Cellulolytic Enzymes from Sheep-Rumen Liquor Micro-organisms

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The enzymic conversion of cellulose into glucose is incompletely understood. The state in the rumen may be expected to be complex, since there are different organisms present and the enzyme may vary with the organism. Yet differences exist concerning even single and particular organisms: Grassmann, Zechmeister, Tóth & Stadler (1933) separated cellobiase as a distinct entity from extracts of Aspergillus oryzae, and indirect evidence for cellobiose as a separate enzyme in filtrates of Myrothecium verrucaria culture has been obtained by its selective inactivation on heating (Kooiman, Roelofsen & Sweers, 1953; Aitken, Eddy, Ingram & Weurman, 1956). Whitaker (1953), however, obtained an enzyme preparation from Myrothecium verrucaria that was electrophoretically homogeneous and active towards both cellulose and cellobiose; he claims (Whitaker, 1956) that differential inactivation of cellobiase by heating represents a structural change in the enzyme which affects its activity in relation to the chain-length of the substrate. Evidence for both single- and multiple-enzyme components in extracts of Aspergillus niger has been found by chromatography (Stone, 1957), the fractions separated including one with activity towards two cellulose substrates as well as towards salicin, and others with activity towards either one cellulose substrate only or salicin only.

In the present investigation, the cellulolytic enzymes from sheep-rumen micro-organisms have been studied with cellobiose and carboxymethylcellulose as substrates. Substituted soluble derivatives of cellulose such as carboxymethylcellulose (Holden & Tracey, 1950), cellulose sulphate (Levinson, Mandels & Reese, 1951) and methyl cellulose (Aitken et al. 1956) have been extensively used as substrates for cellulase, but Siu (1951) and Halliwell (1957a, b) claim that preparations actively hydrolysing these substances do not necessarily attack native cellulose. In particular, the type of preparation used in the present investigation (obtained by butanol extracts of sheep-rumen micro-organisms) as well as an extract of the limpet Patella vulgata (found to be a good source of the enzyme) hydrolyse carboxymethylcellulose, but according to Halliwell (1957a, b) have negligible activity on filter paper and cotton fibres. To avoid confusion in this respect the enzyme attacking the soluble cellulose derivative will be termed carboxymethylcellulase rather than cellulase.

Conchie (1954) studied β-glucosidase from rumen liquor by using o-nitropheryl β-glucoside as a substrate and showed gluconolactone to be a powerful inhibitor of the enzyme. The use of this inhibitor has been extended to the present study, a preliminary account of which has already been given (Festenstein, 1957).

EXPERIMENTAL

Materials. Sodium carboxymethylcellulose containing an average of 0-5 carboxymethyl/anhydroglucose (Cellophane B, Imperial Chemical Industries Ltd.) was kindly provided by Dr G. Halliwell. Cellobiose and glucono-1,4-lactone were obtained from L. Light and Co. Ltd. Both cellobiose and sodium carboxymethylcellulose were found to be chromatographically free from glucose and short-chain oligosaccharides.

Enzyme assay. The following procedure was usually followed: 0-2 ml. of each of 0-1 M-citric acid-0-2 M-NaHPO₄ buffer (pH 5-8; McIlvaine, 1921), substrate, inhibitor (where added) and enzyme were incubated at 37° in a total volume of 1 ml. (pH 6-1) in 15 ml. centrifuge tubes fitted with glass bulbs. After 1 hr. the reaction was stopped by adding the following reagents according to the particular method used for estimation of reducing sugar: 0-25 ml. of 0-5% K₃Fe(CN)₆ in 10% (w/v) Na₂CO₃ for the ferricyanide method; 0-27 ml. of 0-2 M-NaOH for the hypiodiose method; 1 ml. of 0-3 M-Ba(OH)₄ and 1 ml. of 5% (w/v) ZnSO₄ (standardized against each other as specified by Nelson, 1944) for the cuprimetric method. It was not found necessary to deproteinate the incubation mixtures for estimations by the ferricyanide and hypiodiose methods under the conditions used, provided that enzyme blanks were done. The cuprimetric method did not give consistent results for cellobiose in the presence of protein, so protein was removed as described.

Methods for estimating reducing sugar

Ferricyanide method. The method of Levvy (1946) was used.

