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# STUDIES OF MOLECULAR-WEIGHT DISTRIBUTION FOR HYDROLYSIS PRODUCTS FROM SOME *Klebsiella* CAPSULAR POLYSACCHARIDES*

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ABSTRACT

Gel-permeation chromatography has been used to determine the molecular-weight distribution of the products at various stages of acid hydrolysis of some capsular polysaccharides from *Klebsiella* bacteria. Structurally significant oligosaccharides, which are believed to correspond closely to the chemical repeating units in the polysaccharide molecules, were detected together with products having higher molecular weights, which are clearly aggregates of these oligosaccharides. This constitutes good supporting evidence for the view that relatively simple sequences of sugars are repeated throughout the entire molecular structure of these polysaccharides, and quantitative information for the polysaccharides from three different *Klebsiella* strains (serotyped as K54, K4, and K64, respectively) has been obtained by this procedure. The study of the polysaccharide from *Klebsiella* K-type 54 has afforded both independent corroboration and some extension of available data.

## INTRODUCTION

The technique whereby the course of acid hydrolysis of polysaccharides is monitored by gel-permeation chromatography has been extensively used in this laboratory in studies of the breakdown patterns of plant-gum polysaccharides[1–4]. Recently, this procedure has been applied to capsular polysaccharides from different strains of bacteria of the genus *Klebsiella*; in studies to be described in the present paper and elsewhere[5]. This relatively simple technique involves less expenditure of both time and money than do other established methods, such as radiochemical[6] and enzymic[7] techniques, used in the investigation of polysaccharide structure.

## EXPERIMENTAL

*General methods.* — Gel-permeation chromatography was carried out on calibrated columns of Sagavac 6F (6% agarose), Bio-Gel P-300, or Bio-Gel P-10 as

*Dedicated to Dr. Horace S. Isbell, in honour of his 75th birthday.

appropriate, with M sodium chloride eluent, as described previously[1,8]. The solvent systems used in paper chromatography were (*a*) ethyl acetate–pyridine–water (8:2:1), (*b*) butan-1-ol–acetic acid–water (2:1:1), and (*c*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4). Optical rotations were measured at 18° on a Perkin–Elmer Model 141 polarimeter. Reducing power of hydrolysates was determined by the Nelson modification[9] of the Somogyi method[10], due allowance being made for the different responses[6] to the reagents of the various sugars present in the polysaccharides.

Molar proportions of neutral sugars in hydrolysates (2M trifluoroacetic acid, 100°, 8 h) were as determined by means of a Technicon AutoAnalyzer system, using gradient elution, at pH 7.0, with borate buffers[11], and an automated cysteine–sulphuric acid method[12] to assay the sugars. Equivalent weights of the polysaccharides were determined by titration with 0.01M sodium hydroxide, in conjunction with atomic absorption spectroscopy of the cations present in their neutralised forms, and, residually, in the acidic polysaccharides. Pyruvate was estimated both colorimetrically[13] and by p.m.r. spectroscopy (Varian XL-100 instrument) of deuterium oxide solutions of the sodium salts of the polysaccharides after repeated deuterium exchange[14–15]; $\tau'$ values are given relative to that for sodium 4,4-dimethyl-4-silapentanesulphonate, taken as 10. Spectra were run at temperatures ranging from 28 to 90°. The latter method was used also to ascertain the presence of acetate groups[15].

