# FUNGAL CELLULASES

## I. GENERAL PROPERTIES OF UNPURIFIED ENZYME PREPARATIONS FROM *ASPERGILLUS ORYZAE*

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*Summary*

The culture filtrate from *Aspergillus oryzae* contains enzymes capable of depolymerizing sodium carboxymethyl cellulose (SCMC) and of splitting all $\beta$-glucosides tested. These enzymes are produced by the mould in the absence of cellulose or any other carbon source containing $\beta$-glucosidic linkages.

The action of the enzyme degrading SCMC (the $C_x$ enzyme of Reese, Siu, and Levinson 1949) and the $\beta$-glucosidase activities were followed iodometrically. The $\beta$-glucosidase action was also followed colorimetrically using *p*-nitrophenyl-$\beta$-glucoside as a substrate.

The variation in the activity of the $C_x$ enzyme with temperature, pH, and enzyme and substrate concentrations, and in the presence of inhibitors, was measured and some kinetic data are also given for the $\beta$-glucosidase activities. On the basis of these results it is concluded that the physicochemical properties of SCMC solutions make this compound an unsatisfactory substrate for kinetic studies. The true pH optimum of the $C_x$ enzyme is about 3.5; the apparent temperature optimum is very strongly influenced by reaction time.

The behaviour of the $C_x$ enzyme towards metal-complexing, oxidizing, and reducing agents can be explained if it is activated by ferric ions but the method of enzyme estimation used and the complexing properties of sodium carboxymethyl cellulose make proof of this hypothesis difficult. The enzyme activity is also strongly affected by various organic bases, notably caffeine and quinine. The behaviour of a $\beta$-glucosidase (salicinase) towards various reagents affecting the $C_x$ enzyme has also been tested.

## I. INTRODUCTION

Studies that have been commenced in this laboratory on the biochemistry of the cellulolytic fungi required the development of methods for the estimation of cellulase and related enzymes. For this purpose it seemed that the pool of enzyme protein from the culture filtrate of *A. oryzae*, which was already available (Crewther and Lennox 1950; Gillespie and Woods, unpublished data), might serve as a suitable source of material. This paper embodies a description of the methods finally adopted for enzyme estimation and their application to determining the general properties of the unpurified enzyme preparation.

It has long been known that taka-diastase and other products of *A. oryzae* and related species contain an active $\beta$-glucosidase ("mould emulsin"). Although the *flavus-oryzae* group of the aspergilli are usually classified among the non-cellulolytic fungi (Marsh *et al.* 1949), strong cellulase activity was found

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in culture filtrates by Grassmann and Rubenbauer (1931) and Freudenberg and Ploetz (1939). Modern concepts of enzyme specificity having reduced much of the earlier work on "cellulase" to a purely descriptive value, the general uncertainty that has so often in the past surrounded the definition of the substrate and activity of "cellulase" makes it worth while to confirm and extend such reports.

Its was not until the observations of Freeman, Baillie, and MacInnes (1948) on sodium carboxymethyl cellulose (SCMC) that a soluble substrate was readily available which permitted attack on the $\beta$-glucosidic linkages of a cellulose-like material in a homogeneous system. SCMC has been used in the work of Holden and Tracey (1950), Tracey (1950; 1951), Reese, Siu, and Levinson (1949), and Levinson and Reese (1950). It is water-soluble at D.S.* 0.4 and upwards. Calculation shows that at D.S. 0.4 about 40 per cent. and at D.S. 0.6 about 20 per cent. of the $\beta$-glucosidic linkages of the SCMC chains are between unsubstituted glucose residues. The figures of Holden and Tracey for the limiting breakdown of SCMC by snail cellulase are in agreement with the idea that these are the linkages attacked.

The breakdown of SCMC is thus an index of the presence of an enzyme that is capable of hydrolysing polymeric $\beta$-glucosidic linkages. Reese, Siu, and Levinson (1949) point out that this enzyme is not identical with "cellulase" as it has been understood in the past, since it is produced by fungi incapable of attacking native cellulose although capable of attacking degraded and solubilized celluloses. They therefore postulate that apparent "cellulase" activity in fungi is really the combined activity of two enzymes, "$C_1$," produced by cellulolytic organisms and capable of bringing native cellulose, perhaps by breaking cross links, into a form sufficiently soluble to be attacked by "$C_x$," an enzyme produced by both cellulolytic and many non-cellulolytic organisms. Reese and Downing (1951) have shown that the aspergilli can be broadly divided into two sections—those possessing "$C_x$" and "$C_1$" and able to attack native cellulose, and those possessing "$C_x$" alone and able to attack only soluble cellulose derivatives. The *flavus-oryzae* group falls into the latter section.

## II. Materials

### (*a*) *p-Nitrophenyl-β-D-glucoside*

This is as prepared from p-nitrophenol and $\beta$-glucose penta-acetate by the method of Helferich and Schmitz-Hillebrecht (1933) as white crystals of m.p. 164°C. (Goebel and Avery (1929) give 165°C.).

### (*b*) *Modified Celluloses*

Reprecipitated cellulose was prepared from cotton wool dissolved in phosphoric acid; cellodextrin by the ethanol fractionation of cotton wool degraded by solution in 72 per cent. sulphuric acid to a water-soluble form. The cellodextrin used had a degree of polymerization of about 100.

* D.S. = degree of substitution = average number of carboxymethyl groups per glucose residue.

### (c) *Sodium Carboxymethyl Cellulose*

A sample of Cellofas B (blend 874, medium viscosity) was obtained from Imperial Chemical Industries Ltd., Steventon, Ayrshire, through the kind offices of Dr. G. G. Freeman. The degree of substitution was 0.65 as determined by the sodium salt method of McLaughlin and Herbst (1950).

Stock solutions of SCMC were freshly made up at 1 per cent. strength by one of two methods as required:

(i) The SCMC was thoroughly mixed with 1/20 of the final volume of distilled water and allowed to stand overnight. The resulting jelly was carefully diluted by stirring in successive small additions of water until a clear viscous solution resulted and this was made up to volume.

(ii) The SCMC was beaten up with a little less than the required volume of water at about 80°C. in a Waring Blendor until the mixture was homogeneous and the solution allowed to cool and made up to volume.

Solutions made up by method (ii) were attacked by the mould enzyme about twice as fast as those made up by method (i) but were unstable, the rate of attack falling off to the lower level after 2-3 days. All solutions aged slowly at room temperature with separation of gel-like material and increased resistance to enzyme attack. Storage in a refrigerator accelerated these undesirable changes.

### (d) *"Mould Enzyme"*

The general properties of this material have been described by Crewther and Lennox (1952). A single batch of about 30 g. was used in all experiments. It contained some 10 per cent. of carbohydrate material as determined by the anthrone method of Morris (1948). Hydrolysis with 3 per cent. nitric acid and paper chromatography showed roughly equal amounts of mannose and galactose. This carbohydrate was not hydrolysed by the enzyme since prolonged incubation of enzyme solutions revealed no rise in reducing value or liberation of free sugars.

## III. Methods

### (a) *Estimation of* $C_x$ *Enzyme*

The method used is based on the iodine-reducing action of the aldehyde groups liberated by the enzyme (see Hinton 1940; Jermyn and Tomkins 1950, for the application of similar methods to pectin). As standard conditions, SCMC solution (1 per cent.), McIlvaine citrate-phosphate buffer (pH 5.0), and enzyme solution are mixed in the volume ratio 10 : 3 : 2 and incubated at 87°C. The iodine consumption (ml. 0.1N $I_2$) when 10 ml. of 0.1N $I_2$ reacts with 15 ml. of the digest under alkaline conditions is an index of the reducing groups liberated by the enzyme after any period of time. The total hydrolysis of 1 ml. of 1 per cent. SCMC solution should liberate reducing groups nearly equivalent to 1 ml. of 0.1N $I_2$; multiplication of the figure for iodine consumption by 10 therefore gives an approximate estimate of the percentage of glucosidic linkages broken in the substrate.

When iodine consumption is plotted against time a smooth curve is obtained. The tangent at the origin is estimated by fitting a parabolic expression to the curve and the slope expressed as ml. of 0.1N $I_2$ consumed per hour. This initial rate of iodine consumption is taken as the measure of enzyme activity.