Hypiodiose method. The method of Willstätter & Schudel (1918) was adapted as follows: after the addition of 0-27 ml. of 0-2 M-NaOH and 0-5 ml. of 0-04 M-iode (freshly diluted from 0-1% iodine in 10% (w/v) KI), the tubes were corked and left at room temperature for 20 min., before acidification with 0-2 ml. of 4 N-H₂SO₄ and titration with 4 ml. Na₂S₂O₃ from a 10 ml. microburette. The tip of the burette was immersed in the solution, 3 drops of 1% starch solution
were added near the end point and air-stirring was used to complete the titration. Under these conditions up to 2 mg. of cellulose reacted completely; usually 1 mg. was used as substrate. In experiments where 0-2 ml. of m-citric acid–2 M-NaOH buffer, pH 5-5, was used in the initial incubation mixture (final pH 6-0), the reaction was terminated by the addition of 0-28 ml. of 1 N-NaOH, and the procedure continued as before.

**Curprimetric method.** The method of Nelson (1944), as modified by Somogyi (1952), was used as follows: after addition of 1 ml. each of Ba(OH)₂ and ZnSO₄ as described above (slightly more than sufficient to precipitate all the protein in 0-2 ml. of dialysed enzyme) and centrifuging at 1500 g for 5 min., 1 ml. portions (or fractions thereof) were taken for assay with 1 ml. of copper reagent.

**Comparison of methods of estimating reducing sugars.** Cellobiose gave about 55% of the reducing value of an equal weight of glucose for both the curprimetric and hypooiodite methods; the ferricyanide method gave a much higher value (88%). Determination of reducing sugar after enzymic hydrolysis of carboxymethylcellulose expressed in terms of glucose gave similar values for the curprimetric and hypooiodite methods; the ferricyanide method results were two to three times higher. A similar difference was observed by both Holden, Pirie & Tracey (1950) and Holden & Tracey (1950), when comparing a ferricyanide and a hypooiodite method. In the present investigation cellulose estimations were therefore carried out by the hypooiodite and curprimetric methods; the ferricyanide method was used only in the preliminary experiments involving carboxymethylcellulose.

**Chromatography**

Solutions obtained after enzymic hydrolysis of cellobiose and carboxymethylcellulose were analysed by descending-paper chromatograms on Whatman no. 1 paper. After incubation with enzyme, solutions were either placed in a boiling-water bath for 10 min. or, more generally, deproteinized with Ba(OH)₂ and ZnSO₄ as described above. In some cases the reaction was stopped by addition of 10% of the volume of 0-04 M-HgCl₂ (Crook & Stone, 1957). Where concentrated cellobiose or glucose solutions (≥ 5%, w/v) had been incubated with enzyme, these were either applied to the paper without further treatment or after de-ionizing. All solutions to be concentrated were de-ionized by standing over a mixture of the resins Zeo-Karb 215 and Amberlite 1 R-4 B(OH) before being evaporated to dryness in a vacuum desiccator at room temperature. Generally the carboxymethylcellulose hydrolysates were redissolved in water so as to correspond to 0.5–1% solutions of glucose, as determined by estimation of reducing sugar. The following solvent systems were used: butanol–pyridine–water, (a) 6:4:3, by vol. (Chargaff, Levine & Green, 1948), (b) 6:2:3, by vol.; butanol–pyridine–water–benzene (5:3:3:1, by vol.) (De Whalley, Albon & Goss, 1951); acetic acid–propan-2-ol–water (10:58:5:31:5, by vol.) (Hash & King, 1954). Alkaline AgNO₃ reagent (Trevan, Procter & Harrison, 1950), aniline phosphate and benzidine in trichloroacetic acid (Bacon & Edelman, 1951) were used to detect the sugars on the chromatograms. The Rₚ value of a substance describes its rate of movement relative to p-glucose (Rₚ 1-0), determined on the same chromatogram.

**Preparation of enzyme extract**

The butanol-extraction method of Morton (1955), first used for rumen micro-organisms by Halliwell (1957b), was adapted as follows: a sample of rumen liquor (usually 1 l.), obtained from fistulated Cheviot sheep on a hay diet at 9-30 a.m., was strained through six layers of surgical gauze and centrifuged at 1500 g for 30 min. or 10 000 g for 20 min., depending on the micro-organisms required. After removal of the supernatant layer the precipitate was suspended in 1% NaCl solution, the final volume being chosen to give a concentrated suspension that was not too viscous (5–10% of the original volume of rumen liquor). Butanol was added slowly, with stirring, to 10% (by vol.) over 10 min. and then to 30% (by vol.) over the next 5 min. The resulting mixture was centrifuged at 10 000 g for 20 min. and the aqueous layer (3–5% of the original volume of rumen liquor) re-centrifuged (after precolling to 1°C) until clear.

