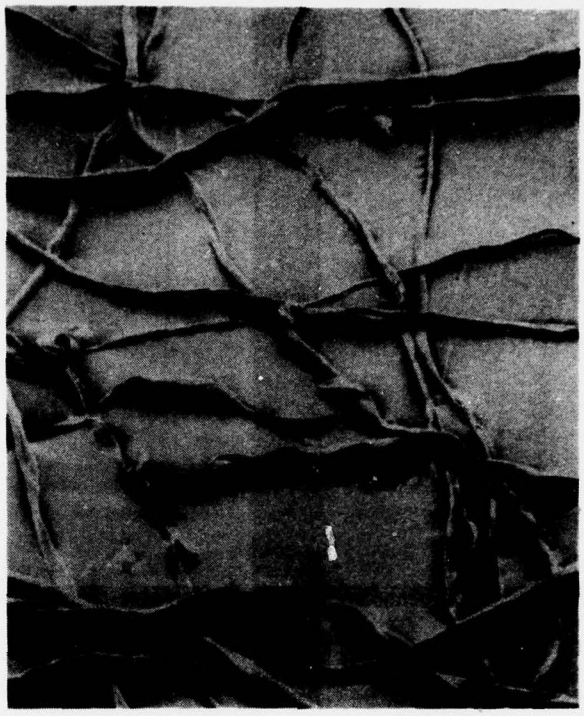


5.6 μm

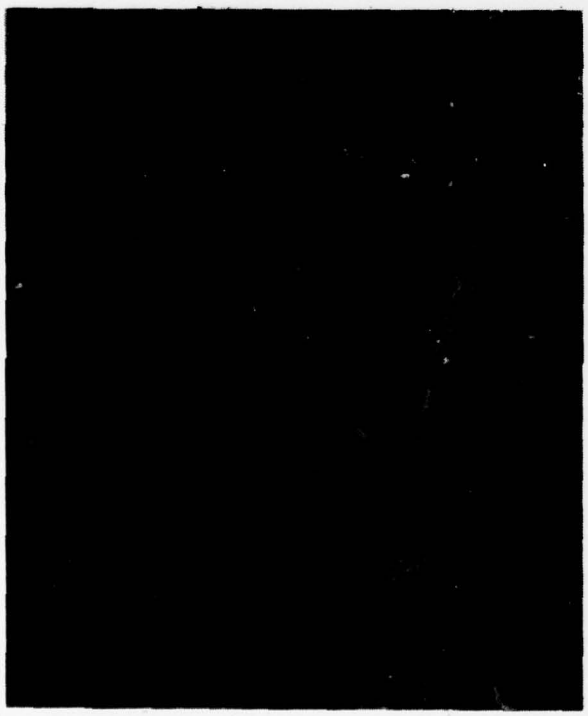


2.9 μm

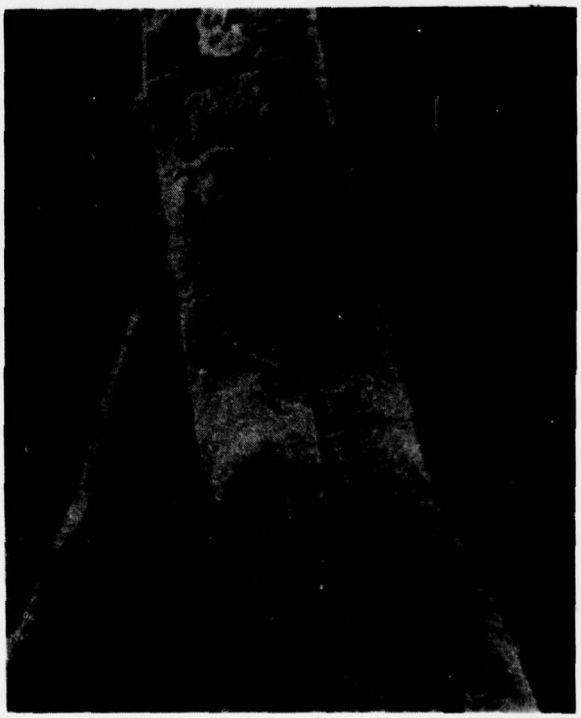
Unground 'University' plugging cotton
Sample 2X-F - Total enzyme from Pestalotiopsis westerdijkii (7.5 Cx units/ml)
Methylated with Diazomethane for 28 days.



⊢ 56 μm



⊢ 5.6 μm



⊢ 2.9 μm

Unground 'University' plugging cotton
Sample 2EM-F - Total enzyme system from T. viride plus Methocel
Methylated with Diazomethane for 28 days.

