

lysing casein and had a specific activity about twice as high as that of the crude venom when hydrolysing casein or gelatin; its benzoylarginine amidase specific activity was about the same as that of the crude venom.

3. The venom fraction precipitated between 0.7 and 0.8 saturation with ammonium sulphate had a specific activity about four times higher than the crude venom when hydrolysing benzoylarginine amide; it was insensitive to Ca^{2+} ions when hydrolysing casein and its caseinase and gelatinase activities were lower than those of the crude venom.

4. An isolation technique has been described which leads to the preparation from the venom of a heat-resistant proteolytic enzyme which is precipitated between 0.7 and 0.8 saturation with ammonium sulphate and has a benzoylarginine amidase specific activity 52 times higher than the crude venom.

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A Microdetermination of Cellulose in Studies with Cellulase

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Cellulose preparations may contain other polysaccharides as impurities (cf. Huffman, Rabers, Spriesterbach & Smith, 1955). When such preparations are used as substrates for cellulase care must be taken to ensure that the degradation observed is of the cellulose and not of the impurities (Halliwell, 1957*a, b*). This will be so if a substantial degradation of the cellulose is allowed to take place, and a measure of such degradation is the rate of solubilization of the polysaccharide, a process which does not necessarily involve complete degradation to sugars. This rate can be determined by measuring the loss in weight of the

cellulose. Until recently microprocedures have not been available for the determination of cellulose. Schramm & Hestrin (1954) describe an acetolytic micromethod which is time-consuming and involves a critical alkali neutralization. More recently, Dearing (1957) has reported a chromogenic reaction with concentrated sulphuric acid which can be used in the quantitative estimation of cellulose. Both these methods, however, involve preliminary and lengthy centrifuging of the cellulose and it has been found that the cellulose often fails to precipitate even in the presence of talcum powder. This failure of the cellulose to

sediment was particularly prominent when 3.8N-hydrochloric acid, 0.7N-ammonia solution, 1% (w/v) Teepol XL and redistilled absolute ethanol, the reagents used to remove bacteria from cellulose fermentations, were employed (Halliwell, 1957a).

The present paper describes a new micromethod for the quantitative determination of cellulose in enzyme reactions and is based upon the use of sintered-glass filter sticks for the separation and washing of the cellulose followed by oxidation of the cellulose in the presence of dichromate. The excess of dichromate is then determined colorimetrically or titrimetrically. The method has the advantage that filtration is quicker and more convenient than repeated centrifugings, cellulose is not lost in supernatant-fluid fractions and, finally, the reaction is stoichiometric. The procedure has been applied to swollen cellulose, cellulose powder, a hydrocellulose prepared from absorbent cotton and to cotton fibres.

MATERIALS AND METHODS

Reagents

The dichromate procedure of Johnson (1949) for the determination of non-volatile organic matter was modified for application to cellulose.

Potassium dichromate solution. Potassium dichromate (A.R., 5 g.) is dissolved in 20 ml. of water and diluted to 1 l. with 98% H_2SO_4 (M.A.R., British Drug Houses Ltd.). The reagent is kept in a glass-stoppered vessel protected from dust.

Sodium sulphite. To make this solution (20%, w/v) 4 g. of $Na_2SO_3 \cdot 7H_2O$ (A.R.) is dissolved in water and made up to 20 ml. (40 mg./0.2 ml. of soln.). The solution is kept in a vessel with a high ratio of volume to surface area and stored at 1°. If necessary it is prepared fresh each week.

Standard glucose solution. A 3.5% (w/v) aqueous solution of glucose (A.R.) is prepared and stored at 1°. For use in constructing a glucose calibration curve the stock glucose solution is diluted 10 times to 0.35% (3.5 mg./ml.) and suitable samples are pipetted out to cover the range 0.1–1.4 mg. of glucose (0.09–1.26 mg. of cellulose).

