The Extracellular Polysaccharide of *Aerobacter Aerogenes* A3 (S1) (Klebsiella Type 54)

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Although it has long been known that organisms of the Aerobacter–Klebsiella group produce very mucoid growths, few attempts have been made to investigate the nature of their capsules and slime. Recently, attention has been drawn to the extracellular polysaccharides of these organisms by the work of Edwards & Fife (1952), who showed that these bacteria exist in a large number of immunologically distinct types. Edwards & Fife identified fifty-seven types, but this has since been increased to nearly 100 (Edmunds, 1954; Henriksen, 1954). These extracellular polysaccharides may occur as loose slime or as distinct capsules and the amount produced varies considerably according to the nature of the growth medium (Duguid & Wilkinson, 1953; Wilkinson, Duguid & Edmunds, 1954). They are also responsible for the distinctive antigenic specificity of strains in the mucoid phase (Wilkinson *et al.* 1954).

Chemical studies on the nature of *Aerobacter aerogenes* extracellular polysaccharides have been carried out on untyped strains. Schardinger (1902*a*, *b*) isolated a galactan from a 6-week broth culture of *Aero. aerogenes* but the product may have been an intracellular polysaccharide. Tomcsik (1927) isolated a polysaccharide by treatment of capsulated cells with potassium hydroxide, and Warren (1950) obtained a slime fraction, but neither author identified the component sugars.

No attempts have been made to determine the influence of different carbon sources on the relative amounts of the component sugars in a heteropolysaccharide. Morgan & Beckwith (1939) cultured a strain of *Escherichia coli* on media containing sucrose, glucose, rhamnose or xylose as the carbon source and showed that the immunological specificity of the strain was unaffected. However, this may only mean that a certain immunologically determinant group is present in the extracellular polysaccharide and the overall composition may have varied. Forsyth & Webley (1949) grew various species of the genus *Bacillus* on media containing sucrose, fructose, glucose, galactose or arabinose, and found that the component sugars of the extracellular polysaccharide remained unaltered although no quantitative determinations were carried out.

In the present study, the composition of the extracellular slime polysaccharide of *Aero. aerogenes* strain A 3 (S 1) (Klebsiella Type 54) has been studied after growth on media containing different carbon sources. The constituent sugars have been identified and the different polysaccharides compared.

EXPERIMENTAL AND RESULTS

Growth of organism

Organism. *Aero. aerogenes* A3 (S1) is a non-capsulate organism producing an abundance of extracellular polysaccharide as a viscous slime. Immunologically it belongs to Type 54 of the Edwards classification, and its origin and biochemical characteristics are described by Wilkinson *et al.* (1954).

Growth medium. The organism was grown in a medium containing a relatively high sugar and low nitrogen-source concentration which gives maximal polysaccharide production (Wilkinson *et al.* 1954). Growth was carried out in 1 l. screw-capped round bottles, each containing 200 ml. of the following medium: 1·0% Na_2HPO_4; 0·3% KH_2PO_4; 0·03% $(NH_4)_2SO_4$; 0·1% K_2SO_4; 0·1% NaCl; 0·02% $MgSO_4, 7H_2O$; 0·002% $CaCl_2, 6H_2O$; 0·0001% $FeSO_4$; 1% carbohydrate (glucose, sucrose, galactose, mannitol, xylose, rhamnose, fucose or glucurone; in the case of glucurone, sufficient NaOH was added to adjust the pH to 7·3). The medium was sterilized by steaming and inoculated from a 24 hr. culture previously grown and adapted to the sugar to be used in the growth medium. The bottles were then filled with O_2 through sterile plugged tubing for 2 min. In order to maintain adequate oxygenation of the medium during growth, the bottles were rotated horizontally on a drum of horizontal axis capable of holding six bottles at a time and mounted in a 35° incubator. The cultures were grown for 48 hr.

General analytical methods

Total nitrogen was determined by the micro-Kjeldahl method. Total phosphorus was determined by the Fiske & Subbarow (1925) method. The ash content was determined by incinerating a little of the material in a Pt boat to constant weight, a drop of 60% (w/v) $HClO_4$ being added after the first ignition.

The reducing value of the unhydrolysed and hydrolysed polysaccharides was determined using Somogyi's (1945) copper reagent in conjunction with a colorimetric reagent (Nelson, 1944) calibrated against glucose. The results were expressed in terms of percentage glucose.