The enzyme extracts stored at 1°C retained their carboxymethylcellulase activity for at least several months. Butanol was usually removed by dialysis in sacs of Visking cellulose tubing against distilled water at 1°C, for 18–24 hr. before use. The organisms centrifuged at 1500 g (described microscopically by Karunasairatnam & Levy (1951)) were found to contain about 85% of the total extractable carboxymethylcellulase, so extracts of this particular fraction were generally used, except for determination of the pH optimum of the enzyme (see below) where a sample of total organisms was extracted. Conchie (1954) found a similar distribution of o-nitrophenyl β-glucosidase activity, with fractions of intact organisms.

The mean protein content of six enzyme preparations from three different sheep, dialysed extracts obtained from organisms centrifuged at 1500 g, was 2-6 mg./ml. (range 0-8–3-5), as determined by the method of Lowry, Rosebrough, Farr & Randall (1951), standardized against crystalline bovine-plasma albumin. For one particular sample of rumen liquor the protein content of the dialysed extract from the smaller organisms (centrifuged between 1500 and 10 000 g) was one-third of that of the dialysed extract from the organisms centrifuged at 1500 g.

**RESULTS**

**Effect of pH on enzyme activity.** The variation of carboxymethylcellulase activity with pH is shown in Fig. 1. The multi-peaked curve resembles that obtained by Conchie (1954) for o-nitrophenyl β-glucosidase, with intact mixed rumen microorganisms. A limpet–enzyme preparation provided by Dr J. Conchie (cf. Conchie, Levy & Marsh, 1956; Conchie & Levy, 1957), which was found to possess carboxymethylcellulase activity, showed a similar pH–activity curve (Fig. 1). The optimum pH determined for cellobiose (0-1% substrate concentration) was found to lie between pH 5 and 6.

**Differentiation of cellobiose and carboxymethylcellulose by glucono-1,4-lactone**

**Inhibition of cellobiose by gluconolactone.** By the use of short incubation periods (30–60 min.) and concentrations of substrate up to 0-4%, 2-5 mm-
lactone was found sufficient to produce complete inhibition of cellobiase; for concentrations up to 0.2%, 0.5 mM-lactone was mostly sufficient. For 5% cellobiose solutions, 2.5 mM-lactone produced nearly complete inhibition of enzymic hydrolysis, as determined chromatographically (see later).

The effect of time on inhibition by lactone, studied in previous experiments, is shown in Fig. 2. It can be seen that inhibition was not sustained for periods longer than 60 min. and investigations have to be carefully controlled on this account.

An evaluation of $K_i$ for gluconolactone was not possible because of non-linear hydrolysis in the presence of lactone, similar to the lag phase sometimes observed for hydrolysis of cellobiose by diluted enzyme (see Fig. 2).

**Inhibition of carboxymethylcellulase by gluconolactone.** At a substrate concentration of 0.4%, inhibition was detected with 0.02 mM-lactone and rose rapidly with lactone concentration to 60%, where it remained constant for lactone concentrations $\geq$ 2.5 mM (Fig. 3). When 2.5 mM-lactone was used for a range of substrate concentrations from 0.4 to 0.04%, a value of about 50% inhibition was obtained for practically the whole range.

**Effect of preheating of enzyme on inhibition of carboxymethylcellulase by gluconolactone.** When an

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**Fig. 1.** Effect of pH on carboxymethylcellulase activity. ●, Results for an enzyme extract obtained from a sample of total rumen micro-organisms, washed with NaCl. Reaction mixtures (1 ml.) contained 0.2% of carboxymethylcellulose, 1:100 enzyme (corresponding to about fivefold dilution of rumen liquor) and 0.2 ml. of 0.1M-citric acid–0.2M-Na$_2$HPO$_4$ buffer of appropriate pH. An activity of 1.0 corresponds to liberation of reducing substances equivalent to 0.06 ml. of 11 mM-Ce(SO$_4$)$_2$ (or 24 $\mu$g. of glucose). ○, Results for a limpet extract (30 g. of wet tissue/100 ml.) diluted 1:2000 in reaction mixtures as above; in this case an activity of 1.0 corresponds to a titre of 0.095 ml. of 11 mM-Ce(SO$_4$)$_2$.