Cellulose samples. Cellulose powder (Whatman) is the standard-grade ashless powder for chromatography. Swollen cellulose is prepared from Whatman cellulose powder by soaking it in 90% phosphoric acid (A.R.) at 1° for 2 hr. and then by washing until it is acid and phosphate-free (Walseth, 1952). Hydrocellulose is prepared from absorbent cotton by immersing it in 75 vol. of 11N-HCl (A.R.) for 48 hr. at 18°. After rejecting the supernatant fluid the fine powder is poured into water and then filtered off on a Büchner funnel, washed with water until acid- and chloride-free and finally dried *in vacuo* at room temperature. Native cotton fibres (Peruvian Tanguis, kindly supplied by the British Cotton Industry Research Association) are dewaxed as follows. The fibres are first extracted in a Soxhlet apparatus with redistilled ethanol for 8 hr. and then with ether for 6 hr. They are next refluxed under nitrogen in 1% (w/v) NaOH soln. for 8 hr. After washing the fibres with hot, boiled water until they are neutral,

washing is continued repeatedly with cold water, followed by 1% (v/v) acetic acid and then water. The product is finally dried in air. All the above cellulose products are typical substrates in enzymic work with cellulase (see Halliwell, 1957a).

Cellulose weights. Samples of cellulose are weighed on a microbalance reading to the nearest $1 \mu g.$, which represents 0.5% of the smallest weight of cellulose determined. Hence results in the text are quoted to the nearest 1%. Where necessary, figures are corrected for moisture (dried at 105°) and ash content (incinerated at 750°) of cellulose samples.

Filter sticks. Sintered-glass filter sticks (10 mm., porosity 3; Pyrex, Sunderland, England) are employed in all filtrations. For some forms of cellulose, particularly the 'swollen' variety, the faster-flowing filter sticks (of those available of porosity 3) are used.

Filtration apparatus. The equipment and technique described by Halliwell (1950) for the microdetermination of oxalic acid by means of filter sticks are used throughout with the exception that glass-to-glass connexions with the filter stick are effected with polythene tubing.

Analytical procedure

Determination of cellulose. The sample of cellulose in a test tube of size slightly larger in both width and height than the filter stick is washed either with water or successively with 3.8N-HCl, aq. 0.7N-NH₃ soln., 1% (w/v) Teepol XL (British Drug Houses Ltd.), redistilled ethanol and water (see Halliwell, 1957a). The reagents are removed in turn by slight vacuum applied indirectly to the filter stick through a Büchner flask. After several washings (8 × 3 ml. of water) the cellulose is sucked as dry as possible with the filter stick and all water removed from inside the filter stick by applying maximal vacuum through the Büchner flask: 0.9 ml. of water and 2 ml. of dichromate solution are then added to each tube with its contained filter stick and also to two empty 'blank' tubes. The dichromate solution rapidly passes through the sintered plate (if slow, it may be assisted with slight vacuum) and, when the plate is covered, compressed air followed by vacuum is applied to expel the dichromate from, and subsequently attract it back through, the sintered plate. The tubes are then covered with glass 'pear bulbs' so that the bulb closes the neck of the tube and does not rest on the upper end of the filter stick. The tubes are then heated in a boiling-water bath for 1.5 hr. and then cooled in ice-water: 3 ml. of water is added to each tube and the contents are mixed, allowed to cool and the solution is sucked rapidly through the filter stick under vacuum into a test tube contained in a Büchner-type Quickfit boiling tube with side arm for vacuum. A further 1 ml. of water is likewise sucked rapidly through the filter stick; speed is necessary to reduce possible contact with the polythene connexion. This has not caused trouble. Additional washings are made with suitable volumes of water until a total of exactly 10 ml. of washing water has been added to the original 2.9 ml. of dichromate mixture. After the first two rapid washings, subsequent washes are used to rinse the filter stick and the sides of the test tube, after which the liquid is sucked slowly through the filter stick into the receiver. The latter is then closed with a ground-glass stopper and the contents are mixed by inversion: 10 ml. of water is added directly to each of the 'blank' tubes and, after mixing each in turn, 0.2 ml. (40 mg.) of 20% (w/v)

$\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ is added to one of the blanks, which is again mixed. All tubes are then read against the reduced blank in the Spekker photoelectric absorptiometer (Hilger) with the violet (no. 601) filters. If desired, the excess of dichromate, after dilution, may be determined titrimetrically with KI and thiosulphate (Johnson, 1949) or by using ferrous ammonium sulphate (1 ml. of $\text{N-K}_2\text{Cr}_2\text{O}_7 \equiv 6.75$ mg. of cellulose). In the present procedure for the determination of enzymic hydrolysis of cellulose the colorimetric method is preferred for its convenience and rapidity. The amount of cellulose present is read from a glucose calibration curve, the factor 0.9 being used to obtain the unknown equivalent cellulose values.