The anthrone value. The modified anthrone method of Fairbairn (1953) was adopted. The method, which is finding increasing application as a rapid and sensitive means of estimating polysaccharides, is of little direct value in composition studies. However, it was found to be useful as an independent check on the composition data derived by the methods described below. This was done by comparing the experimental anthrone value of a polysaccharide with the anthrone value calculated from the composition data. The 'anthrone equivalents' of the component sugars (colour given by 100 μg. of the sugar/colour given by 100 μg. glucose) were as follows: galactose, 0·60; fucose, 0·92; glucurone, 0·05. All results were expressed in terms of percentage glucose.

Determination of component sugars

Glucose, galactose and fucose. These sugars were estimated after hydrolysis and chromatographic separation on paper according to the general principles described by Flood, Hirst & Jones (1948). The polysaccharides were found to be polyuronides and resistant to the normal methods of hydrolysis such as N-H_2SO_4 at 100° for 24 hr. The conditions finally adopted as the standard method for quantitative studies which gave complete hydrolysis as indicated by a maximal reducing value, were 24 hr. in 96% (w/v) formic acid at 100°, followed by 6 hr. in N-H_2SO_4 at 100°. Samples of polysaccharide (20–40 mg.) were hydrolysed for 24 hr. with 96% formic acid (2 ml.) in a sealed tube in a boiling-water bath. (Preliminary experiments showed that a polysaccharide concentration within the range 10–20 mg./ml. gave minimal destruction.) A weighed quantity (15–30 mg.) of reference sugar (arabinose), shown chromatographically to be absent from the polysaccharide hydrolysate, was added and the formic acid distilled off under reduced pressure. Further hydrolysis was carried out for 6 hr. at 100° after the addition of N-H_2SO_4 (2 ml.). The contents of the tube were neutralized by the addition of $BaCO_3$. It was assumed that the destruction of the reference sugar and the component sugars of the polysaccharide took place at the same rate.

Because of the difficulties introduced by lactone trails on chromatograms and of the anomalous yield of formic acid from periodate oxidation, it was necessary to remove uronic acids from the hydrolysate. Preliminary experiments were made using anion-exchange resins, but these were discontinued because of the possibility that free sugars might be differentially adsorbed on to the resin (Roseman, Abeles & Dorfman, 1952). Finally it was found that the uronic acid could be removed from the neutral hydrolysate without disturbing the ratio of free sugars, by precipitation of the Ba salt. Ethanol (2 vol.) was added to the supernatant after $BaCO_3$ treatment, the tube was left to stand overnight at 4°, and the precipitated Ba salt of the uronic acid was removed by centrifuging. The supernatant was treated with Amberlite cation-exchange resin IR-120 and evaporated to dryness. The syrup was dissolved in an appropriate small volume of water and suitable amounts were applied to the starting line of a strip of Whatman no. 1 filter paper. The solvent used for separation in early experiments was a benzene–butanol–pyridine–water mixture (1:5:3:3, v/v) for 44 hr. at 15°. This gave good separation of sugars but often interfered in the estimations because of erratic high blank values. It was replaced in later experiments by butanol–ethanol–water mixture (5:1:4, v/v), which was found to give equally good separation after 120 hr. at 15° while having reproducible small blank values. After drying, the side strips were sprayed with saturated aqueous aniline oxalate, and the colours developed by heating; it was often found that spots invisible by daylight could be observed under ultraviolet radiation. The sugars were eluted from the corresponding central portion of the chromatogram by the method of Laidlaw & Reid (1950). The amounts of the sugars were estimated by periodate oxidation (Hirst & Jones, 1949).

Fucose. This was also determined separately on the unhydrolysed polysaccharide by the colorimetric method for methylpentoses (Dische & Shettles, 1948). The method was claimed by the authors to be specific for methylpentoses but in trial experiments it was found that all the sugar components of the polysaccharide would affect the result. The relative colour-producing powers of fucose, glucose and glucurone were 1·00, 0·058 and 0·021, respectively, and in each case the colour was proportional to the sugar concentration. Experiments with mixtures of these sugars showed that when the colours produced by glucose and glucurone were subtracted from the total colour, fucose (6–44 μg.) could be estimated in the presence of glucose (68 μg.) and glucurone (37 μg.) with recoveries of 95–112%. By correcting the results for the amounts of glucose and uronic acid (assumed to be glucurone) known to be present in the polysaccharide, the fucose content determined by this method was found to give results comparable to the chromatographic method.