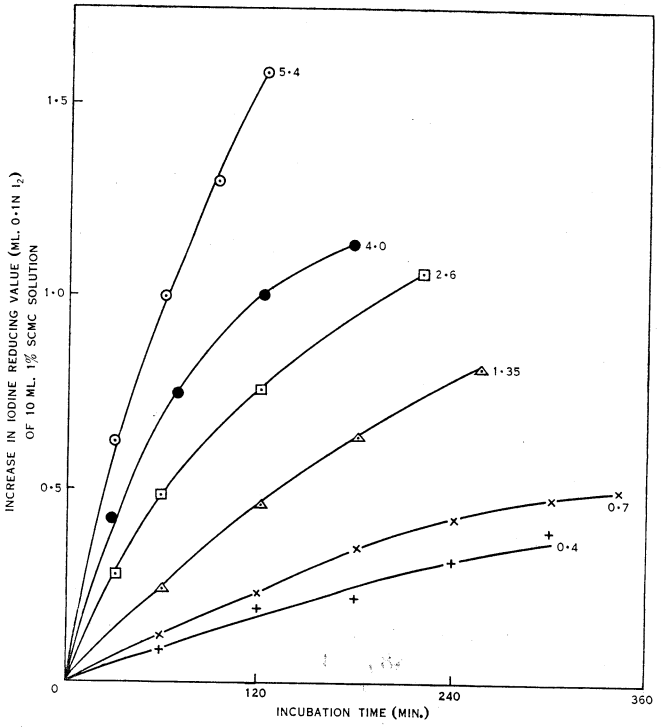


Fig. 1.—Progress curves for the hydrolysis of SCMC by crude *A. oryzae* enzyme under standard conditions. Final enzyme concentrations are indicated as mg./ml.

Figure 1 shows that the initial slope of the iodine consumption curve is very little different from that of the straight line joining the zero and 30-min. points over a wide range of enzyme concentrations. The iodine consumption during the first 30 min. of incubation was therefore routinely used ("short method") as a measure of enzyme activity. This simplified procedure is not valid under conditions departing very widely from those used as the standard.

### (*b*) *Iodometric Estimation of β-glucosidase*

The iodometric method can be used to determine β-glucosidase activity using salicin, aesculin, cellobiose, β-methyl glucoside, *p*-nitrophenyl-β-glucoside, and cellodextrin as substrates. The procedure is that used in the short method for the $C_x$ enzyme with modifications to allow for the peculiarities of individual substrates. With phenolic glucosides the volume of iodine consumed is not a measure of the glucose liberated since the liberated phenols also react with hypoiodite solutions. The method is therefore not directly quantitative but

is reproducible under the conditions stated, and the amount of iodine consumed can therefore be used as a purely comparative index of enzyme activity.

### (*c*) *Colorimetric Estimation of β-glucosidase*

The optical density at pH 8.5-9.0 due to liberated *p*-nitrophenol is determined after incubation of the enzyme at 28°C. with 0.001M *p*-nitrophenyl-*β*-glucoside. Provided the breakdown of substrate does not exceed about 20 per cent., optical density after 20 min. incubation is proportional to enzyme concentration. The standard pH of incubation for routine activity measurements is 5.0. This procedure is essentially the same as those of all authors who have followed Aizawa (1939) in using nitrophenyl glycosides as enzyme substrates.

### (*d*) *Visual Methods of Estimating SCMC Breakdown*

When three volumes of 1 per cent. SCMC solution are mixed with one volume of 10 per cent. lead acetate solution the mixture sets immediately to a stiff gel. If the SCMC has been previously partially degraded by small amounts of enzyme for varying periods the appearance passes through the stages of thick gel, thin gel broken up on shaking, curdy precipitate, and flocculent precipitate. Finally only the faint residual precipitate due to the enzyme protein is left. The breakdown has in general progressed well into the curdy precipitate stage before there is a measurable increase in iodine consumption.

Another visual index of SCMC breakdown is given by its power of forming complexes with iodine. On adding an equal volume of 0.1N iodine solution to a 1 per cent. solution of SCMC, making alkaline, and reacidifying, a thick blue-black precipitate is formed in the solution. With increasing degradation of the SCMC this passes to a blue-black solution and then through brown-black and dark brown solutions to the normal iodine colour. The change is complete at about 1.0 per cent. breakdown. This behaviour complicates the iodometric method of estimating $C_x$ activity although the iodine-SCMC bonding is not sufficiently strong to affect the accuracy of the titration if this is done slowly with vigorous shaking in the first stages.

Both these tests are sufficiently sensitive to detect the presumably microbiological deterioration of SCMC solutions that have been standing for some time at room temperature.

## IV. Enzyme Characteristics

### (*a*) *Enzyme Production and Culture Conditions*

The strain of *A. oryzae* (292-4795) used in this laboratory by Maxwell (1952) for production of the crude enzyme was grown in shake culture (120 cycles/min.) in parallel with *Stachybotrys atra,* a recognized cellulolytic organism. Three carbon sources were used—sucrose, SCMC, and ground cotton wool (60 mesh), the mineral source being the Waksman-Carey medium as modified by Fahraeus (1947) with the addition of 12 μg./l. of biotin required for the optimal growth of *S. atra.* The growth of *S. atra* on all three carbon sources was good, that of *A. oryzae* good on sucrose, scanty on SCMC, and

only demonstrable by staining and microscopy on ground cotton wool. The results are incorporated in Table 1. The $\beta$-glucosidase was measured colorimetrically, and protease by the gravimetric method of Crewther (1952). The activity values for $\beta$-glucosidase are given on a subjective scale of visual intensity; those for $C_x$ and protease may be taken as arbitrary units in this context. Values less than 1.0 for protease activity are not significant.

TABLE 1

GROWTH AND ENZYME PRODUCTION BY *ASPERGILLUS ORYZAE* AND *STACHYBOTRYS ATRA* GROWN IN SHAKE CULTURE AT 28°C. ON MODIFIED WAKSMAN-CAREY MEDIUM

| Carbon Source | Period of Incubation (days) | Sucrose (2% w/v) | | SCMC (2% w/v) | | Cellulose (ground cotton wool) (2% w/v) | |
|---|---|---|---|---|---|---|---|
| | | *A. oryzae* | *S. atra* | *A. oryzae* | *S. atra* | *A. oryzae* | *S. atra* |
| $C_x$ enzyme | 3 | – | – | 0·35 | 0·23 | 0·04 | 0·24 |
| $\beta$-Glucosidase | | ± | – | + | ++++ | + | +++ |
| $C_x$ enzyme | 5 | – | – | 0·47 | 0·42 | 0·27 | 0·06 |
| $\beta$-Glucosidase | | – | ± | ++++ | + | ± | + |
| $C_x$ enzyme | 10 | – | – | 0·11 | 0·57 | 0·15 | – |
| $\beta$-Glucosidase | | – | – | ++++ | ++ | + | ++ |
| Protease | | 0·2 | 2·3 | 5·3 | 1·2 | 1·7 | 1·1 |
| $C_x$ enzyme | 16 | – | 0·24 | 0·44 | 0·57 | – | 0·30 |
| $\beta$-Glucosidase | | – | – | +++ | +++ | ± | ++ |
| Protease | | 0·5 | 1·0 | 6·7 | 0·6 | 1·2 | 0·0 |
| $C_x$ enzyme | 21 | 0·10 | 0·59 | 0·34 | Not done | – | – |
| $\beta$-Glucosidase | | + | ± | ++ | ++ | ± | ± |

### (*b*) *General Properties of the Enzymes*

The complex nature of the mixture in which the $C_x$ enzyme and $\beta$-glucosidase of *A. oryzae* appear as minor components render certain lines of investigation unprofitable unless extensive purification can be accomplished. No work therefore has been carried out on inactivation by heat and pH change or on the nature of the end-products of enzyme action. In the first case, attack by proteases renders any conclusions worthless, and in the second, enzymes are present capable of attacking any end-products other than simple monomers. Nevertheless, other data allow conclusions on these subjects to be drawn in some instances. Chromatographic examination of the products of hydrolysis of cellodextrin by crude *A. oryzae* enzyme showed that in the early stages of the hydrolysis products containing one, two, and three glucose residues were about equally prominent. This observation is incompatible with a single mechanism of hydrolysis with cellobiose units being removed from the polymeric $\beta$-glucoside chain and subsequently being hydrolysed to glucose, but is compatible with the simultaneous action of more than one mode of hydrolysis.

The conclusions of Reese, Siu, and Levinson (1949) on the existence of at least two enzymes ($C_1$ and $C_x$) in the cellulase complex are borne out by the behaviour of the *A. oryzae* enzyme. Although attack on SCMC and cellodextrin is vigorous there is little or no attack on native cellulose or insoluble derived celluloses. Table 2 illustrates these points.