Glucose calibration curve for cellulose determination. Suitable volumes of the 0.35% standard glucose solution, to cover the range 0.1–1.4 mg. of glucose, are pipetted into Quickfit test tubes and all tubes are made up to 0.9 ml. with water. Two blank tubes are also included with 0.9 ml. of water. The dichromate solution (2 ml.) is added and the reactants are carefully mixed. The tubes are closed with pear bulbs and heated in a boiling-water bath for 30 min., after which they are cooled in ice-water and 10 ml. of water is added to each tube. After fitting the tubes with ground-glass stoppers and mixing, 0.2 ml. of 20% (w/v) sodium sulphite is added to one of the blanks and mixed. The remaining tubes are read against the reduced blank with violet filters (no. 601) in the Spekker photoelectric absorptiometer (Hilger).

RESULTS

Quantitative recovery of cellulose from water suspensions. Table 1 shows the recoveries of cellulose powder over the range from 0.2 to 1.1 mg. of cellulose. Recoveries similar to those given in the table were also obtained with the other forms of cellulose, namely swollen cellulose, hydrocellulose and dewaxed cotton fibres. In the earlier experiments recoveries tended to be lower, but this was overcome by using the faster-flowing filter sticks of those available of porosity 3 (average pore diam. 20–30 μ) and by the compressed air-vacuum treatment described above ('Analytical procedure'). The latter treatment, carried out before putting the filter sticks in the water bath, prevented carbonization on the sintered plate, which sometimes occurred with the slower-flowing filter sticks.

No improvement in recoveries was obtained by drying (105° for 15 hr.) the cellulose and filter sticks before digestion with dichromate. At the 0.2 mg. level of cellulose, recoveries after oven-drying were identical with those in Table 1, where the cellulose was merely sucked dry on the filter stick. On the other hand, at the 0.8 mg. level of cellulose, oven-drying regularly gave low recoveries (90% of theory).

The results obtained by washing the cellulose with water before the dichromate treatment were the same as those obtained by washing with the selected reagents (HCl, aq. NH_3 soln., Teepol, ethanol) described above. This confirms earlier

work (Halliwell, 1957a) in which it was found that the selected reagents acted principally in removing bacterial debris from bacterial fermentations of cellulose.

Recoveries of cellulose were made worse by using a higher concentration of H_2SO_4 in the final dichromate and cellulose mixture. When the 0.9 ml. of water was omitted and 2 ml. of dichromate added to the cellulose, there was a greater tendency for carbonization to occur on the sintered plate. On the other hand, in the glucose determination the presence of water (up to 0.9 ml. in the final dichromate and glucose mixture) had negligible effect on the results, apart from the expected dilution of colour intensity.

Recovery of cellulose from fermentation media. In order to avoid errors in correcting for moisture, ash, etc., recovery experiments were done by comparing the cellulose obtained after filtration (from a culture medium) with the figure for a similar

Table 1. *Recovery of cellulose powder after filtration and washing*

Each weight represents the average of seven determinations. Figures are corrected for moisture and ash content. Results are calculated as anhydroglucose.

Cellulose taken (average, mg.)	Cellulose found (average, %)	Range of recovery (%)
1.084	100	98–102
0.663	99	96–102
0.353	98	96–101
0.190	97	95–100

Table 2. *Recovery of added cellulose from fermentation media*

Cellulose used was cellulose powder (Whatman). For each of the four average weights given, five pairs of samples of cellulose were weighed out. One sample of each pair was estimated for cellulose directly (not filtered) with dichromate and the other was placed in a tube with 3 ml. of phosphate buffer (pH 5.5, 0.067 M- Na_2HPO_4 + 0.067 M- KH_2PO_4) and 5 ml. of heat-inactivated cellulase from *Myrothecium verrucaria* (Halliwell, 1957b). The cellulose-enzyme mixture was filtered off and washed on filter sticks and the cellulose determined by the dichromate procedure. The figures give the recoveries of the cellulose filtered out from fermentation medium expressed as a percentage of the value found for the unfiltered cellulose.