Uronic acid. This was determined on the unhydrolysed polysaccharide by the decarboxylation method of McCready, Swenson & Maclay (1946).

Isolation and purification of the polysaccharide

The culture was centrifuged at 13000 rev./min. for 30 min. and the cellular centrifugate discarded. High-speed centrifuging is necessary because of the viscosity of the slime polysaccharide; the culture supernatant gave a relative viscosity of 22 at 15° as determined in an Ostwald viscometer. Acetone (1·5 vol.) was added to the supernatant and the mixture stirred with a spatula. The polysaccharide gel adhered to the spatula and could be washed and dehydrated by gradually increasing concentrations of acetone until the gel was converted into a stringy white solid. This resulted in a considerable purification of the polysaccharide, since many of the impurities present in the culture supernatant did not adhere to the spatula and were not precipitated by 1·5 vol. acetone (e.g. the extracellular 0-polysaccharide). The dehydrated polysaccharide was dissolved to give a 0·5% solution in an acetate buffer containing 4% (w/v) sodium acetate and 2% (v/v) acetic acid. It was then deproteinized by shaking each 100 ml. with six to eight successive lots of a mixture of $CHCl_3$ (20 ml.) and *n*-butanol (4 ml.) (Sevag, 1934). The final aqueous solutions were combined, recentrifuged and precipitated by acetone (1·5 vol.) as described previously. The dried precipitate was redissolved in water to give a 0·2% solution and was dialysed against running tap water for 96 hr. The purified polysaccharide was finally precipitated from solution by one of two methods.

Method A

The solution was shaken alternately with small portions of anion and cation-exchange resins (Amberlite IR-4B and

IR-120). This produced the free acid form of the polysaccharide which was no longer precipitable by acetone. Instead, it was lyophilized.

Method B

Sodium acetate (1%) was added followed by precipitation with acetone (1·5 vol.). The precipitate was washed with acetone and ether and dried in a vacuum desiccator over P_2O_5.

In the procedures described above, it was difficult to remove protein effectively from the polysaccharide preparation because of its high viscosity in solution. It was found that if a solution was boiled at a neutral pH, the viscosity fell to a low value with a consequent increase in the ease of handling. Consequently, the following method was adopted for later preparations.

Method C

The initial precipitate from the culture supernatant was dissolved in distilled water to give a 0·5% solution, the pH was adjusted to 7·0 and the solution was boiled for 30 min. The further stages of deproteinization and purification were as described in method *B*, except that the polysaccharide required the addition of 3 vol. of acetone followed by a period of 12 hr. at 4° to allow complete precipitation.

The yields of polysaccharide isolated by the three methods were of the order of 1 g./3·6 l. of culture.

Separation and identification of the component sugars of the polysaccharide

Polysaccharide (2 g.) was hydrolysed by heating on a boiling-water bath for 24 hr. in 96% formic acid (200 ml.). After removal of the formic acid by distillation under reduced pressure, the resultant syrup was heated at 100° for 6 hr. in N-H_2SO_4 (100 ml.). The product was neutralized with $BaCO_3$, concentrated to 30 ml. and methanol (60 ml.) was added. The mixture was left for 24 hr. at 4° and the precipitate of the Ba salt of the uronic acid was centrifuged. Chromatographic analysis showed that the supernatant contained glucose, fucose and a small amount of uronic acid. There was also a slight trace of an unknown sugar travelling on a chromatogram faster than fucose. This may have been fucoketose produced by prolonged contact with $BaCO_3$, since fucose treated with $BaCO_3$ for 24 hr. at room temperature produced a spot similar in its chromatographic behaviour. (Rate of movement relative to 2:3:4:6-tetramethyl glucose (R_G) in *n*-butanol–ethanol–water (5:1:4, v/v) was 0·36.) The supernatant was evaporated to a syrup and redissolved in water to 60 ml. The remaining uronic acid was removed by passage through a column of Amberlite IRA-400 resin previously converted into the carbonate form. The eluate (500 ml.) containing glucose and fucose, was concentrated and dried over P_2O_5 to give a syrup (1·5 g.), which was fractionated on a cellulose column at 37° by using as the solvent butanol saturated with water as described by Hough, Jones & Wadman (1949). Every tenth tube was examined for the presence of carbohydrate by the anthrone method and for the nature of the sugar chromatographically. The following results were obtained: tubes 1–54, no carbohydrate; tubes 55–95, fucose; tubes 96–124, fucose and glucose; tubes 125–260, glucose.