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**Fig. 2.** Progress of enzymic hydrolysis of cellobiose and the effect of gluconolactone. ○—○ and ●—● represent the hydrolysis of 0.1% cellobiose in the absence and the presence of 10 mM-gluconolactone respectively; ○—○ and ●—● represent the hydrolysis of 0.05% cellobiose by diluted enzyme (1:5) in the absence and the presence of 0.5 mM-gluconolactone respectively. Incubation was carried out in 0-2M-citric acid–0.4M-Na$_2$HPO$_4$ buffer, pH 6.0. An increase in reducing power of 1.40 ml. of 4 mM-Na$_2$S$_2$O$_4$ corresponds to the liberation of 1 mg. of glucose.

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**Fig. 3.** Effect of concentration of gluconolactone on the inhibition of carboxymethylcellulase. Enzyme activity was determined by measurement of the reducing substances liberated from 0.4% sodium carboxymethylcellulose after incubation for 1 hr. (as determined by the cuprimetric method).
enzyme preparation was kept at 65° for 10 min., 
cellobiase activity was lost, carboxymethylcellu-
lase activity was reduced to 15 % of its initial value 
and inhibition of carboxymethylcellulase by 
2·5 mM-gluconolactone was completely abolished. 

Chromatographic analysis of cellobiose and car-
boxymethylcellulose hydrolysates, after enzyme action 
in the presence and the absence of gluconolactone. 
Chromatograms of hydrolysates of 0·2 % cellobiose 
(made after about 20 % hydrolysis of substrate, 
and usually concentrated 10–25 times) revealed 
only glucose and cellobiose when examined with 
aniline phosphate; silver nitrate reagent showed 
a trace of a substance, \( R_g \) 0·22 (solvent, butanol–pyridine–water–benzene), 
which was probably formed by a transferase reaction. Transferase 
activity could be readily detected by incubating 
enzymes with 5 % (w/v) solutions of cellobiose (cf. 
Crock & Stone, 1957); three other substances 
besides glucose were produced, which can be 
tentatively identified as \( \beta \)-glucosylcellobiose, 
gentiobiose and gentiobiose (arising in that order), 
on the basis of the \( R_g \) values in different solvent 
systems compared with the data of Crock & Stone 
(1957). Gluconolactone (2·5 mM) inhibited trans-
ferase activity as well as greatly diminishing 
production of glucose.

Chromatograms of carboxymethylcellulose hy-
drolysates sprayed with aniline phosphate or 
benzidine in trichloroacetic acid revealed glucose 
as the predominant product, along with traces of 
cellobiose. Occasionally traces of a substance 
producing a pink colour with aniline phosphate 
\( R_g \) 1·4, butanol–pyridine–water–benzene) were 
found; possibly this was xylose. The intensity of 
the glucose spot on the chromatogram generally 
corresponded to less than would be expected if the 
total reducing material was glucose. If 2·5 mM-
gluconolactone was present during the incubation 
of carboxymethylcellulose with the enzyme, 
the amount of glucose, although very much reduced, 
was still detectable; cellobiose sometimes showed 
a slight increase but no other substances were found.

The more sensitive silver nitrate reagent de-
tected several other substances on the chromato-
grams, including gluconolactone; up to three 
different spots with \( R_g \) values varying from 0 to 
0·11 (butanol–pyridine–water–benzene) were found 
but when gluconolactone \( (R_g \) 0·14) was present it 
overlapped with and possibly obscured intensity 
changes in these spots. Gluconolactone was only 
partially removed by Amerlite 1R·4 B(OH) resin 
added after deproteinization with barium hydro-
oxide–zinc sulphate, even when this resin was the 
first added and the pH rose to 8·6. In acetic acid– 
propan-2-ol–water as developing solvent gluco-
nolactone produced a spot which almost coincided 
with glucose, but did not interfere with any of the 
others [it was found advisable to deproteinize with 
barium hydroxide–zinc sulphate and then to de-
ionize to ensure that the lactone did not interfere]; 
the results obtained with the acetic acid-containing 
solvent are summarized in Table 1.