Cellulose found* (average, mg.)	Recovery from fermentation medium (average, % of cellulose found)	Range of recovery (%)
1.168	100	99–100
0.797	98	97–100
0.296	100	99–100
0.206	98	97–102

* Calculated from calibration curve.

sample determined directly without filtration. Thus in Table 2 recoveries are quoted for experiments in which cellulose was filtered out from a culture medium of *Myrothecium verrucaria*. For each of the average weights 1.168, 0.797, 0.296 and 0.206 mg., five pairs of samples of cellulose powder were weighed out. One sample of each pair was estimated for cellulose directly by adding 0.9 ml. of water and 2 ml. of dichromate solution. The second sample of each pair was placed in a test

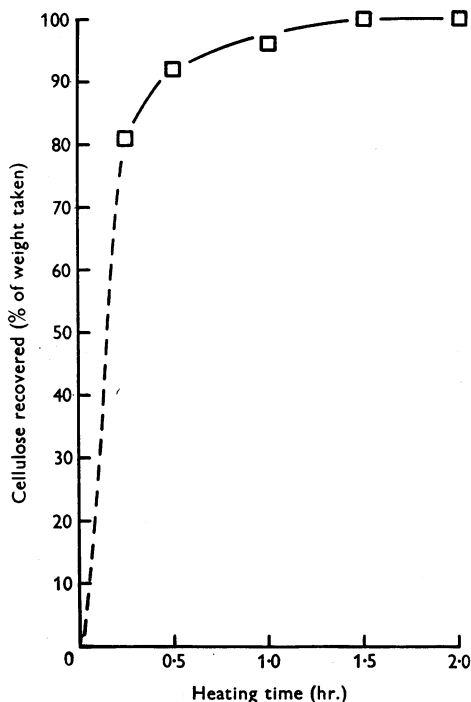


Fig. 1. Effect of heating time on the recovery of cellulose. Cellulose used was cellulose powder (Whatman). Cellulose (1.197 mg., average of seven replicates for each point) was washed with water on filter sticks and sucked dry; 0.9 ml. of water and 2 ml. of dichromate were added and all tubes heated in a boiling-water bath for the times shown. The oxidation mixture was then washed through the filter sticks with a total of 10 ml. water in portions. The tubes were read colorimetrically and results calculated as anhydroglucose; they are given as percentages of the weights of cellulose taken.

tube, to which was added 3 ml. of phosphate buffer (pH 5.5, 0.067M- Na_2HPO_4 and 0.067M- KH_2PO_4) and 5 ml. of heat-inactivated, cell-free cellulase from *M. verrucaria* (Halliwell, 1957b). The cellulose-enzyme mixture was filtered off immediately and washed with water in the usual manner. After sucking the cellulose dry on the filter stick it was treated with dichromate solution as described above ('Analytical procedure').

Effect of heating time with dichromate on the recovery of cellulose. Fig. 1 shows that maximal recoveries were obtained by oxidizing the cellulose with dichromate for 1.5 hr. In some experiments shorter times of heating (1 hr. and even 0.5 hr.) gave complete recovery, but in no case has longer than 1.5 hr. been required.

Application of the method to the determination of cellulase. Finally, the present micromethod for cellulose determination has been applied to the determination of the cellulase of *Myrothecium verrucaria*. Cell-free preparations of the cellulase were examined for activity on cellulose powder and on swollen cellulose (Halliwell, 1957b). The enzyme was incubated with each of the substrates at 37° and pH 6.0 in phosphate buffer (0.067M- Na_2HPO_4 and 0.067M- KH_2PO_4). Table 3 shows that the cellulose powder was solubilized to an extent of about 11% of its initial weight, whereas the swollen cellulose was almost completely solubilized (88%). These figures confirm results achieved on the semi-micro scale for a similar enzyme preparation on similar substrates (Halliwell, 1957b). Swollen cellulose, although attacked more rapidly than cellulose powder, resembles native cellulose more so than does carboxymethyl-cellulose. Varying the duration of hydrolysis (Fig. 2) and varying the enzyme concentration (Fig. 3) provide non-linear curves. As one would expect, the figures illustrate that the amorphous regions were attacked more readily than the less-reactive centres in the cellulose.

DISCUSSION

For some time the microprocedure of Schramm & Hestrin (1954) for the determination of cellulolysis by enzyme preparations from micro-organisms was used and highly satisfactory results were obtained

Table 3. Cellulolysis by cell-free enzymes of *Myrothecium verrucaria* as determined by the present method

M. verrucaria cell-free enzyme preparation (Halliwell, 1957b) was incubated in phosphate buffer (as Table 2), pH 6.0, at 37° for the times stated. Substrate consisted of cellulose powder (Whatman) or swollen cellulose powder (Whatman) (Halliwell, 1957b).