Identification of L-*fucose.* The syrup (93 mg.) from tubes 55–95 had $[\alpha]_D^{15} - 69°$ in water (*c*, 2) (literature value $-76°$) and paper chromatography showed the presence of fucose only. The identity of the sugar as L-fucose was confirmed by the formation of L-fucose phenylhydrazone (m.p. and mixed m.p. 156–159°).

Identification of D-*glucose.* Syrup (300 mg.) from tubes 125–245 had $[\alpha]_D^{15} + 50°$ in water (*c*, 2) (literature value, $+52·5°$) and paper chromatography showed the presence of glucose only. The identity of the sugar as D-glucose was confirmed by the formation of β-D-glucose penta-acetate (m.p. and mixed m.p. 124–127°).

Identification of the uronic acid. This will be dealt with in a later communication. The fact that a lactone was formed, indicated that the uronic acid was not galacturonic acid.

Analysis of the extracellular polysaccharide from glucose-grown Aerobacter aerogenes

The basic results of the quantitative analysis of the polysaccharide prepared by methods *A*, *B* and *C* after growth on glucose are shown in Table 1. The main components are glucose, fucose and uronic acid. A small amount of galactose was also present but was most likely due to contamination by some extracellular 0-polysaccharide which is probably a galactan (Dudman & Wilkinson, unpublished results). The colorimetric method for the estimation of fucose was found to give more consistent results than the chromatographic method, possibly because of the rather diffuse nature of the fucose spot on the chromatograms. The anthrone value calculated from the analytical data compares well with the experimental result, showing that all the anthrone-reactive carbohydrates in the polysaccharide had been accounted for. It can be seen that the relative proportions of the main component sugars remain constant irrespective of the mode of preparation, showing that boiling the polysaccharide solution caused no change in composition in spite of the great lowering in viscosity.

Further analysis was carried out on the polysaccharide prepared by method *A*. The specific rotation was $[\alpha]_D^{15} - 50° \pm 5°$ in water (*c*, 0·1). The uronic acid content, determined by the naphthoresorcinol method (Jarrige, 1947), was 22%. It was found over a range of different heteropolysaccharides, that this method gave consistently low values compared with those by decarboxylation, possibly owing to difficulty in obtaining complete hydrolysis of the polysaccharide without decomposition of the uronic acid. The acid equivalent was 760, corresponding to a uronic acid content of 25·5%. The reducing value after hydrolysis was 78%. Amino acids and hexosamines were absent as indicated by negative results after paper chromatography using respectively, ninhydrin and the Elson & Morgan (1933) reagent as developers. Ketoses were absent, as indicated by negative results after paper chromatography using naphthoresorcinol reagent as developer and also by the Seliwanoff reaction on the intact polysaccharide.

Analysis of the extracellular polysaccharide of Aero. aerogenes *after growth on various carbon sources*

The results of analysis of the extracellular polysaccharide produced by *Aero. aerogenes* after growth on a variety of carbohydrates as the sole carbon and energy source and isolated by method *C*, are shown in Table 1. The carbohydrates were chosen to give representative types of sugars as well as those normally occurring in the polysaccharide. The proportions of the component sugars, glucose, fucose and uronic acid, can be seen to be identical within the limits of experimental error in each of the polysaccharides.

It has been reported that *Bacillus polymyxa* when grown on monosaccharides produces an extracellular polyuronide while after growth on sucrose it produces a polyuronide together with an extracellular levan (Hestrin, Avineri-Shapiro & Aschner, 1943; Forsyth & Webley, 1949). Consequently, *Aero. aerogenes* was grown on sucrose as the sole source of carbon and energy, and the polysaccharide isolated according to method *C*. A full analysis was not made but fructose was shown to be absent by paper chromatography after mild acid hydrolysis and by the Seliwanoff reaction on the intact polysaccharide.

DISCUSSION

All extracellular heteropolysaccharides produced by bacteria from widely different species and examined with the aid of modern techniques, have been found to contain uronic acid or hexosamine components. The extracellular polysaccharide of *Aero. aerogenes* A 3 (S 1) is a polyuronide and therefore no exception to this rule. An unusual feature is the presence of L-fucose. Until a recent report by Norris, de Sipin, Zilliken, Harvey & György (1954) that fucose was a component of the polysaccharide of a mucoid mutant of *Lactobacillus bifidus*, fucose has never been found in a bacterial polysaccharide. However, another naturally occurring methylpentose, rhamnose, has been identified in the polysaccharides of a variety of strains (see Whistler & Smart, 1953).