56 μm

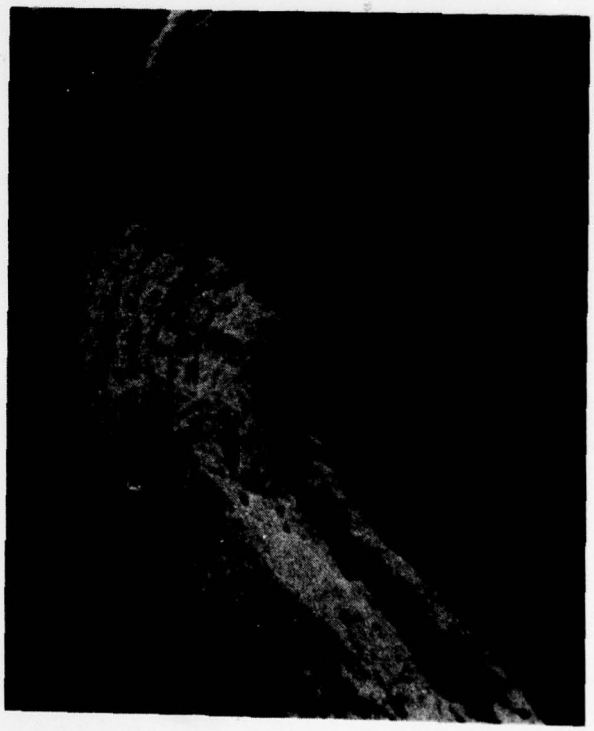
5.6 μm

2.9 μm

Unground 'University' plugging cotton
Sample 2E-F - Total enzyme system from T. viride
Methylated with Diazomethane for 28 days.

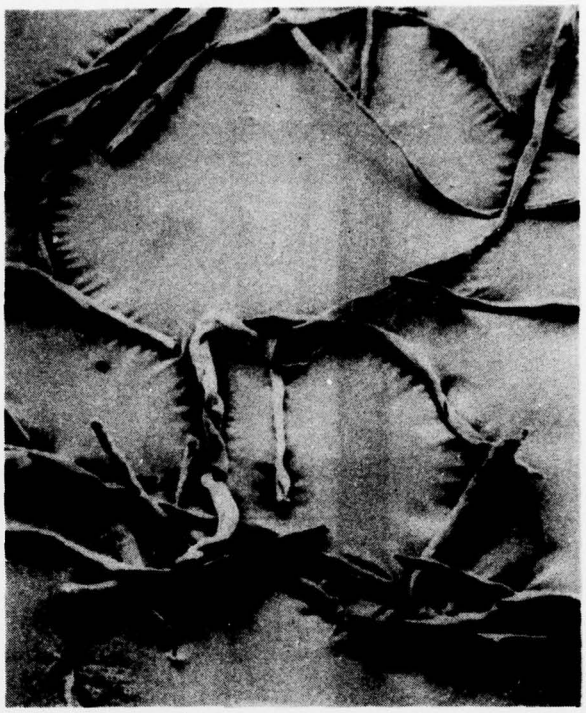
56 μm

5.6 μm

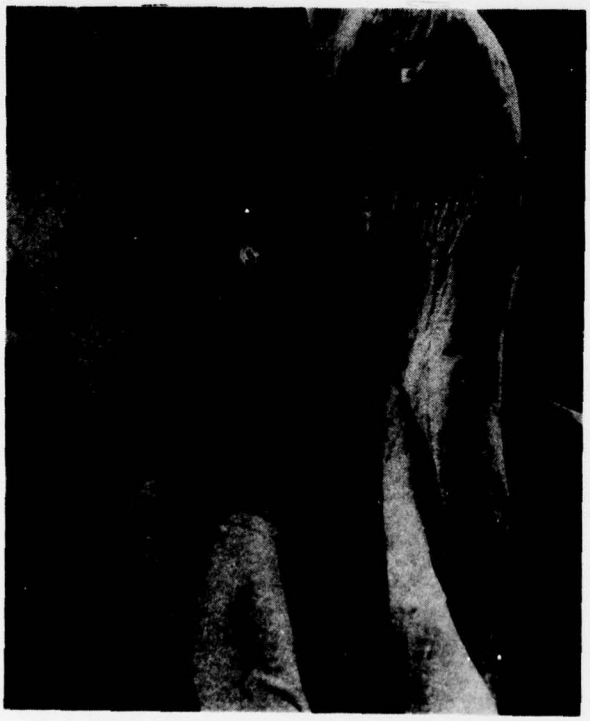


2.9 μm

Wiley-milled 'University' plugging cotton
Sample 2C - Control, no enzyme
Methylated with Diazomethane for 28 days.