TABLE 2

ATTACK ON CELLULOSE AND CELLULOSE DERIVATIVES BY *A. ORYZAE* ENZYME

Substrate (500 mg.) in 15 ml. pH 5.0 McIlvaine buffer containing 0.2 per cent. sodium benzoate; enzyme concentration 5 mg./ml.; incubation temperature 37°C.; incubation time 24 hr.

| Substrate | Ground Cotton Wool (60 mesh) | Ground Whatman No. 1 Filter Paper (60 mesh) | Ground Cellophane (60 mesh) | Reprecipitated Cellulose | Cellodextrin | SCMC |
|---|---|---|---|---|---|---|
| Reducing groups liberated (as mg. glucose) | 0·95 | 1·08 | 0·56 | 0·95 | 39·5 | 28 |

No statistically significant effect could be demonstrated on the strength of cotton sewing thread incubated in 10 per cent. enzyme solution at pH 5 at room temperature and 37°C. for periods up to a week. None the less, electrophoretic and chromatographic experiments have shown a marked tendency for break-up of the filter paper to occur at places where the enzyme has been in contact with the paper for considerable periods. Miss M. E. Maxwell has reported to the author that, during the filtering of certain batches of culture, rapid disintegration of the filter papers was observed. It is possible therefore that a little $C_1$ enzyme was originally present but was more liable to storage and protease action than the $C_x$ enzyme.

Besides the compounds with $\beta$-glucosidic linkages that were further investigated the following $\beta$-glucosides were split by the enzyme: rutin, quercitrin, arbutin, phenyl-$\beta$-glucoside, $\alpha$-naphthyl-$\beta$-glucoside. The phenyl and naphthyl-$\beta$-glucosides were split at about the same rate as salicin. Rutin, quercitrin, and arbutin behaved rather like aesculin but because of erratic results in the iodometric method were not further investigated.

Holden and Tracey (1950) found that the attack of snail cellulase on SCMC did not proceed to completion but only to a point corresponding to the breakage of all linkages between two adjacent unsubstituted glucose residues. Calculations following the method of Spurlin (1939) show that in a SCMC of D.S. 0.65 about 23 per cent. of the $\beta$-glucosidic linkages should be between such unsubstituted glucose residues. The degree of breakdown of cellodextrin should on this theory be 100 per cent. This analysis assumes an enzyme of the $\alpha$-amylase or polygalacturonase type which attacks all the linkages of a straight-chain substrate at random. Enzymes with properties similar to $\beta$-amylase, splitting the cellulose chain to cellobiose units, such as those studied

by Levinson, Mandels, and Reese (1951) would give different results when acting alone but the presence of active cellobiase in the *A. oryzae* enzyme would give the same final results in both cases.

It was found that only by using very strong enzyme solutions over relatively short periods was a figure for the limiting decomposition of SCMC attained. Similar phenomena were observed using cellodextrin as substrate. The highest degree of breakdown observed with cellodextrin was 57 per cent., using an enzyme concentration of 24 mg./ml. Increasing difficulties due to the large blanks caused by the iodine consumption of the enzyme solutions prevented the method being pushed beyond this point but the steady increase in maximum decomposition with increasing enzyme concentration made it probable that 100 per cent. decomposition was attainable if the enzyme concentration were raised sufficiently. Figure 2 illustrates these points.

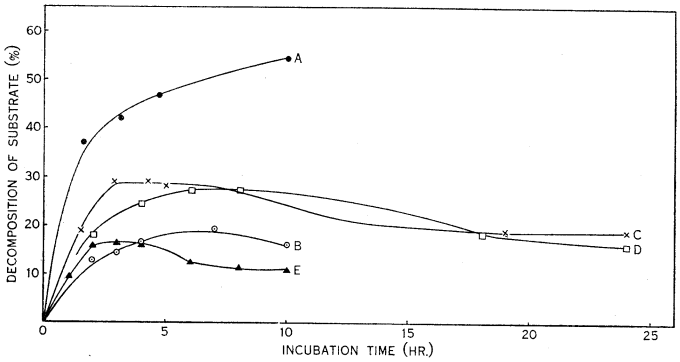


Fig. 2.—Effect of various factors on the breakdown of cellodextrin and SCMC by the *A. oryzae* enzyme, under the standard conditions with toluene and 0.2 per cent. sodium benzoate added.
*A*, cellodextrin 0.33 per cent., enzyme 24 mg./ml.
*B*, cellodextrin 0.67 per cent., enzyme 6 mg./ml.
*C*, SCMC 0.13 per cent., enzyme 25 mg./ml.
*D*, SCMC 0.13 per cent., enzyme 15 mg./ml.
*E*, SCMC 0.67 per cent., enzyme 6 mg./ml.

The addition of sodium benzoate and toluene inhibited gross microbiological contamination for 24 hr. at least, and destruction of substrate through this agency was negligible. The disappearance of iodine-reacting material could therefore best be explained if synthesizing enzymes were in competition with the hydrolytic enzymes and the effects of the synthesizing enzymes became apparent when the hydrolytic enzymes were inactivated. Interference with the normal course of hydrolysis would also occur in the presence of trans glucosidase systems, and Pazur and French (1951) have in fact found a trans glucosidase in *A. oryzae* culture filtrates that acted on $\alpha$-glucosidic substrates. Takano and Miwa (1950) have shown that the $\beta$-glucosidase and glucotransferase activities of apricot emulsin are probably identical. Incubation at room temperature of the enzyme (2.5 mg./ml.) with 1 per cent. solutions of glucose and cellobiose followed by paper chromatography of samples withdrawn at

intervals showed that in the cellobiose solutions, besides glucose and residual cellobiose, higher molecular weight material, presumably oligosaccharides, could be demonstrated between 18 and 90 hr. of incubation. The matter was not investigated further.

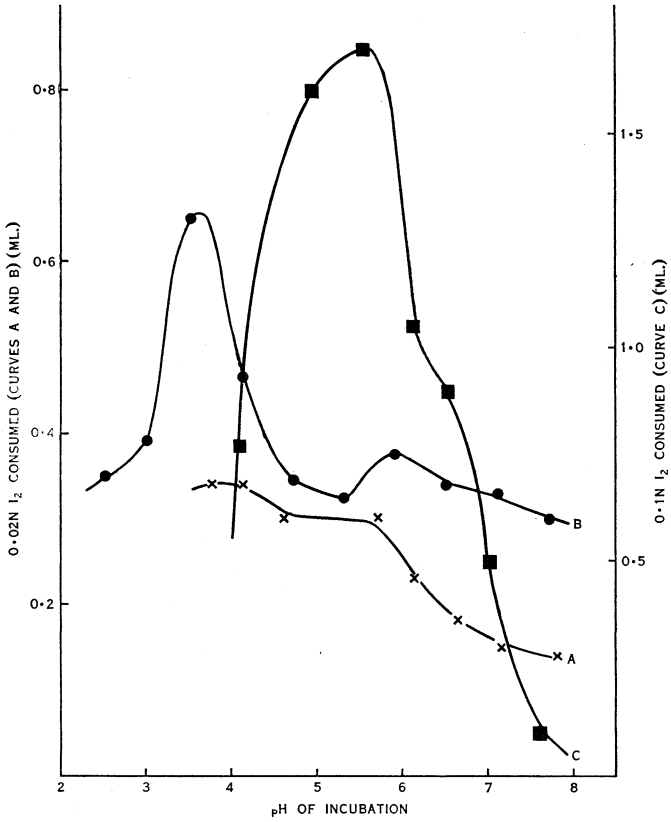


Fig. 3.—pH-activity curves of the *A. oryzae* enzyme using SCMC as substrate in citrate-phosphate buffers at 37°C.

*A*, SCMC 0.67 per cent., enzyme 0.14 mg./ml., incubated 30 min.

*B*, SCMC 0.10 per cent., enzyme 0.36 mg./ml., incubated 30 min.

*C*, SCMC 0.67 per cent., enzyme 10 mg./ml. incubated 6 hr.

The value of approximately 29 per cent. for the maximum breakdown of SCMC seems rather high to agree with the calculated value of 23 per cent., but until less complex enzyme mixtures are available it cannot be decided whether other linkages than those between unsubstituted glucoses are attacked. Kristiansson (1950) suggests that barley malt "cellulase" attacks the linkages between all the glucose residues of hydroxyethylcellulose with equal ease.