DISCUSSION

The presence of a multi-peaked pH–activity curve 
for carboxymethylcellulase suggests either differ-
ences in respect of the same enzyme for different 
organisms or alternatively more than one type 
of enzyme. Jermyn (1952a) found more than one 
peak in the pH–activity curves for \( \text{Aspergillus oryzae} \) 
enzyme acting on cellobiose, salcin and 
carboxymethylcellulose; he subsequently showed 
(Jermyn, 1952b) by chromatographic and electro-
phoretic analysis that there were at least eight 
components, with varying degrees of specificity 
towards different \( \beta \)-glucosidic substrates. In 
contrast with this finding a \( \text{Stachybotrys atra} \) pre-
paration, which showed a multipeaked pH– 
activity curve for \( p \)-nitrophenyl \( \beta \)-glucoside as sub-
strate, yielded only one component on chromatogram 
and electrophoresis (Jermyn, 1955a, b).

The inhibition of carboxymethylcellulase by 
gluconolactone does not exceed 60 %, as deter-
mined by estimation of reducing sugar. The maxi-
mum value is attained by 2·5 mM-lactone (see

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<th>Substances detected</th>
<th>Incubation with lactate</th>
<th>Incubation with lactate</th>
<th>Notes</th>
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<tr>
<td>( R_g ) values</td>
<td></td>
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<tr>
<td>0·23</td>
<td>-</td>
<td>+</td>
<td>Found consistently</td>
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<tr>
<td>0·58</td>
<td>+</td>
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<tr>
<td>0·71</td>
<td>+</td>
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<td>Did not always occur</td>
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<tr>
<td>0·80</td>
<td>+ + +</td>
<td>+ + + (+)</td>
<td>Cellobiose</td>
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Fig. 3), which is sufficient to produce complete inhibition of cellobiase for concentrations of cellobiose up to 5%. This suggests that two types of enzyme are involved in the production of reducing sugar from carboxymethylcellulose, one of which is inhibited by lactone in a similar manner to cellobiase. In accord with this interpretation, Reese & Mandels (1957) found that the carboxymethylcellulase activity of culture filtrates of *Myrothecium verrucaria*, *Pestalotiopsis vesterdijkii* and *Trichoderma viride* was unaffected by gluconolactone when measured under conditions where the effects due to cellobiase were eliminated.

The results obtained by treating chromatograms of carboxymethylcellulose hydrolysates with aniline phosphate or benzidine spray are in agreement with the findings of Kitte & Underkofer (1954), who chromatographed carboxymethylcellulose hydrolysates obtained with rumen micro-organisms and cell-free preparations and found glucose to be the chief product, with traces of cellobiose and xylose. Since gluconolactone inhibits cellobiase, it might be expected that the products obtained from extracts of rumen micro-organisms incubated with carboxymethylcellulose in the presence of lactone would be intermediates formed in the normal process of hydrolysis. It was found (Table 1) that cellobiose increased only slightly and not invariably, and small amounts of a substance of Rₐ 0-23 (in the acetic acid-containing solvent) were produced, as detected by the silver nitrate reagent. From the data of Hash & King (1954) cellotetraose has Rₐ 0-35 in the same solvent system, and interpolated values for cellopentaose and cellohexaose are Rₐ 0-22 and 0-14 respectively. On this basis it would appear that the additional substance appearing in the presence of lactone may be cellopentaose. The substances of Rₐ 0-58 and 0-71 (Table 1) did not always occur; the former may be cellotriose (Rₐ 0-53) and the latter a product of transferase activity, inhibited by gluconolactone.

Koimman et al. (1953) found that a soluble cellulose dextrin hydrolysed by *Myrothecium verrucaria* enzyme produced a range of sugars from glucose to cellotetraose, with traces of cellopentaose and cellohexaose, as identified by paper chromatography. When the enzyme was preheated at 100° for 15 min., it still produced the sugars, but cellobiose was increased in amount, and so apparently was cellopentaose which, previously recorded as doubtful, now appears as definitely present. These findings accord with the results of the present investigation.