56 μm

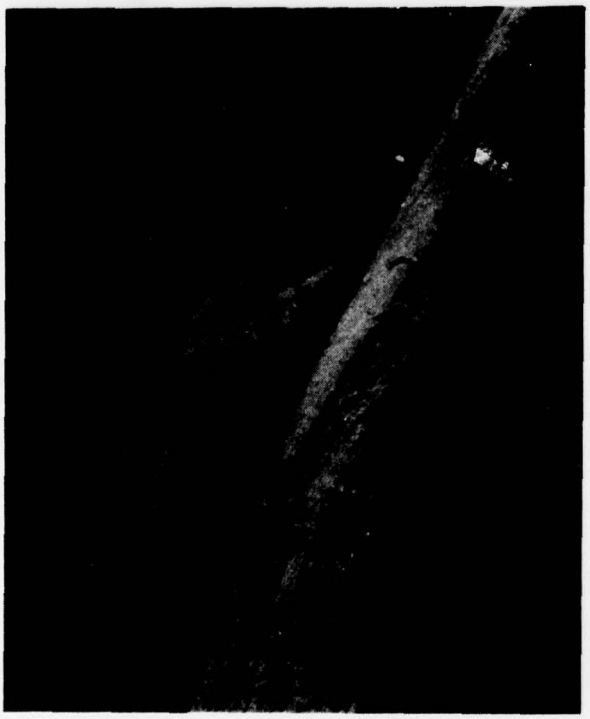


5.6 μm

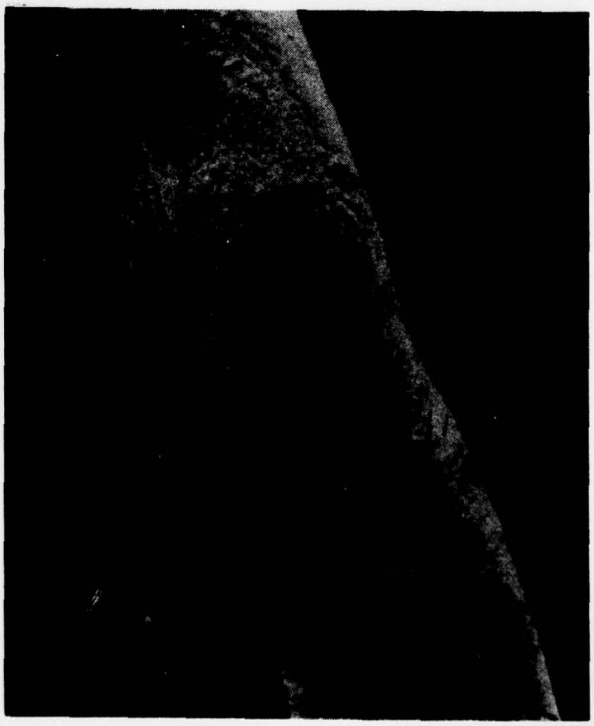
2.9 μm

Wiley-milled 'University' plugging cotton
Sample 2XP - Total enzyme from P. westerdijkii (75 Cx units/ml)
Methylated with Diazomethane for 28 days.

56 µm



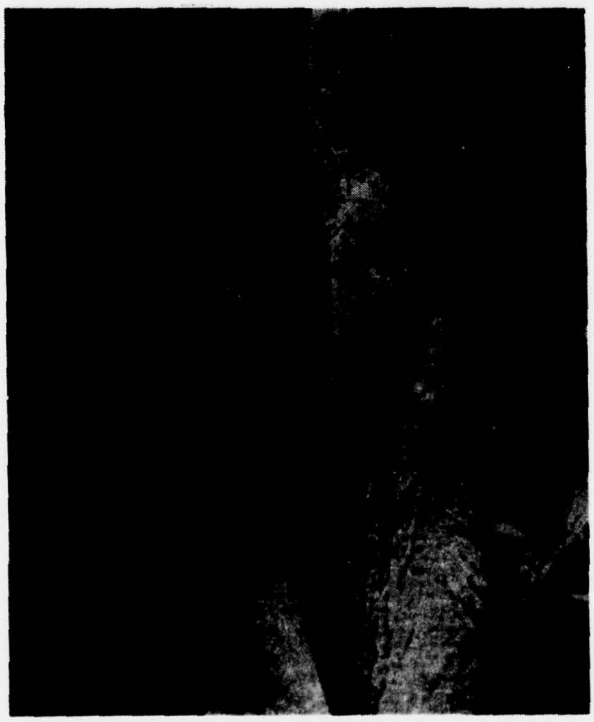
5.6 µm



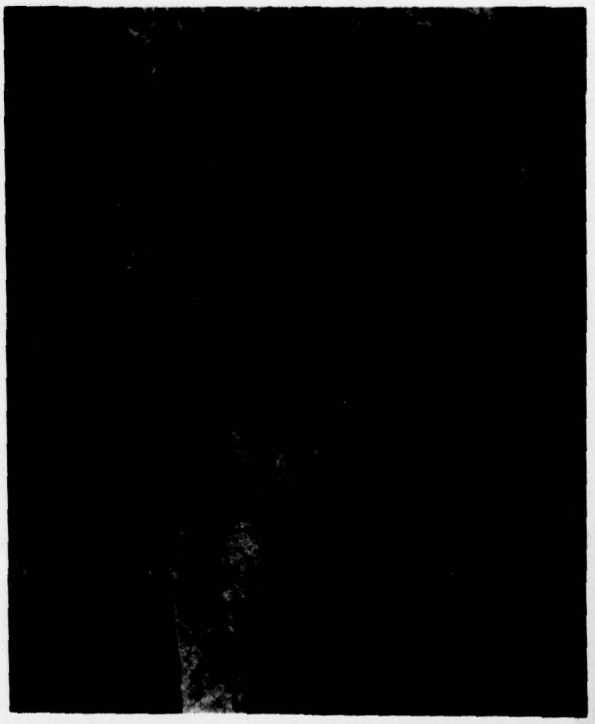
2.9 µm

Wiley-milled 'University' plugging cotton
Sample 2EM - Total enzyme from T. viride plus Methocel
Methylated with Diazomethane for 28 days.