Substrate	Incubation time (hr.)	Enzyme (ml.)	Cellulose (mg.)		Cellulose solubilized (% of initial)
			Initial	Final	
Cellulose powder	45	1	0.88	0.78	11
Swollen cellulose powder	18	0.5	0.94	0.11	88

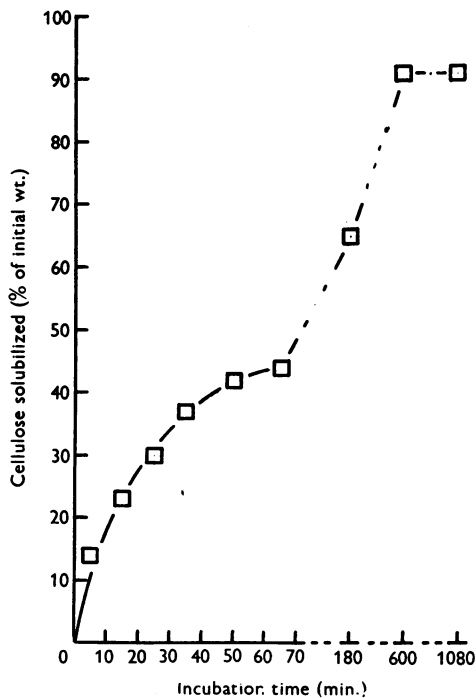


Fig. 2. Effect of period of incubation at 37° on the hydrolysis of cellulose by a cell-free extract of cellulase of *M. verrucaria*. Substrate, swollen cellulose powder (Whatman) (Halliwell, 1957*b*). Other conditions were as described for swollen cellulose powder in Table 3.

when filter sticks were substituted for centrifuging. The original procedure, however, with repeated centrifugings of cellulose was time-consuming and often gave rise to losses of cellulose in supernatant fractions, particularly when several different solutions were required for cleansing the cellulose before its determination. The introduction of filter sticks in the method overcame some of the delay, but not that associated with the acetolysis of the cellulose.

The method described in the present paper with filter sticks and dichromate is relatively rapid and well adapted to general use for cellulose determination. The reaction is stoichiometric and may be followed by titration or more conveniently by colorimetry. In the procedure presented here 0.2 mg. of cellulose appears to be the lower limit for reasonable accuracy. This weight is probably the lower limit for most laboratories in view of the error involved in weighing on the microbalance.

SUMMARY

1. A micromethod is described for the quantitative determination of cellulose. The cellulose is washed with water or other solvents on a sintered-glass filter stick. The cellulose is then oxidized

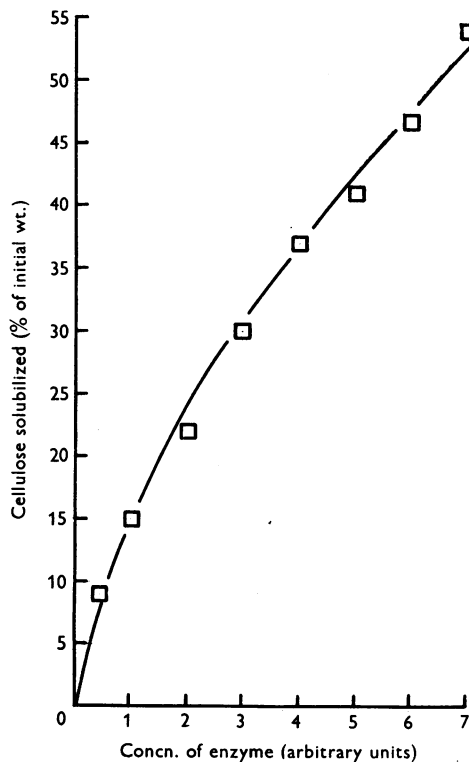


Fig. 3. Relationship between decomposition of cellulose and concentration of enzyme of *M. verrucaria*. Substrate, swollen cellulose powder (Whatman) (Halliwell, 1957*b*), incubated at 37° for 50 min. Other conditions were as for swollen cellulose powder and described in Table 3. The activity value at concentration 7 is that of 0.7 ml. of cell-free extract of *M. verrucaria*. Other enzyme concentrations were obtained by dilution of this extract, 0.7 ml. of the diluted solution being used for each assay.

stoichiometrically with sulphuric acid-dichromate reagent, the excess of dichromate being determined titrimetrically or, more conveniently, colorimetrically. The cellulose is calculated as anhydroglucose from a glucose calibration curve.