### (c) *Effects of pH*

The pH-activity curve for the hydrolysis of SCMC is strongly influenced by the concentration of the substrate and the length of the incubation period. Figure 3, curve *B* seems to show the effect of pH on the enzyme activity with the least inactivation and disturbance by colloid effects.

Figure 4 confirms the choice of pH 5.0 as a reasonable average pH at which to measure a series of $\beta$-glucosidase activities.

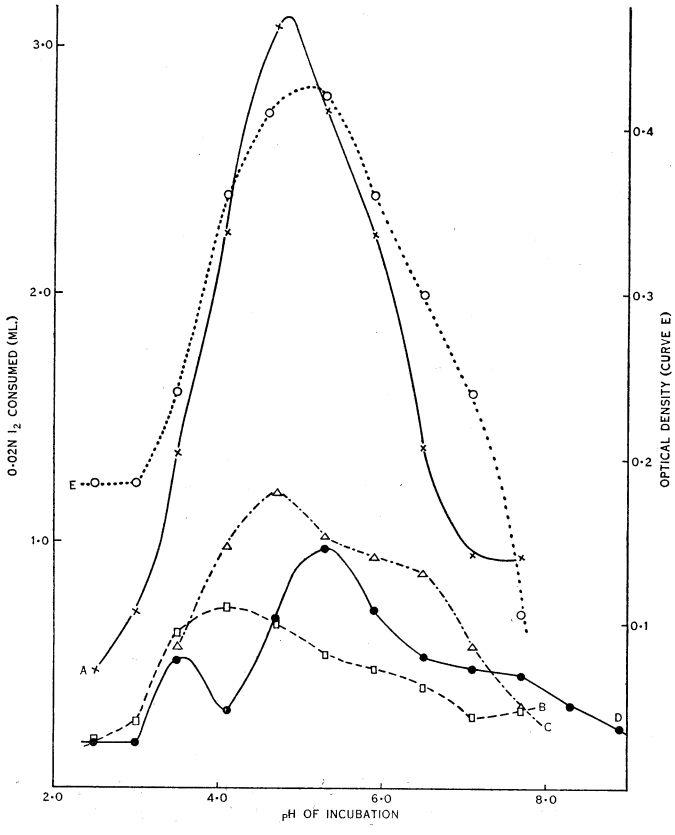


Fig. 4.—pH-activity curves for the *A. oryzae* enzyme acting on various $\beta$-glucosidic substrates in citrate-phosphate buffers at 37°C., incubation time 30 min.

*A*, 0.0013M aesculin, enzyme 0.13 mg./ml.
*B*, cellodextrin 0.13 per cent., enzyme 0.13 mg./ml.
*C*, 0.0067M salicin, enzyme 0.13 mg./ml.
*D*, 0.0067M cellobiose, enzyme 0.13 mg./ml.
*E*, colorimetric $\beta$-glucosidase, standard conditions, enzyme 0.4 mg./ml.

### (d) *Effects of Temperature*

Figure 5*B* shows that the basic assumption on which the "short method" is based—that the slope of the curve of iodine consumption against time is nearly

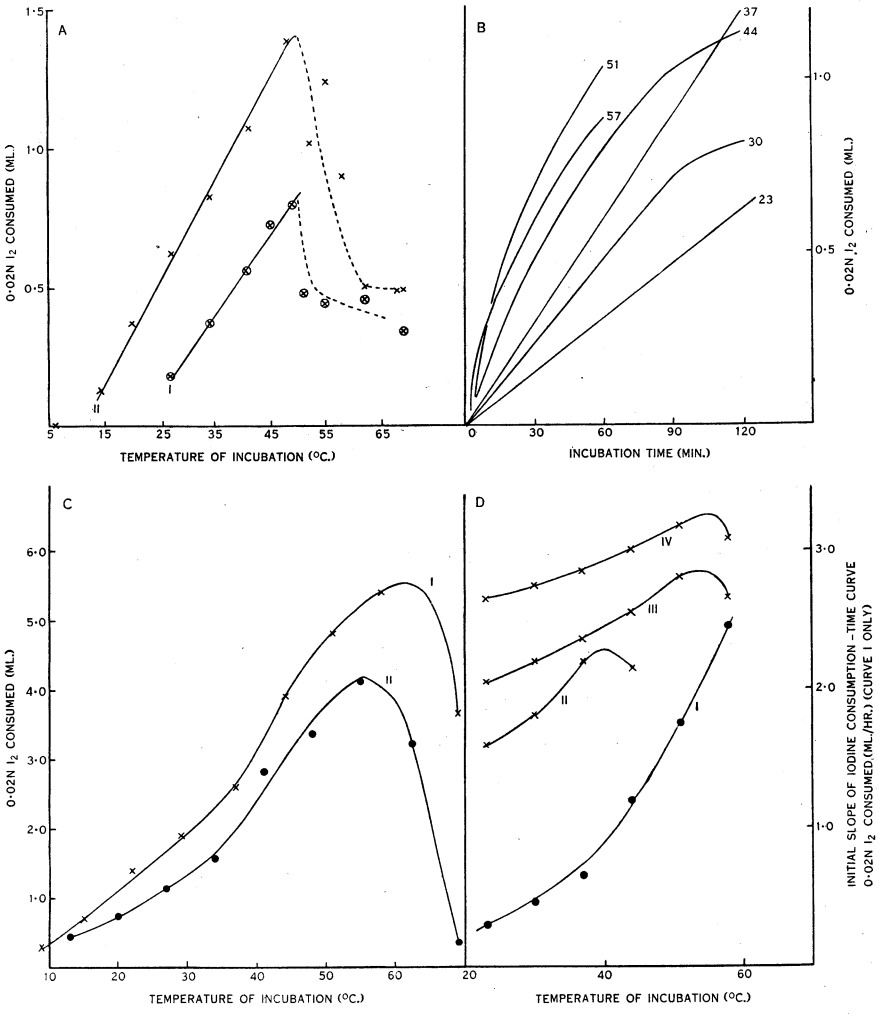


Fig. 5.—Effect of temperature on *A. oryzae* enzymes.

*A*, activity of the $C_x$ enzyme in decomposing SCMC at various temperatures measured by the short method. I, enzyme 0.2 mg./ml., added in solution. II, enzyme 0.4 mg./ml., added dry.

*B*, progress of the decomposition of SCMC by the $C_x$ enzyme at various temperatures: SCMC 0.10 per cent., enzyme 0.2 mg./ml. Incubation temperature is indicated in °C.

*C*, decomposition of cellodextrin and salicin at various temperatures, enzyme 0.4 mg./ml. I, cellodextrin 0.67 per cent. II, 0.0067M salicin.

*D*, effect of temperature on decomposition of SCMC (from data of Figure 5*B*). I, initial slope of iodine consumption curves plotted against temperature. II, III, IV, iodine consumption after various incubation times plotted against temperature: the curves have been displaced arbitrarily along the abcissa; II, 2 hr. incubation; III, 1 hr.; IV, 0.5 hr.

linear for the first hour or so—is only valid up to about 40-45°C. In conformity with this, in Figure 5*A*, curve I shows a sharp drop in apparent enzyme activity above this temperature to a low and fairly constant level. Since it seemed that this behaviour might be due to inactivation on mixing cold enzyme and hot substrate solutions the method was modified by adding the enzyme as a dry powder to the substrate solution with vigorous mixing. Curve II (Fig. 5*A*), shows relatively higher but still erratic values in the region of 50-55°C.

The method was therefore varied by reducing the final SCMC concentration to a level (0.10 per cent.) where the curve relating substrate concentration to activity (Fig. 7*C*) showed that the effect of substrate concentration on enzyme kinetics was appreciable, adding the enzyme as the dry powder and working according to the long method. None the less, inactivation above 60°C. was too rapid to make it possible to plot the breakdown with time; even at lower temperatures in the region 40-60°C. inactivation was sufficiently rapid to give a marked fall in the apparent optimum temperature as the length of the incubation period was increased.

The plot of $\log_{10}$ (rate of reaction) against the reciprocal of absolute temperature should be linear with a slope of $E/2.303R$ ($E$ = energy of activation,

TABLE 3

ACTIVATION ENERGY OF CERTAIN HYDROLYTIC ENZYMES OF *A. ORYZAE*

| Activity | Enzyme Concentration (mg./ml.) | Substrate Concentration | pH (McIlvaine buffer) | Temperature Range (°C.) | Activation Energy (cal./g. mol.) |
|---|---|---|---|---|---|
| Hydrolysis of SCMC | 1·0 | 0·10% | 5·0 | 23 - 57 | 10800 |
| Hydrolysis of cellodextrin | 0·20 | 0·67% | 5·0 | 13 - 54 | 10250 |
| Hydrolysis of salicin | 0·20 | 0·067M | 5·0 | 15 - 51 | 9800 |

$R$ = gas constant). Figure 6 shows that the relationship for three substrates is in fact linear and in Table 3 the calculated energies of activation are given. The differences between the various figures are within the experimental errors of the determination.