⊢ 56 μm

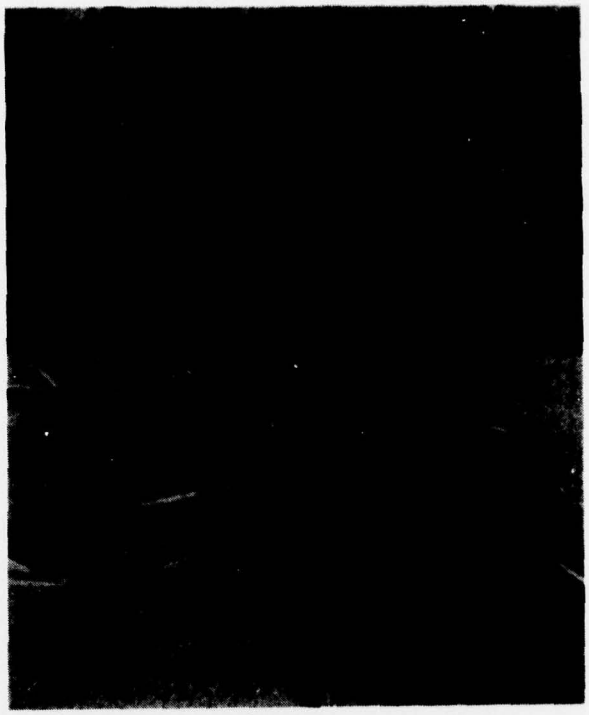


⊢ 5.6 μm

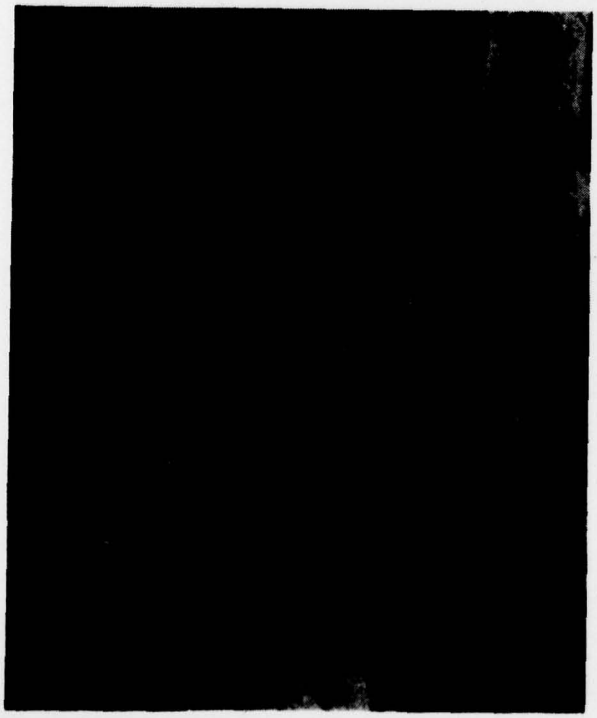


⊢ 2.9 μm

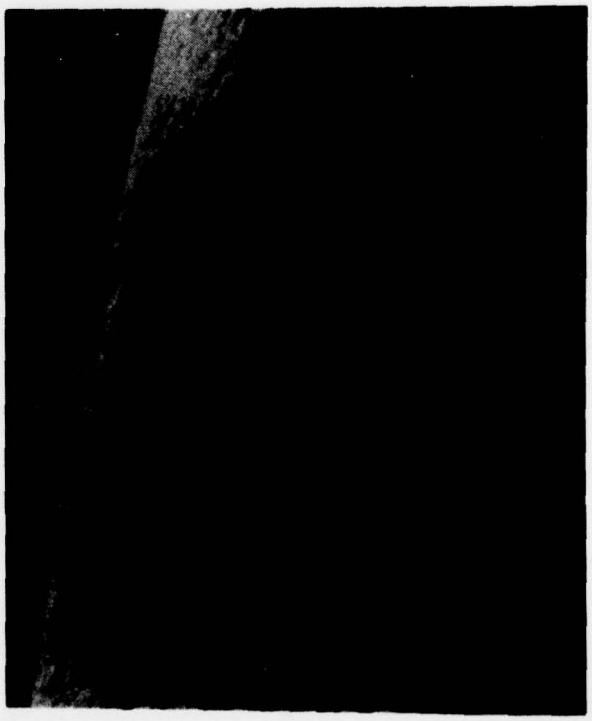
Wiley-milled 'University' plugging cotton
Sample 2E - Total cellulase from T. viride
Methylated with Diazomethane for 28 days



⊢ 56 μm



⊢ 5.6 μm



⊢ 2.9 μm

Scoured Acala Cotton - Sample A (Dr. E.T. Reese)
Control - no enzyme.