Levinson et al. (1951) detected cellobiose as the initial product of hydrolysis of cellulose sulphate by *Myrothecium verrucaria* and *Trichoderma viride* culture filtrates which were low in 'β-glucosidase'. Aitken et al. (1956) similarly established that heating of *Myrothecium verrucaria* enzyme (to abolish cellobiase activity) diminished production of glucose from methyl cellulose, but the absolute amount of 'cellobiose etc.' as determined by estimation of reducing sugar after yeast fermentation, was not substantially increased. It would appear that this heating of enzyme produced a similar result to inhibition by lactone in the present study.

The conclusion to be drawn here is that production of glucose from carboxymethylcellulose practically ceases if a cellobiase-type of enzyme is inhibited; cellobiose does not increase markedly under these conditions, suggesting either that glucose is split off from carboxymethylcellulose directly, and that this mechanism is inhibited by lactone, or else that inhibition of hydrolysis of cellopentaose and possibly higher homologues occurs and that this hydrolysis gives rise to glucose as well as cellobiose as described by Whitaker (1956). The rate of hydrolysis of cellobiose by *Myrothecium verrucaria* enzyme was shown by Whitaker (1956) to be extremely slow, and that of cellotriose to be moderately fast, in comparison with the extremely fast rate of hydrolysis of cellopentaose and cellohexaose. Grassmann et al. (1933) showed that, for *Aspergillus oryzae*, 'polysaccharase' (as distinct from 'oligosaccharase' or cellobiase) hydrolysed cellohexaose to a certain extent but had hardly any effect on cellotetraose and lower oligoglucosides.

**SUMMARY**

1. Cell-free extracts of sheep-rumen micro-organisms have been used to study the hydrolysis of cellobiose and sodium carboxymethylcellulose, by determination of the reducing sugar liberated.

2. Controlled inhibition studies with gluconolactone have shown cellobiose to be completely inhibited by 2-5 mM-lactone, for concentrations of cellobiose up to 5% (w/v). Inhibition of hydrolysis of sodium carboxymethylcellulose reaches a constant value of 60% at a lactone concentration where cellobiase is completely inhibited.

3. Chromatographic analysis of cellobiose hydrolysates has shown that transferase activity is barely detectable in 0-2% cellobiose solutions, but becomes appreciable in 5% cellobiose, and three oligosaccharides have then been detected. Transferase activity is inhibited by gluconolactone.

4. Chromatographic analysis of sodium carboxymethylcellulose hydrolysates has shown glucose to be the predominant product, along with traces of cellobiose, and possibly xylose. In the presence of gluconolactone, production of glucose is almost completely inhibited and the amounts of cellobiose are sometimes slightly increased; a small amount of a substance which may be cellopentaose has been detected with a silver nitrate reagent.
Thanks are due to Dr G. A. Levvy for his stimulating and critical interest in this work; to Dr G. Halliwell for suggesting the butanol method of enzyme extraction, assistance at the commencement of the investigation and for providing the samples of rumen liquor used; to Dr B. H. Howard for advice on chromatography; to Mr I. Strachan and Miss E. F. Wallace for technical assistance. Acknowledgement is also due to several colleagues for comments on the manuscript.

REFERENCES


Studies on Sulphatases

21. THE ANOMALOUS KINETICS OF ARYLSULPHATASE A OF HUMAN TISSUES: THE ANOMALIES*

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The anomalous kinetics shown by partially purified preparations of the arylsulphatase A of ox liver when acting on dipotassium 2-hydroxy-5-nitropheryl sulphate (nitrocatechol sulphate, NCS) were first noted by Roy (1953a, b). When measured over an incubation period of 1 hr. the velocity of the enzymic reaction was linearly related to enzyme concentration only when the latter was raised to the power of 3/2. This effect was attributed to the ability of the enzyme to exist in several polymeric forms. Similar anomalous kinetics were observed by Dodgson, Spencer & Wynn (1956) for the corresponding enzyme of human liver, and it was subsequently possible to show (Dodgson & Spencer, 1956a) that the fundamental anomaly was that the enzymic reaction was not of zero order when low concentrations of enzyme were used. These findings were later confirmed for the ox enzyme (Roy, 1956). Meanwhile, it was noted (Roy, 1956; Roy & Kerr, 1956) that the NCS used in these studies contained appreciable amounts of a sulphated impurity which was identified as nitropyrogallol disulphate. When NCS which had been ‘purified’ via the methylene-blue salt or by paper electrophoresis was used as assay substrate, the ox-liver enzyme no longer exhibited anomalous kinetics.

* Part 20: Spencer (1956).