2. The method covers the range from 0.2 mg. of cellulose, with an average recovery of 97% (range 95–100%), to 1.1 mg. of cellulose, with an average recovery of 100% (range 98–102%).

3. The applicability of the method to different forms of cellulose (cellulose powder, swollen cellulose, hydrocellulose and dewaxed cotton fibres) has been verified.

4. The procedure has been used to determine cellulolysis by the cellulase of *Myrothecium verrucaria*.

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The Relationship Between Glucuronidase and Galacturonidase Activity in the Limpet and in Mammalian Tissues

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In the first systematic study of the enzyme β -glucuronidase, Masamune (1938) showed that ox-kidney preparations did not hydrolyse (-)-menthyl α -D-glucuronide, and this compound was also not hydrolysed by mouse-liver β -glucuronidase (Levvy & Marsh, 1952) and by a snail preparation rich in β -glucuronidase and in other simple glycosidases (Utusi, Huzi, Matumoto & Nagaoka, 1949). Nevertheless, although it is not so ubiquitous as the β -glucuronide residue, there is evidence for a naturally occurring α -conjugated glucuronic acid, for example in wheat straw (Bishop, 1953; Roudier, 1953). It has been inferred that uridine diphosphoglucuronic acid also has the α -configuration (Storey & Dutton, 1955; Strominger, Maxwell, Axelrod & Kalckar, 1957).

The synthesis of chromogenic α -glucuronides (see Appendix) made a search for an enzyme hydrolysing these compounds feasible, and a rich source was found in the visceral hump of the common limpet (*Patella vulgata*), which was already known to contain β -glucuronidase (Dodgson, Lewis & Spencer, 1953) as well as many other simple glycosidases (Conchie & Levvy, 1957). α -Glucuronidase activity was also detected elsewhere, but not in mammalian tissues.

During this investigation it was observed that limpet preparations hydrolysed β -D-galacturonides and, to a lesser extent, α -D-galacturonides. Hydrolysis of both types of β -glucuronide was inhibited by saccharo-1:4-lactone and less powerfully by boiled mucate solution, suggesting that a single enzyme was responsible for both reactions. Since these inhibitors act similarly on mammalian β -glucuronidase (Levvy, 1952; see also Conchie & Levvy, 1957), it was considered possible that this enzyme should also hydrolyse β -galacturonides, and this has been verified. Previous evidence from experiments with competing substrates had sug-

gested that β -galacturonides are unaffected by β -glucuronidase (Levvy & Marsh, 1952), although the enzyme was known to be inhibited by D-galacturonic acid as well as by D-glucuronic acid (Levvy, 1952).

The method of synthesis employed for aromatic glucuronides and galacturonides (see Appendix) was extended to the preparation of phenyl α - and β -N-acetylglucosaminuronides. These are compounds of particular interest in that the sugar moiety is said to occur in bacterial polysaccharides (Park, 1951; Webster, Clark & Freeman, 1954) and is closely related to one of the structures proposed for neuraminic acid and sialic acid (Klenk, Faillard, Weygand & Schöne, 1956), although this structure is open to doubt (Cornforth, Daines & Gottschalk, 1957). Neither glucosaminuronide was, however, hydrolysed by the appropriate glucuronidase or glucosaminidase.

EXPERIMENTAL

Materials

Phenolphthalein glucuronide (Talalay, Fishman & Huggins, 1946) and veratroyl glucuronide (Sammons & Williams, 1946) were biosynthetic. The synthesis of other aromatic glucuronides employed and of borneol α -D-glucuronide are described in the Appendix (Marsh & Levvy, 1958); aliphatic glucuronides were prepared as described by Marsh (1952). D-Mannurone and D-mannuronic acid were prepared from methyl α -D-mannuronide. Sources of other compounds used are given in a recent paper (Conchie & Levvy, 1957).

Two types of solution of the inhibitory saccharo-1:4-lactone were employed: (a) the freshly dissolved pure lactone; (b) aqueous mM-potassium hydrogen saccharate maintained at 100° for 30 min. to convert one-third of the anion into saccharo-1:4-lactone as described by Levvy (1952). Where the latter was employed it is referred to as 'boiled saccharate' in the text. Similarly, 'boiled mucate'