### (*e*) *Effect of Enzyme Concentration*

When the initial rate of the iodine consumption curve is plotted against enzyme concentration for the decomposition of SCMC, a straight line is obtained as in Figure 7*A*. Hence it is possible, using the long iodometric method and a final SCMC concentration of 0.67 per cent., to estimate the $C_x$ enzyme concentration in a solution of unknown strength. A solution will be said to contain *one unit of* $C_x$ *enzyme per ml.* when under the standard conditions it

gives a curve for the rise in iodine consumption with time, with an initial slope of 0.1 ml. 0.1N $I_2$/hr. Using the unit so defined the crude enzyme preparation from *A. oryzae* contains 4.9 units/mg.

In Figure 7*B* are shown initial rate plots for cellobiose and cellodextrin done under the same conditions as SCMC; they are both linear, in agreement with normal enzyme behaviour. The plots of iodine consumption in the short method against enzyme concentration for aesculin and salicin were in effect used as calibration curves for measuring enzyme activity against these substrates; the kinetics were not investigated exactly and no explanation will therefore be attempted for the non-linear relation found with salicin.

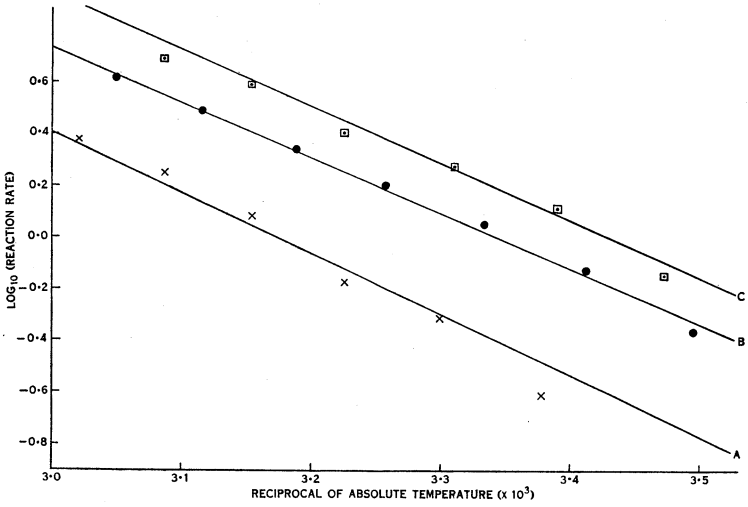


Fig. 6.—Transposition of temperature-activity curves from Figure 5. Substrates: *A*, SCMC; *B*, salicin; *C*, cellodextrin.

### (*f*) *Effect of Substrate Concentration*

When an attempt was made to follow the variation of $C_x$ enzyme activity with the concentration of SCMC solution by making up SCMC solutions of relatively high concentration (1.0, 1.5 per cent.) and preparing serial dilutions, it was found that the results obtained for enzyme activity were extremely erratic and depended on the previous history of the solution. In the range 0.15-1.0 per cent. final concentration of SCMC the values for the activity were higher or lower than the initial value and were not reproducible at successive attempts. On standing 3-5 days the activity values tended to rise or fall to a value nearly the same as that of the original undiluted solution. Below 0.15 per cent. final concentration the activity fell away towards zero. The following method was finally found to give reproducible results. SCMC was made up directly as a 0.15 per cent. solution and a series of dilutions prepared. These and the original solution were allowed to stand 3 days and the activity then measured.

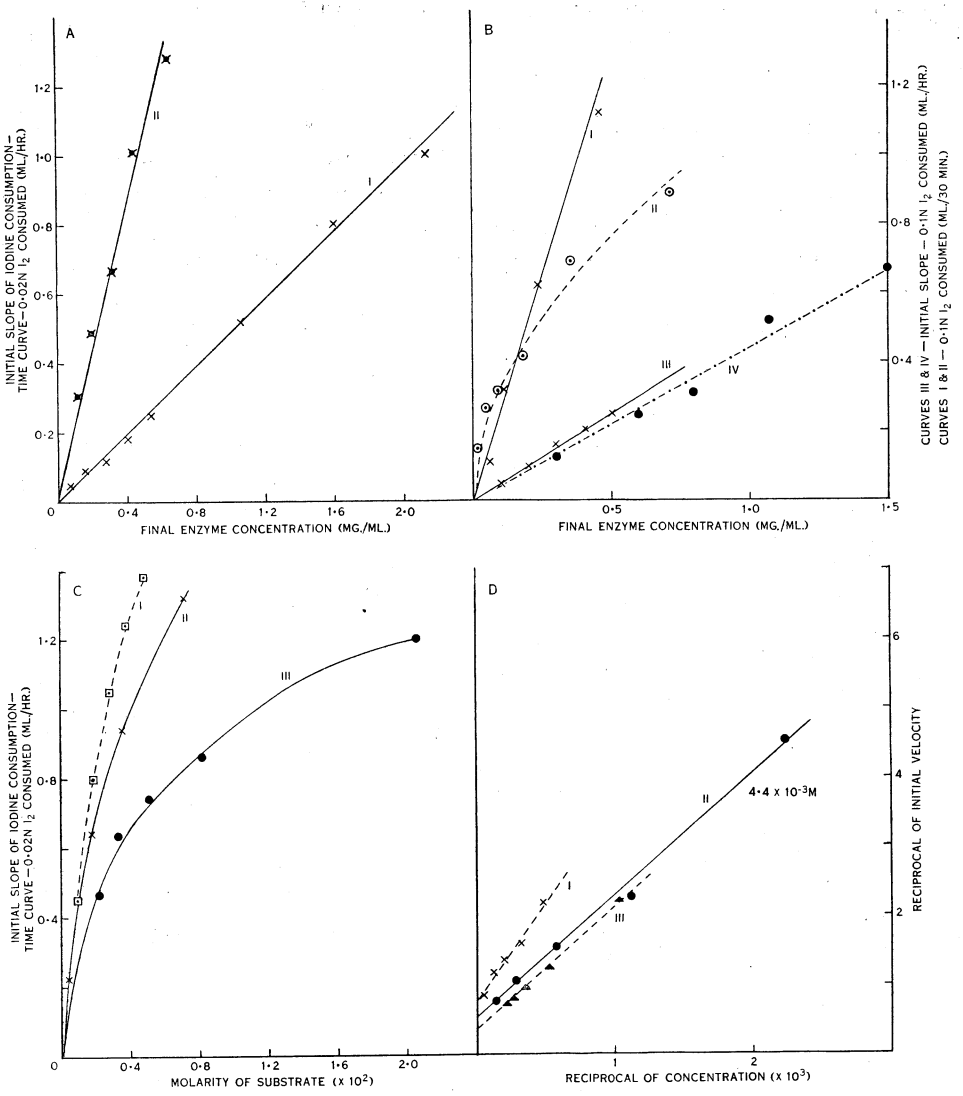


Fig. 7.—Effect of enzyme and substrate concentration on the activity of *A. oryzae* enzymes.

*A*, enzymic hydrolysis of SCMC at 37°C. I, 0.67 per cent. SCMC, pH 5.5, 0.1N $I_2$. II, 0.10 per cent. SCMC, pH 5.1, 0.02N $I_2$.

*B*, enzymic hydrolysis of various substrates at 37°C. and pH 5.0 at varying enzyme concentrations. I, 0.0013M aesculin. II, 0.0013M salicin. III, 0.0067M cellobiose. IV, 0.33 per cent. cellodextrin.

*C*, enzymic hydrolysis of various substrates at 37°C. and pH 5.0 at varying substrate concentrations. I, SCMC, enzyme 0.66 mg./ml. II, cellodextrin, enzyme 0.2 mg./ml. III, salicin, enzyme 0.66 mg./ml. SCMC and cellodextrin molarities are those of the calculated glucose equivalents.

*D*, transposition of Figure 7*C*. I, salicin. II, cellodextrin. III, SCMC.

The results for the hydrolysis of SCMC, salicin, and cellodextrin have been used to determine the Michaelis constants for the three enzymic reactions according to the method of Lineweaver and Burk (1934).