⊢ 18 μm

⊢ 5.6 μm

⊢ 1.8 μm

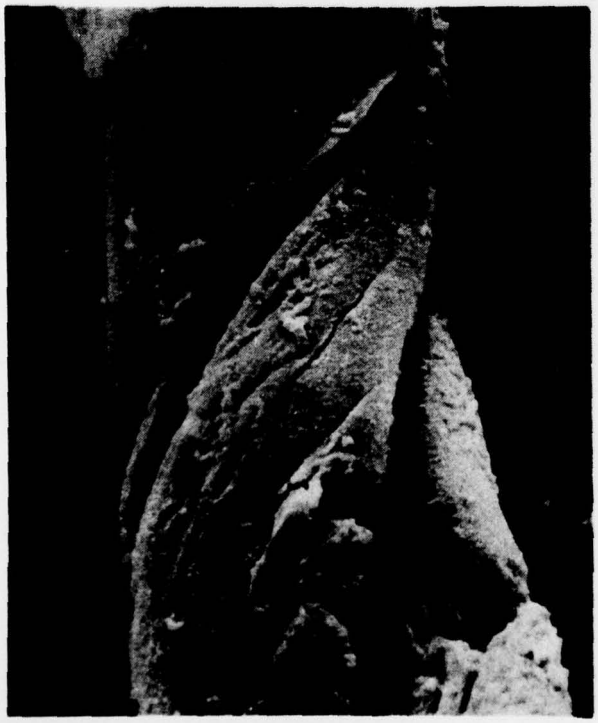
Fig. 48

Scoured Acala Cotton - Sample B (Dr. E.T. Reese)

Treated with Total Cellulase Complex from T. viride (50 C_x-Iu/ml)

18 μm

5.6 μm



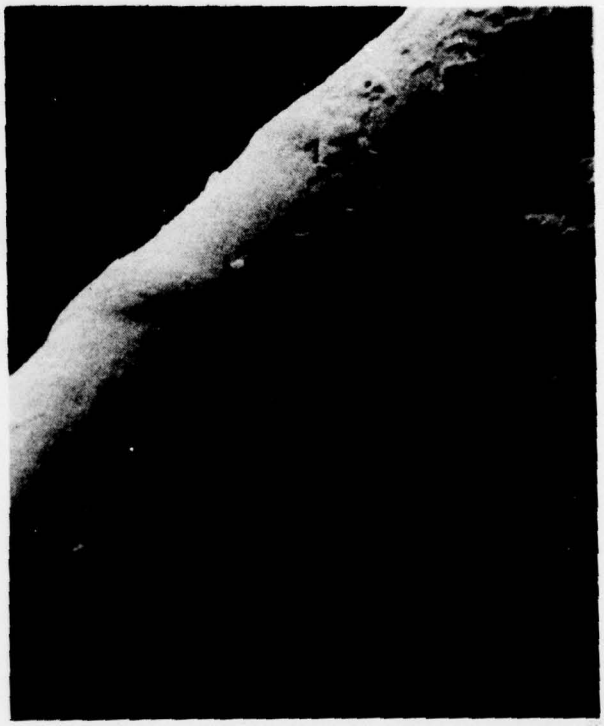
1.8 μm

1.8 μm

Scoured Acala Cotton - Sample C (Dr. E.T. Reese).
Treated with Total Cellulase Complex from T. viride (12.5 C_x-Iu/ml)

18 μm

5.6 μm



1.8 μm

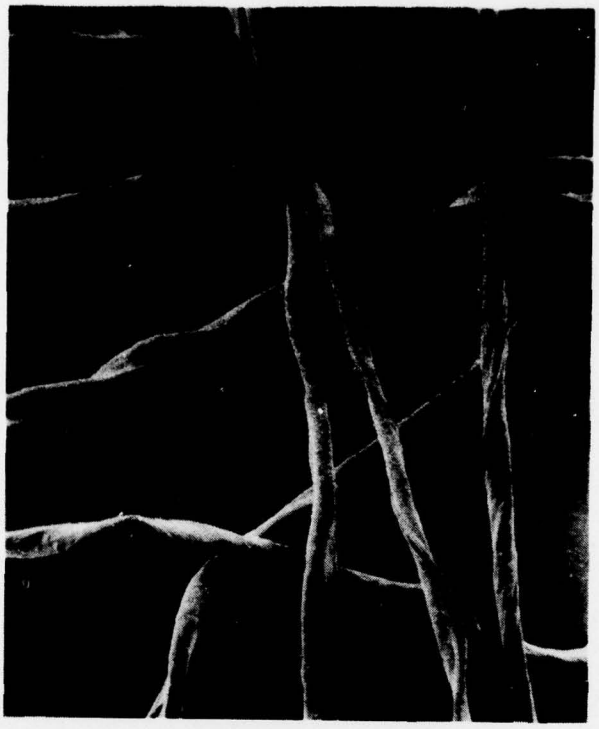
Scoured Acala Cotton - Sample D (Dr. E.T. Reese)
Treated with Total Cellulase Complex from T. viride (50 C_x-Iu/ml)
plus Methocel.