An ultracentrifugal examination of the polysaccharide, prepared by method *A* after growth on glucose, indicated the presence of only one component with a narrow molecular weight distribution, thus suggesting the presence of a single polysaccharide rather than a mixture of two or more molecular species (C. T. Greenwood, 1954; unpublished results).

The amount of the three main components, glucose, fucose and uronic acid, was found to be independent of the carbon source used in the growth medium. This has a bearing on the mode of

Table 1. *Analytical results on the polysaccharide of* Aerobacter aerogenes *after growth on various carbon sources*

	Sugar in growth medium										
	Glucose			Galactose	Mannitol	Xylose	Rhamnose	Fucose	Glucurone
Method of preparation ...	*A*	*B*	*C*	*C*	*C*	*C*	*C*	*C*	*C*
Glucose (%)	46	46	50	45	43	49	47	44	46
Galactose (%)	2	2	1	1	2	2	2	2	1
Fucose (%)									
(*a*) Chromatographically	10		10	7	4	8	8	7	7
(*b*) Colorimetrically	9		10	9	9	10	9	9	9
Uronic acid (%)	27	28	29	28	25	28	26	27	29
Total % sugar components	84	85	90	83	79	89	84	82	85
Fucose relative to glucose as 100	20	20	20	20	21	20	19	20	20
Uronic acid relative to glucose as 100	58	61	58	61	58	58	56	61	62
Reducing value of unhydrolysed polysaccharide	1·8	1·6	0·7	0·8	1·4	1·3	0·8	1·2	1·3
Anthrone value (%)	54	55	64	59	53	60	52	55	57
Anthrone value theoretical (%)	56	56	60	54	53	60	57	55	56
Ash (%)	1·3	4·0	7·6	7·0	6·3	4·5	4·0	3·7	2·9
N (%)	1·05	0·18	0·17	0·15	0·15	0·39	0·79	0·65	0·89
P (%)	0·10	0·12	0·04	0·04	0·06	0·19	0·49	0·45	0·43

synthesis of such heteropolysaccharides. Work on homopolysaccharides summarized by Barker & Bourne (1953) and by Hassid (1954) has shown that they are synthesized according to the general equation:

$$Gt\text{–}O\text{–}X + H\text{–}O\text{–}Gr \rightleftharpoons Gt\text{–}O\text{–}Gr + X\text{–}O\text{–}H,$$

where Gt–O and X are, respectively, the sugar residue and the aglycone portion of a glycoside donor (Gt–O–X) which serves as the substrate for an enzyme, and Gr–O–H is the carbohydrate receptor molecule. Two main types of compounds have been demonstrated as glycoside donors, (1) disaccharides and higher saccharides where X is a carbohydrate residue, (2) sugar phosphates where X is a phosphate residue. However, no evidence has been obtained as to the nature of the glycoside donors in heteropolysaccharide formation although they may be assumed to function in a similar manner. By varying the nature of the carbon source during growth and polysaccharide formation, one can be assumed to vary the equilibrium concentrations of these glycoside donors so that the relative proportions are different. This follows unless all the carbon sources are metabolized to a common intermediate from which all the glycoside donors are formed. If polysaccharide synthesis is carried out by a comparatively non-selective enzyme or enzymes which string together glycoside radicals according to the availability of glycoside donors, then the nature of the polysaccharide should vary according to the concentration of glycoside donors and thus according to the nature of the carbon growth source. This was not so in the case of the extracellular heteropolysaccharide of *Aero. aerogenes* and therefore a mechanism of this type is unlikely to occur.

Rather, there appear to be two possible mechanisms:

(1) Synthesis carried out by a series of completely specific enzymes. The number of enzymes involved would depend on the complexity of the polysaccharide; even for the simplest type of regular structure (e.g. a linear chain with regularly spaced branching points) at least three specific enzymes would be required. Preliminary results of a structural investigation of the extracellular polysaccharide of *Aero. aerogenes* indicate a highly branched complex molecule, and therefore that a large number of enzymes would be required in order to synthesize this polysaccharide invariably.

(2) Synthesis carried out by a template mechanism as envisaged for protein synthesis. Such a template would probably involve deoxyribonucleic acid in view of the evidence of type transformation in the pneumococcus and other organisms (summarized by Austrian, 1952).