### (g) *Inhibition and Activation of the* $C_x$ *Enzyme*

There appears to be very little in the literature on the inhibition of cellulase; the scattered literature on the inhibition of $\beta$-glucosidase has been summarized by Veibel (1950, p. 617), known inhibitors being heavy metals, oxidizing agents, formaldehyde, and carbonyl reagents. A few substances are listed by Ziese (1931) as inhibitors of the hydrolysis of hydroxyethyl cellulose by snail cellulase. A systematic preliminary survey under standard conditions was therefore instituted to find what classes of substances inhibit the *A. oryzae* enzyme.

TABLE 4

MICHAELIS CONSTANTS FOR THE *A. ORYZAE* ENZYMES HYDROLYSING CERTAIN $\beta$-GLUCOSIDIC SUBSTRATES (FROM FIGURE 7*D*)

| Substrate | Enzyme Concentration (mg./ml.) | Temperature (°C.) | pH (McIlvaine buffer) | $K_m$ (g.-mol/l.) |
|---|---|---|---|---|
| Salicin | 0·066 | 37 | 5·0 | $3{\cdot}6\times10^{-3}$ |
| Cellodextrin (D.P. 100) | 0·2 | 37 | 5·0 | $3{\cdot}2\times10^{-3}$ |
| SCMC | 0·66 | 37 | 5·0 | $4{\cdot}4\times10^{-3}$ |

It was found that SCMC solutions prepared by method (i) (dissolution in the cold) gave highly erratic results for the various reagents tried. Solutions made by method (ii) were therefore freshly prepared every 2 days. As a standard procedure the short method for estimating cellulase was employed, the final concentration of crude enzyme being 0.4 mg./ml., at 37°C., and 1 ml. of reagent solution being added to each 15 ml. of the enzymic digest. The iodometric method employed imposed a severe limitation on the concentration of any reagent that itself reacted with iodine or liberated iodine from potassium iodide, and $10^{-3}$M was usually the maximum permissible concentration to give a workable blank (titration was with 0.02N reagents throughout). With each series of reagents tested a blank experiment was run with only water added as a check on alteration in properties of the SCMC solution or small changes in enzyme concentration. The velocity found in the presence of the reagent was reported as percentage of the velocity in the blank experiment.

When it was found that 8-hydroxyquinoline alone amongst metal chelating agents had an inhibitory effect on the enzyme, it seemed possible that this might be due to a competition between the chelating agent and the citrate and phosphate ions of the buffer. Acetate buffer (0.1M) of pH 5.0 was therefore used in place of the McIlvaine buffer. The final pH of the mixture under the standard conditions was now 5.1 instead of 5.5 as found with the McIlvaine buffer, but the iodine consumption using SCMC solution freshly prepared by method (ii) was almost identical (1.20 ml. 0.02N iodine).

TABLE 5

ACTIVITY OF VARIOUS REAGENTS AS ACTIVATORS OR INHIBITORS OF THE ENZYMIC DECOMPOSITION OF SCMC, USING TYPE II SCMC SOLUTIONS AND McILVAINE BUFFER

| Reagent Type | Reagent | Subsidiary Effect | Concentration | Percentage of Original Reaction Velocity |
|---|---|---|---|---|
| Metal ions | $Co^{++}$ | | $10^{-3}$M | 119 |
| | $Cu^{++}$ | | $10^{-3}$M | 92 |
| | $Cd^{++}$ | | $10^{-3}$M | 119 |
| | $Cr^{+++}$ | | $10^{-3}$M | 112 |
| | $Ni^{++}$ | | $10^{-3}$M | 120 |
| | $Zn^{++}$ | | $10^{-3}$M | 108 |
| | $Al^{+++}$ | | $10^{-3}$M | 109 |
| | $Mn^{++}$ | | $10^{-3}$M | 94 |
| | $Fe^{+++}$ | Oxidizing agent | $10^{-3}$M | 134 |
| | $Fe^{+++}$ | | $10^{-2}$M | 115 |
| | $Mg^{++}$ | | $10^{-3}$M | 128 |
| | $Mg^{++}$ | | $10^{-2}$M | 120 |
| | $Cu^{++}$ | | $10^{-3}$M | 129 |
| | $Pb^{++}$ | Thiol reagents | $10^{-3}$M | 103 |
| | $Ag^{+}$ | | $10^{-3}$M | 94 |
| Metal complexing reagents | 8-Hydroxyquinoline | | $10^{-3}$M | 9 |
| | Sodium diethyldithiocarbamate | | | Decomposed by enzyme |
| | Nitroso R salt | | $10^{-3}$M | 108 |
| | $\alpha$-Nitroso-$\beta$-naphthol | | $10^{-3}$M | 96 |
| | Diphenylcarbazide | | $10^{-3}$M | 146 |
| | Ferron | | $10^{-3}$M | 125 |
| | Cupferron | | $10^{-3}$M | 54 |
| | Dimethylglyoxime | | $10^{-3}$M | 121 |
| | BAL-intrav. | Thiol | $10^{-4}$M | 129 |
| | Quinalizarin | | $10^{-4}$M | 90 |
| | Titan yellow | | $5 \times 10^{-4}$M | 106 |
| | Rubeanic acid | Thiol | $10^{-4}$M | 112 |
| | Formaldoxime | | $10^{-3}$M | 112 |
| | Rhodizonic acid | | $10^{-3}$M | 49 |
| | Salicylaldoxime | | $10^{-3}$M | 90 |
| | $\alpha$-Benzoinoxime | | $10^{-3}$M | 137 |
| | Cyanide | | $10^{-3}$M | 0 |
| | Thiocyanate | | $10^{-3}$M | 103 |
| | Hexametaphosphate | | 1% | 131 |
| Thiol reagents | Iodosobenzoic acid | Oxidizing agent | $10^{-3}$M | 176 |
| | $p$-Chloromercuribenzoic acid | | $10^{-3}$M | 126 |
| $-NH_2$ reagents | Nitrite | | | No end point |
| | Formaldehyde | Reducing agent | $10^{-3}$M | 90 |
| Carbonyl regeants | Hydrazine | Reducing agents | $10^{-3}$M | 124 |
| | Phenylhydrazine | | $10^{-3}$M | 146 |
| | Semicarbazide | | $10^{-3}$M | 121 |
| | Thiosemicarbazide | Thiol | $10^{-3}$M | 101 |

TABLE 5 (*Continued*)

| Reagent Type | Reagent | Subsidiary Effect | Concentration | Percentage of Original Reaction Velocity |
|---|---|---|---|---|
| Sugar complexing Agents | Boric acid | | $10^{-2}$M | 109 |
| | Boric acid | | $3 \times 10^{-1}$M | 121 |
| | Molybdate | Oxidizing agent | $10^{-2}$M | 189 |
| | Tungstate | | $10^{-2}$M | 114 |
| | Tartar emetic | | $10^{-2}$M | 78 |
| Reducing agents | Bisulphite | Carbonyl reagent | $10^{-3}$M | 78 |
| | Dithionite | | $10^{-3}$M | 0 |
| Oxidizing agents | Permanganate | | $10^{-3}$M | 400 |
| | Chromate | | | No end point |
| | Ferricyanide | | $10^{-3}$M | 110 |
| | *p*-Benzoquinone | | $10^{-3}$M | 47 |
| Thiols | Thioglycollate | | $10^{-3}$M | 227 |
| | Cystine | | $10^{-3}$M | 167 |
| | Cysteine | | $10^{-3}$M | 147 |
| | Sulphide | | $10^{-3}$M | 0 |
| Growth hormones | β-Naphthoxyacetic acid | | $10^{-3}$M | 98 |
| | β-Indole propionic acid | | $10^{-3}$M | 62 |
| | β-Indoleacetic acid | | $10^{-3}$M | 35 |
| | 2, 4-Dichlorophenoxy-acetic acid | | $10^{-3}$M | 105 |
| Bases (complex) | Caffeine | | $10^{-1}$M | 39 |
| | Adenine | | $5 \times 10^{-2}$M | 50 |
| | Quinine | | $10^{-2}$M | 40 |
| | Betaine | | $10^{-1}$M | 17 |
| | Choline chloride | | $10^{-1}$M | 105 |
| Simple amines | Methylamine hydro-chloride | | $10^{-1}$M | 72 |
| | Dimethylamine hydrochloride | | $10^{-1}$M | 51 |
| | Trimethylamine hydrochloride | | $10^{-1}$M | 175 |
| | Tetramethyl ammon-ium bromide | | $10^{-1}$M | 104 |
| Miscellaneous | Silicofluoride | | $10^{-1}$M | 97 |
| | Fluoride | | $10^{-2}$M | 107 |
| | Stearate | | $3 \times 10^{-4}$M | 119 |
| | Iodine | | $10^{-4}$M | 116 |
| | Azide | | $10^{-3}$M | 108 |
| | Arsenate | | $10^{-3}$M | 104 |
| | Gluconate | | $10^{-3}$M | 80 |
| | Gluconate | | $10^{-2}$M | 75 |
| | Galactonate | | $10^{-3}$M | 93 |
| | Galactonate | | $10^{-2}$M | 107 |

The data collected for various reagents in McIlvaine buffer are summarized in Table 5. Certain of the classes of reagents tried were suggested by the conclusions of authors working with similar enzyme systems, for instance the inhibition of amylase by plant growth hormones (Volker 1950) and caffeine and other bases (Vincent and Lagreu 1950), and galactosidase inhibition by gluconate and galactonate (Nishizawa 1949). Deviations from the normal reaction rate of ± 10 per cent. or less can be regarded as not significant.