18 μm

5.6 μm

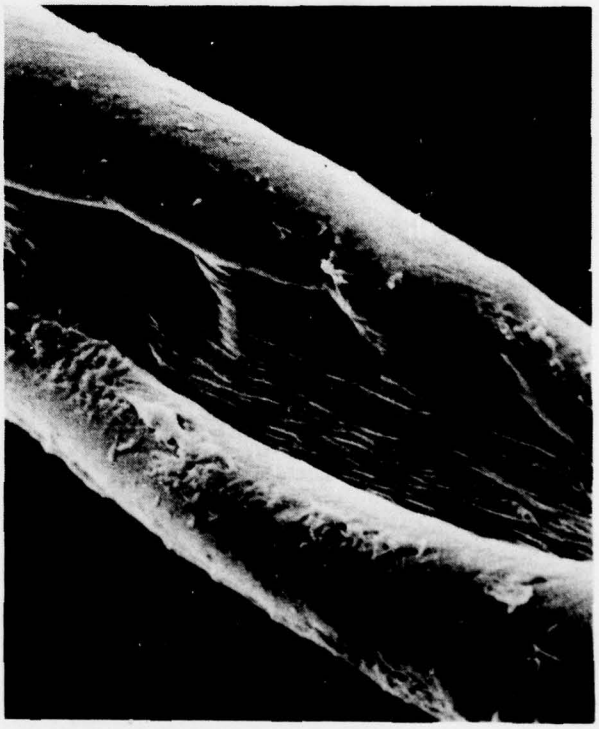
1.8 μm

Scoured Acala Cotton - Sample E (Dr. E.T. Reese)
Treated with Cx fraction from T. viride



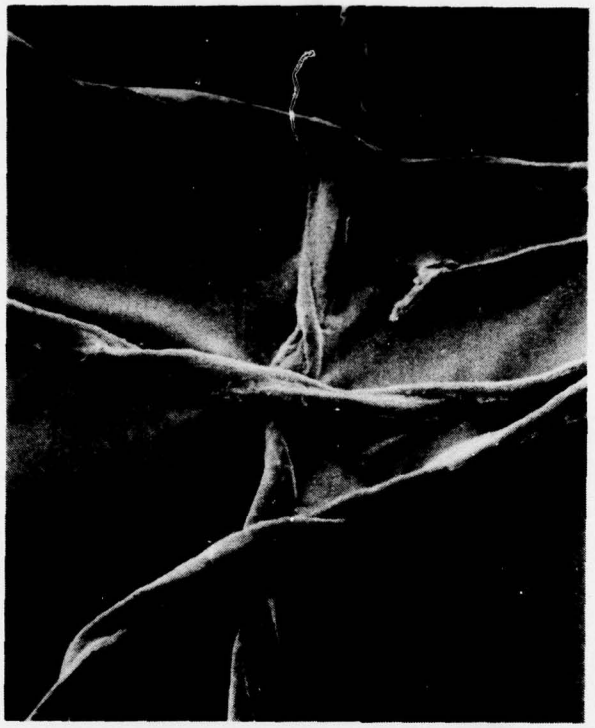
18 μm

5.6 μm

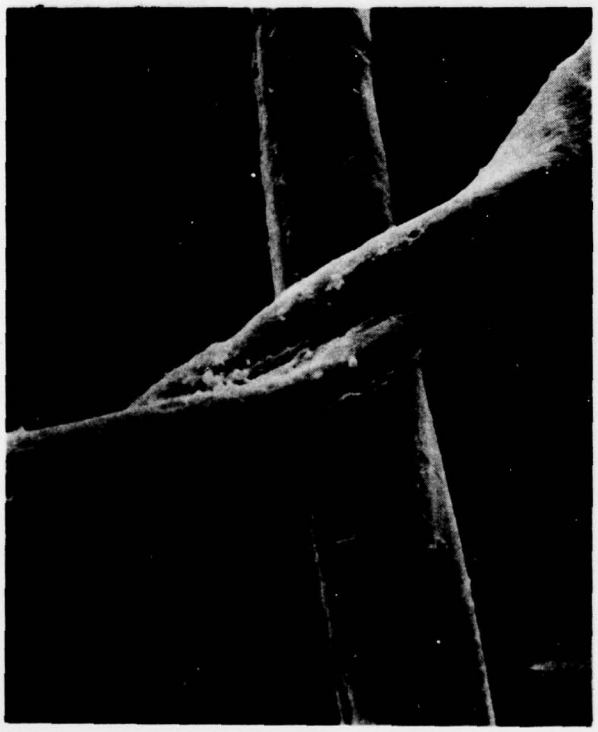


1.8 μm

Scoured Acala Cotton - Sample F (Dr. E.T. Reese)
Treated with C_1 fraction from T. viride