Since the ability to synthesize an extracellular polysaccharide and therefore form a mucoid colony is apparently controlled by a single mutable step, and thus according to current views by one enzyme, the former mode of synthesis involving a large number of enzymes is less likely. Thus the evidence favours the idea that heteropolysaccharide synthesis is carried out by some template mechanism, and is thus different from the mechanism of homopolysaccharide formation.

SUMMARY

1. The extracellular slime polysaccharide of *Aerobacter aerogenes* A 3 (S 1) (Klebsiella Type 54) was isolated and purified and shown to contain three main component sugars: D-glucose (50 %), L-fucose (10 %) and an unidentified uronic acid (29 %).

2. The bacterium was grown in presence of a variety of carbohydrates as the sole carbon and energy source and the polysaccharides produced from each were isolated. The composition was found to be invariable. The implications of this fact upon the nature of heteropolysaccharide synthesis is discussed.

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The Preparation and Properties of β-Glucuronidase

7. ACTION ON ESTER GLUCURONIDES

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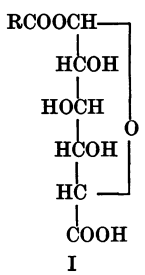
It has long been known that aromatic carboxylic acids can be conjugated with glucuronic acid in the animal body to form ester glucuronides (general formula, I; for review, see Williams, 1949): the best-known example is benzoyl glucuronide (I, R = C_6H_5), but the glucuronide of veratric acid (3:4-dimethoxybenzoic acid) is more easily prepared (Sammons & Williams, 1946). Interest in the ester glucuronides has recently been stimulated by the discovery that the branched-chain fatty acids, α-ethylbutyric acid and α-ethylhexanoic acid, can form derivatives of this type *in vivo* (Kamil, Smith & Williams, 1953). Unlike the ether glucuronides formed by alcohols and phenols (general formula, II), the ester glucuronides in general are unstable in alkaline solution and therefore give a positive reaction in tests for reducing sugars.

The biosynthetic glucuronides of alcohols and phenols are hydrolysed by the enzyme β-glucuronidase, but the action of this enzyme on ester glucuronides has not hitherto been studied, unless the synthetic compound β-glucuronic acid 1-phosphate (II, R = H_2PO_3; Levvy & Marsh, 1952), is regarded as a member of the group. At the suggestion of Professor R. T. Williams, specimens of α-ethylbutyryl, α-ethylhexanoyl and veratroyl glucuronides which he supplied were examined as possible substrates for mouse-liver β-glucuronidase.



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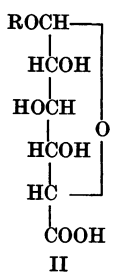

II

EXPERIMENTAL AND RESULTS

Methods

In general, methods followed those used in earlier papers, in particular, Levvy & Marsh (1952) and Levvy (1954).

Inhibition of the hydrolysis of phenolphthalein glucuronide

α-Ethylbutyryl, α-ethylhexanoyl and veratroyl glucuronides were tested as competing substrates in the hydrolysis of phenolphthalein glucuronide by mouse-liver β-glucuronidase (Levvy & Marsh, 1952). As a test of enzyme specificity, this technique is less dependent on the purity of the enzyme preparation than direct measurements of hydrolysis. In the present instance, the difficulty to be avoided was the possible presence in the liver preparations of an esterase capable of hydrolysing ester glucuronides. To conserve supplies of the ester glucuronides, the microprocedure for enzyme assay described in connexion with baicalinase (Levvy, 1954) was adopted. Incubation was for 1 hr. at 37° and pH 5·2 in sodium acetate–acetic acid buffer, final concentration 0·125 N. The phenolphthalein glucuronide concentration ranged from 0·00125 to 0·000031 M.

All three ester glucuronides depressed the release of phenolphthalein and acted competitively, the percentage depression varying inversely with the phenolphthalein glucuronide concentration. Values for K_i, the dissociation constant for the complex formed between enzyme and competing compound, were determined by the method of Lineweaver & Burk (1934). The mean values obtained were: α-ethylbutyryl glucuronide, $7{\cdot}9 \times 10^{-4}$ M; α-ethylhexanoyl glucuronide, $2{\cdot}2 \times 10^{-4}$ M; veratroyl glucuronide, $3{\cdot}5 \times 10^{-5}$ M. The reciprocals of these figures give an approximate measure of the affinities of the respective ester glucuronides for the enzyme (Levvy & Marsh, 1954). For phenolphthalein glucuronide and mouse-liver β-glucuronidase, the