Table 6 records results with the use of acetate buffer. Only certain of the reagents listed in Table 5, which had been found to give marked inhibition or which were useful for comparison purposes, were tested in this medium.

TABLE 6

ACTIVITY OF VARIOUS REAGENTS AS ACTIVATORS OR INHIBITORS OF THE ENZYMIC DECOMPOSITION OF SCMC IN ACETATE BUFFER

| SCMC Solution | Reagent | Concentration | Percentage of Original Reaction Velocity |
|---|---|---|---|
| Type I | Phosphate | $10^{-3}$M | 113 |
| | | $10^{-2}$M | 147 |
| | | $3 \times 10^{-2}$M | 193 |
| | Citrate | $10^{-2}$M | 20 |
| | Oxine | $10^{-4}$M | 120 |
| | | $10^{-3}$M | 0 |
| | Ferron | $10^{-3}$M | 160 |
| | (Reaction with same SCMC solution in McIlvaine buffer) | | 194 |
| Type II | Citrate | $10^{-2}$M | 73 |
| | Phosphate | $10^{-2}$M | 63 |
| | Cyanide | $10^{-3}$M | 46 |
| | Dithionite | $10^{-3}$M | 0 |
| | Cupferron | $10^{-3}$M | 0 |
| | Nitroso R salt | $10^{-3}$M | 66 |
| | Oxine | $10^{-4}$M | 101 |
| | | $10^{-3}$M | 90 |
| | Ferron | $10^{-3}$M | 102 |
| | Caffeine | $10^{-1}$M | 56 |
| | Quinine sulphate | $10^{-2}$M | 44 |
| | $\beta$-Indolepropionic acid | $10^{-3}$M | 59 |
| | Betaine | $10^{-1}$M | 30 |

The system acetate buffer plus cupferron was chosen for further study since it gave a reasonably sharp end-point uncomplicated by colour formation and precipitates and also since the results of Albert and Gledhill (1947) show that only three ions — $Fe^{++}$, $Fe^{+++}$, and $Cu^{++}$ — react with cupferron under "physiological" conditions. The effect of cupferron concentration on the activity of the $C_x$ enzyme is shown in Figure 8, curve *A*. A set of experiments was therefore set up in which the ability of these three ions to reverse the cupferron inactivation of the enzyme was tested. The results are summarized in Table 7. Reactivation by $Fe^{++}$ and by $Fe^{+++}$ cannot be regarded as indepen-

dent of each other in an aerobic system with oxidizing and reducing enzymes present.

Since ferric iron seemed to be the most effective reactivator of the enzyme in the presence of cupferron, the experiments whose results are plotted in Figure 8, curve *B*, were carried out. These show that the inactivation is substantially reversed at a ratio of three $Fe^{+++}$ ions per cupferron molecule and that additional $Fe^{+++}$ leads to a slight activation in accordance with the data of Table 5.

TABLE 7

REVERSAL OF THE ACTION OF CUPFERRON ON THE $C_x$ ENZYME BY CERTAIN IONS

| Experiment | Cupferron Concentration | Concentration of Added Ions | Relative Rate |
|---|---|---|---|
| I | — | — | 100 |
| II | $10^{-3}$M | — | 0 |
| III | $10^{-3}$M | $Cu^{++}$ $3\times10^{-3}$M | 49 |
| IV | $10^{-3}$M | $Fe^{++}$ $3\times10^{-3}$M | 41 |
| V | $10^{-3}$M | $Fe^{+++}$ $3\times10^{-3}$M | 90 |

### (*h*) *Inhibition and Activation of Salicinase*

It seemed of interest to compare the pattern of inhibition of a $\beta$-glucosidase activity with that observed for the hydrolysis of SCMC. For this purpose the hydrolysis of salicin was chosen as most convenient and a number of reagents found to have an effect on the SCMC hydrolysis were tested for their effect on this reaction. Since the change from McIlvaine buffer to acetate buffer

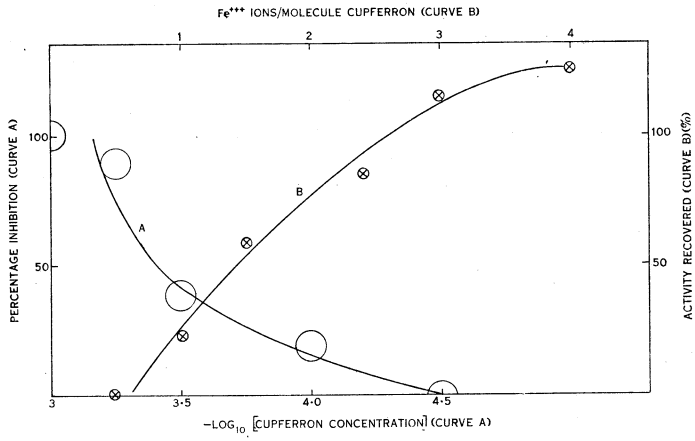


Fig. 8.—Curve *A*; inhibition of $C_x$ enzyme by cupferron; short method conditions; enzyme 0.4 mg./ml., type (ii) SCMC solution.

Curve *B*; reversal of the inhibition by $10^{-3}$M cupferron of the $C_x$ enzyme using $Fe^{+++}$; conditions as in curve *A*.

appeared to affect the rate of SCMC hydrolysis, a number of buffer systems were investigated to see whether there was any effect on the rate of salicin hydrolysis. The results obtained are shown in Table 8. A variation of ± 5 per cent. from the standard probably represents significant activation or inhibition.

## V. Discussion

Reese, Siu, and Levinson (1949) have postulated that the $C_x$ enzyme is only produced by fungi in the presence of a $\beta$-glucosidic substrate. The existence of a powerful activity in the "mould enzyme" used in this study, which had been secreted into a sucrose-tartrate medium, and the data summarized in Table 1 for *A. oryzae* and the cellulolytic fungus S. *atra* show this conclusion to be untenable. By choosing one fungus and one particular period of incubation it is possible to show that the postulate is true; another set of conditions would seem to prove the exact opposite. The production of $C_x$ enzyme and $\beta$-glucosidase by both fungi appears to be adaptive in a general way without showing an absolute dependence on the nature of the substrate. The identity of enzymes produced in the presence and absence of such substrates cannot at present be proved and detailed comparison with the results of other workers will not be attempted.