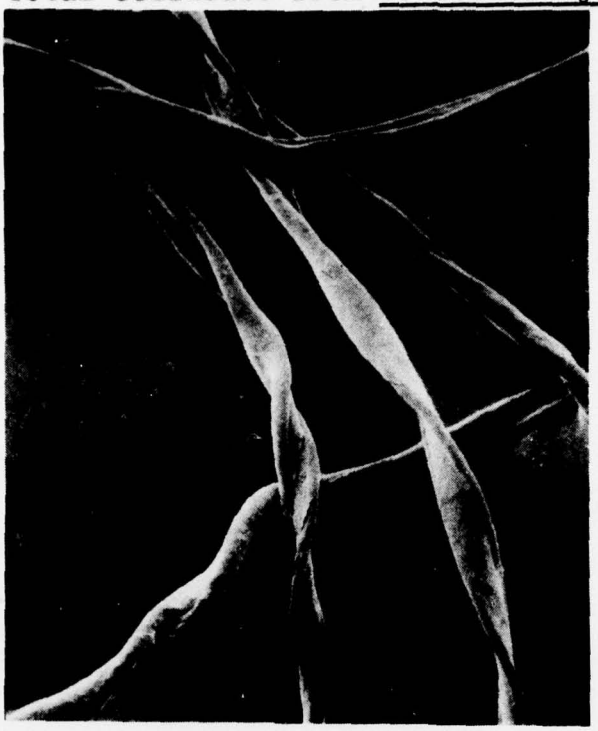
18 μm



5.6 μm

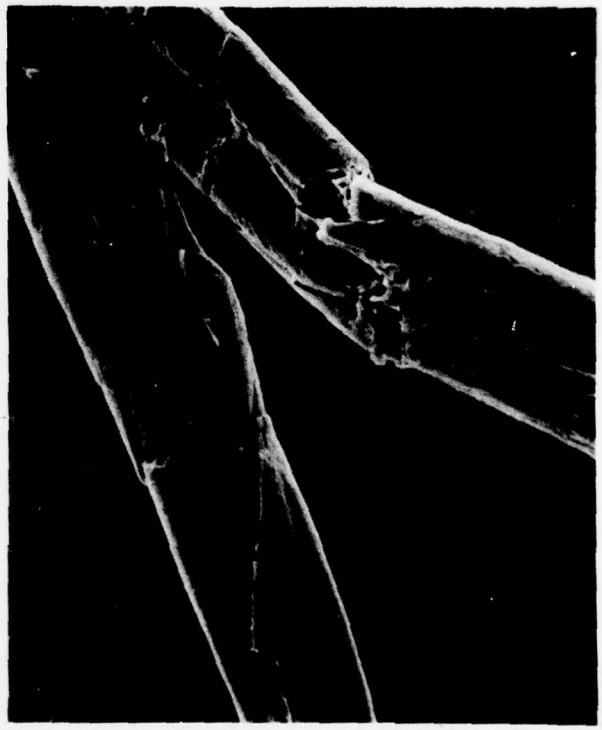
1.8 μm

Scoured Acala Cotton - Sample G (Dr. E.T. Reese)
Treated with Total Cellulase from P. westerdijkii



⊢ 18 μm

⊢ 1.8 μm



⊢ 5.6 μm

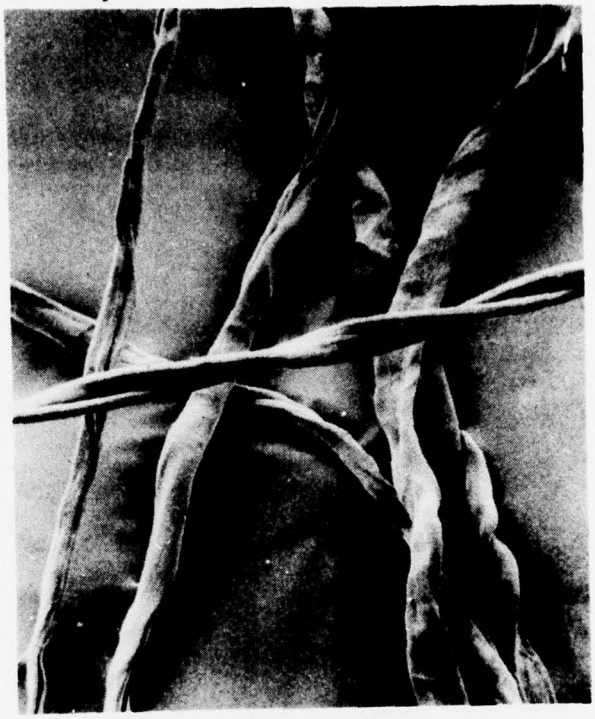
Scoured Acala Cotton - Sample H (Dr. E.T. Reese)
Treated with Total Cellulase Complex from T. viride (Unshaken).