It will be demonstrated in the next paper that the enzyme activities studied are those of up to eight separate components and that the sums of the varying activities of these components towards different substrates constitute the activities here studied as "$C_x$ enzyme" and "$\beta$-glucosidase." This conclusion is borne out by the generally similar properties of the two hypothetical enzymes but is masked by complications arising from the colloidal nature of SCMC. The

Table 8

COMPARATIVE ACTIVITY OF THE *A. ORYZAE* ENZYME IN VARIOUS BUFFERS AND IN THE PRESENCE OF VARIOUS REAGENTS FOR THE HYDROLYSIS OF SALICIN

(*a*) Buffers of pH 5.0

| Buffer | Final Concentration | Comparative Activity (McIlvaine buffer = 100) |
|---|---|---|
| *p*-Hydroxyphenylacetate | $4\times10^{-3}$M | 121 |
| Ethylenediamine tetra–acetate | $10^{-2}$M | 109 |
| Acetate-HCl | $4\times10^{-2}$M (in acetate) | 108 |
| Acetate | $10^{-2}$M | 107 |
| Malate | $10^{-2}$M | 106 |
| Phosphate | $10^{-2}$M | 106 |
| Borate-succinate | $10^{-2}$M | 104 |
| Succinate | $2\times10^{-3}$M | 102 |
| Phthalate | $10^{-2}$M | 102 |
| Citrate | $10^{-2}$M | 101 |
| Citrate-phosphate (McIlvaine) | | 100 |
| Oxalate | $10^{-2}$M | 90 |
| Phenylacetate | $4\times10^{-3}$M | 72 |

TABLE 8 (*Continued*)

(*b*) Reagents in Acetate-HCl Buffer, pH 5.0

| Reagent | Concentration | Comparative Activity |
|---|---|---|
| $Fe^{+++}$ | $10^{-3}$M | 104 |
| $Fe^{++}$ | $10^{-3}$M | 92 |
| $Mg^{++}$ | $10^{-3}$M | 95 |
| $Ca^{++}$ | $10^{-3}$M | 96 |
| $Cu^{++}$ | $10^{-2}$M | 33 |
| | $3 \cdot 2 \times 10^{-3}$M | 38 |
| | $10^{-3}$M | 62 |
| | $3 \cdot 2 \times 10^{-4}$M | 82 |
| | $10^{-4}$M | 101 |
| Cupferron | $10^{-3}$M | 112 |
| Oxine | $10^{-3}$M | 85 |
| Rhodizonic acid | $10^{-3}$M | 68 |
| Ferron | $10^{-3}$M | 99 |
| Cyanide | $10^{-3}$M | 66 |
| Dithionite | $10^{-3}$M | 98 |
| Bisulphite | $10^{-3}$M | 95 |
| Permanganate | $10^{-3}$M | 108 |
| *p*-Benzoquinone | $10^{-3}$M | 104 |
| Thioglycollic acid | $10^{-3}$M | 126 |
| Glutathione | $10^{-3}$M | 85 |
| Cysteine | $10^{-3}$M | 92 |
| Sulphide | $10^{-3}$M | 36 |
| *p*-Chloromercuribenzoic acid | $10^{-3}$M | 85 |
| Quinine | $10^{-2}$M | 83 |
| Caffeine | $10^{-1}$M | 97 |
| Betaine | $5 \times 10^{-2}$M | 103 |
| Semicarbazide | $10^{-3}$M | 90 |
| Phenylhydrazine | $10^{-3}$M | 88 |
| Benzoic acid | $10^{-3}$M | 96 |
| Salicylic acid | $10^{-3}$M | 99 |
| Naphthoxyacetic acid | $10^{-3}$M | 98 |
| 2, 4-Dichlorophenoxyacetic acid | $10^{-3}$M | 103 |
| Benzilic acid | $10^{-3}$M | 100 |
| Benzylmalonic acid | $10^{-3}$M | 99 |
| *p*-Hydroxybenzoic acid | $10^{-3}$M | 102 |
| *p*-Hydroxyphenylacetic acid | $10^{-3}$M | 92 |
| $\beta$-Indolylpropionic acid | $10^{-3}$M | 86 |
| | $4 \times 10^{-3}$M | 114 |
| Phenoxyacetic acid | $10^{-3}$M | 100 |
| Benzyl alcohol | $10^{-2}$M | 97 |

properties of this substrate appear to set a limit to the types of experiment that can usefully be performed. Kagawa and Katsuura (1951) have shown that the apparent hydrogen ion concentration of solutions of SCMC may be very different from the hydrogen ion concentration of the colloid phase and that it is this latter concentration that determines the behaviour of the SCMC micelles and hence bulk properties of the solution such as viscosity. This type

of behaviour would explain the smearing out of the maxima of the pH-activity curve in concentrated SCMC solution since the pH of the environment in which the enzyme acts will be only qualitatively related to the measured pH of the solution.

The linear activity-temperature relationship in concentrated SCMC solutions and the indifference of the activity to changes in substrate concentration suggest that diffusion is the rate-limiting process, as might be expected in a solution containing large colloidal aggregates. Dürig and Banderet (1950) have shown that the behaviour of SCMC solutions is determined largely by the degree to which they are composed of amorphous and semi-crystalline fractions. The amorphous fractions form a stable colloid sol under all conditions but the semi-crystalline fraction slowly deposits from 1 or 2 per cent. solution as a gel and consists of large aggregates when dispersed. In agreement with these results it was found that high-speed centrifuging of aged solutions of the SCMC used in this study resulted in the deposition of a transparent gel. The observation that many characteristics of the enzyme attack on SCMC solutions depend on the concentration and previous history of the solutions is in harmony with the expected behaviour of such a complex colloid mixture.

It may therefore be concluded that, although the linear relationship between enzyme concentration and activity in concentrated SCMC solutions provides a valuable tool for measuring enzyme concentration, the general properties of these solutions make them unsuitable for detailed investigation of the kinetics of enzymes that hydrolyse polymeric $\beta$-glucosidic linkages. If these disturbing effects are disregarded no evidence has been discovered that SCMC is hydrolysed by an enzyme intrinsically different from other $\beta$-glucosidases. The kinetic constants (Michaelis constant, energy of activation) are consistent with the unitary hypothesis; however, if they are in fact averaged figures for a group of enzymes no useful argument can be drawn from them. None of the enzyme preparations is yet pure enough to bring departures from the kinetics of single-enzyme behaviour (e.g. non-linearity of Lineweaver-Burk plots) within the limits of the experimental errors.

The comparative results of experiments on the effect of various reagents on the activity of "salicinase" and "cellulase" are also consistent with the hypothesis that each observed activity is the sum of the activities of a set of enzymes hydrolysing $\beta$-glucosidic linkages but of different specificity towards the two substrates. The effect of copper on salicinase, which does not go beyond about 65 per cent. inhibition at relatively high concentrations of $Cu^{++}$, is a valuable indication of the existence of more than one component in this system. In general the two enzyme activities tend to be affected by the same groups of substances, but the degree to which one is affected by a certain concentration of reagent and even whether the reagent will act as an inhibitor or activator cannot be predicted from the behaviour of the other. Thus both enzymic activities seem to be affected by substances of the general formula $Ar(CH_2)_nCOOH$ and this effect seems to be specific, judged by the ineffectiveness of various plant hormone and saligenin analogues, but the amount and sign of this effect varies with the substrate and the nature and concentration of the reagent.

It is of interest to note that the pattern of inhibitors active against "salicinase" and "cellulase" is in agreement neither with the list given by Veibel (1950) for $\beta$-glucosidases nor with the results of Ziese (1931) for the action of malt and snail cellulases on hydroxyethyl cellulose. Ziese found these cellulases to be unaffected by papain or copper ions, and inactivated by cysteine, glutathione, and ferric chloride. This disagreement is another demonstration of the fact that "cellulase" systems from different organisms cannot be comparable and that a number of different mechanisms may well be operative.

For inhibitors and activators of the hydrolysis of SCMC the whole picture is obviously complicated by the nature of the buffer, the history of the SCMC solution, and the interaction of buffer and SCMC. If it is postulated that the ability of a complexing agent to inhibit the $C_x$ enzyme is conditioned (*a*) by its ability to form a stable complex with the metal activating agent at the pH used, and (*b*) by its ability to form such a complex in competition with the complexing power of buffer and SCMC, most of the results with the heavy metal complexing agents can be interpreted. Only reagents known to give complexes with metals under "physiological conditions"—8-hydroxyquinoline, cupferron, Nitroso R salt (Albert and Gledhill 1947)—inhibit the enzyme.

None the less the data of Table 7 and Figure 8 cannot be unequivocally interpreted as the inhibition of an enzyme activated by $Fe^{+++}$ (or some other heavy metal ion) and the reversal of this inactivation by excess $Fe^{+++}$. Since these observations cannot be readily harmonized with those on the inhibition of salicinase, it is quite possible that the primary effect of the heavy metal ion may be on the SCMC rather than the enzyme. Alternatively, cupferron may be the primary inhibitor whose influence is reversed by complexing with $Fe^{+++}$. The difference between the behaviour of chelating agents and that of more specific inhibitors with change of buffer is hard to reconcile with this hypothesis, and the action of oxidizing, reducing, and certain other reagents is consistent with an activation by $Fe^{+++}$.

Only experiments with purified enzymes and substrates can give any certain results in this type of investigation and the complex patterns of activity are what might be expected from a crude mixture of enzymes. It is impossible therefore to draw any but the most general conclusions.

## VI. Acknowledgments

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