18 μm

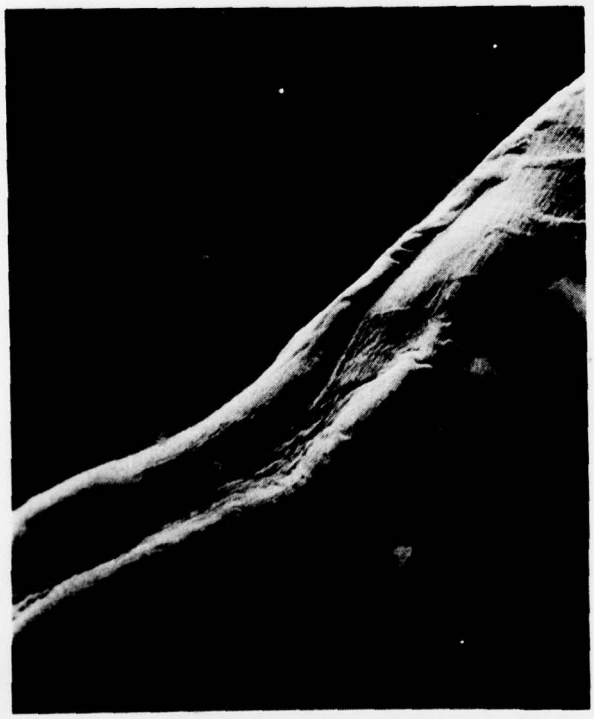
5.6 μm

1.8 μm

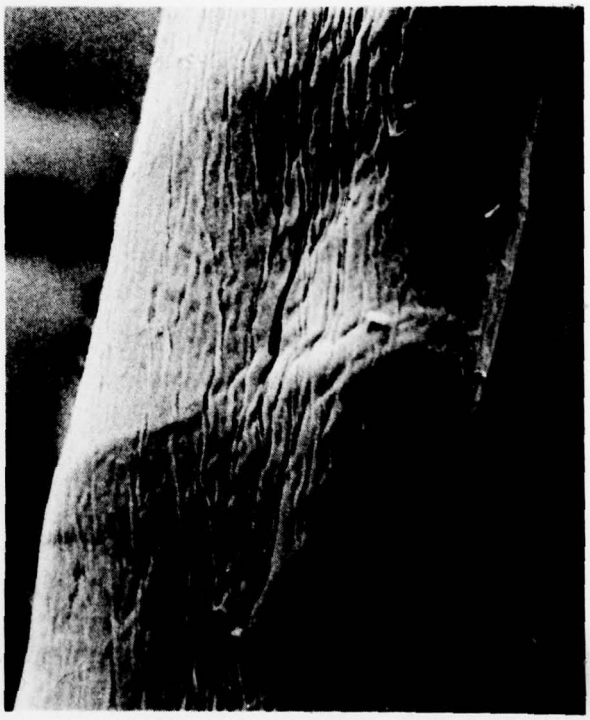
Scoured Acala Cotton - Sample Co (Dr. T.M. Wood)
Control, no enzyme.



⊢ 18 μm



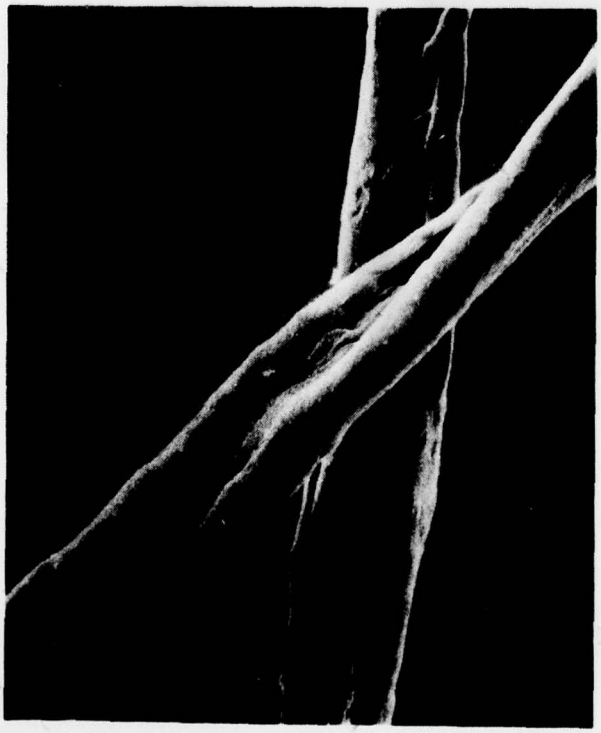
⊢ 5.6 μm



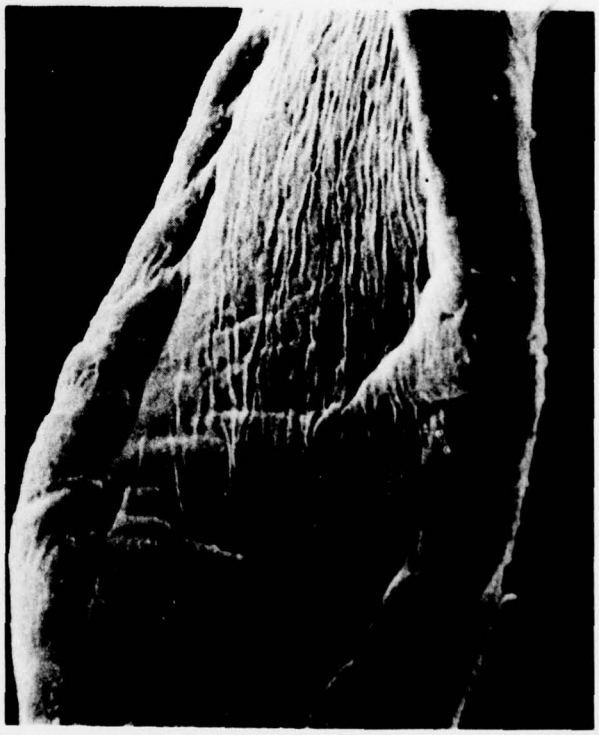
⊢ 1.8 μm

Scoured Acala Cotton - Sample C_x (Dr. T.M. Wood)
Treated with C_x fraction from T.koningii

18 μm



5.6 μm



1.8 μm

Scoured Acala Cotton - Sample C_1 (Dr. T.M. Wood)
Treated with C_1 fraction from T. Koningii

⊢ 18 μm

⊢ 5.6 μm

⊢ 1.8 μm

Scoured Acala Cotton - Sample C_{1+x} (Dr. T.M. Wood)

Treated with mixture of C_1 and Cx fractions from T. koningii

18 μm

5.6 μm

1.8 μm

1.8 μm