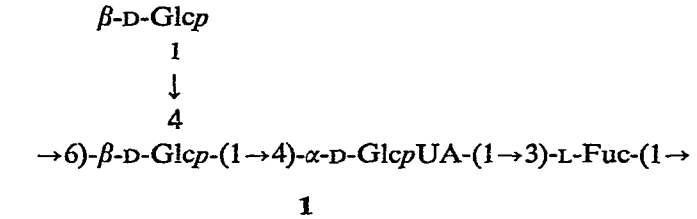
*Preparation and purification of polysaccharides.* — The *Klebsiella* strains, isolated at Groote Schuur Hospital, Cape Town, were serotyped as K54, K4, and K64, respectively, by courtesy of Dr. I. Ørskov (Copenhagen). Cultures were grown in a medium containing agar (10 g), sucrose (30 g), yeast extract (2 g), NaCl (2 g), $KH_2PO_4$ (2 g), $CaCO_3$ (0.8 g), and $MgSO_4 \cdot 7H_2O$ (0.25 g) per litre. After 48 h, the cells were harvested, treated with 5% aqueous phenol, and poured into ethanol. The resulting precipitate, containing both the required polysaccharide and cellular debris, was collected by centrifugation and then resuspended in water. This suspension was centrifuged for 90 min at 27,000 *g*, the supernatant solution yielding the polysaccharide on freeze-drying. Each polysaccharide isolated in this manner was subsequently purified by Cetavlon precipitation (Nimmich's procedure[16]), and the resulting products were redissolved in water, dialysed for several days, and deionized with Amberlite IR-120 ($H^+$) resin before being freeze-dried to yield the samples used in the present work.

*Acid hydrolysis of polysaccharides.* — A sample (100 mg) of each polysaccharide was heated at 100° in 0.01M trifluoroacetic acid (25 ml) for 48 h, samples (2 ml) being removed at intervals. The acid concentration in the solution was then adjusted to 0.1M by addition of M trifluoroacetic acid, and heating was continued, with periodic removal of samples, for a further 24 h. Finally, the remaining solution was evaporated to dryness, and the residue was redissolved in 2M trifluoroacetic acid and heated at 100° for 18 h. Each hydrolysate sample, in aqueous solution (2 ml) after the removal of the trifluoroacetic acid by evaporation, was examined by gel-permeation chromato-

graphy, paper chromatography, and measurements of optical rotation and reducing power, as described in previous papers[1–4].

## RESULTS AND DISCUSSION

*Capsular polysaccharide of* Klebsiella *K-type 54.* — This polysaccharide, which contains residues of glucose, fucose, and glucuronic acid, has been much investigated[6,7,17–20], and all the available evidence suggests the presence of the repeating sequence of sugars shown in **1**.




Substituent acetate (one to every eight sugar residues in the molecule)[7,19] and formate[20] groups have also been reported.

The accumulation and decay of the aldotetraouronic acid believed to correspond to the repeating unit in the molecule, and of its degradation products, at various stages during hydrolysis of the polysaccharide in 0.5M sulphuric acid at 100°, has been studied by Conrad *et al.*[6], using a procedure involving radiochromatography of these products following reduction with $^3$H-labelled sodium borohydride. The present study is an extension of this investigation, since the use of milder conditions of hydrolysis has enabled the gradual formation of the aldotetraouronic acid from oligomers thereof to be followed. The main conclusions have been summarised in a preliminary note[21]; the results are given here in detail, in Tables I and II, and Figs. 1–3.

TABLE I

PARTIAL HYDROLYSIS OF *Klebsiella* K54 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

| *Time* (*h*) | $\overline{M}_w$ | $[\alpha]_D$[b] (*degrees*) | *Degree of scission* (x)[d] |
|---|---|---|---|
| 0 | 1,600,000[a] | −28[c] | |
| 1 | 65,000 | −25 | 0.02 |
| 4 | 2,900 | −10 | 0.05 |
| 7 | 1,100 | −3 | 0.12 |
| 10 | 970 | −1 | 0.14 |
| 24 | 620 | +14 | 0.21 |
| 48 | 600 | +20 | 0.31 |

[a]Single peak (Sagavac 6F). [b]Concentration (*c*), 0·40. [c]In M NaOH. [d]Defined in Ref. 1.

TABLE II

FURTHER HYDROLYSIS OF DEGRADED *Klebsiella* K54 POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

| *Time* (*h*) | $[\alpha]_D^a$ (*degrees*) | *Degree of scission* (x) |
|---|---|---|
| 0 | +20 | 0.31 |
| 1 | +21 | 0.54 |
| 6 | +22 | 0.60 |
| 24 | +24 | 0.66 |

[a]Concentration (*c*), 0·36.

In the Tables, the values of the degree of scission ($x$) at various stages of hydrolysis are those calculated from the reducing power of the corresponding hydrolysates as described previously[1]. The values of $\bar{M}_w$, the weight-average molecular weight, in Table I have been estimated as before[1] from the elution curves

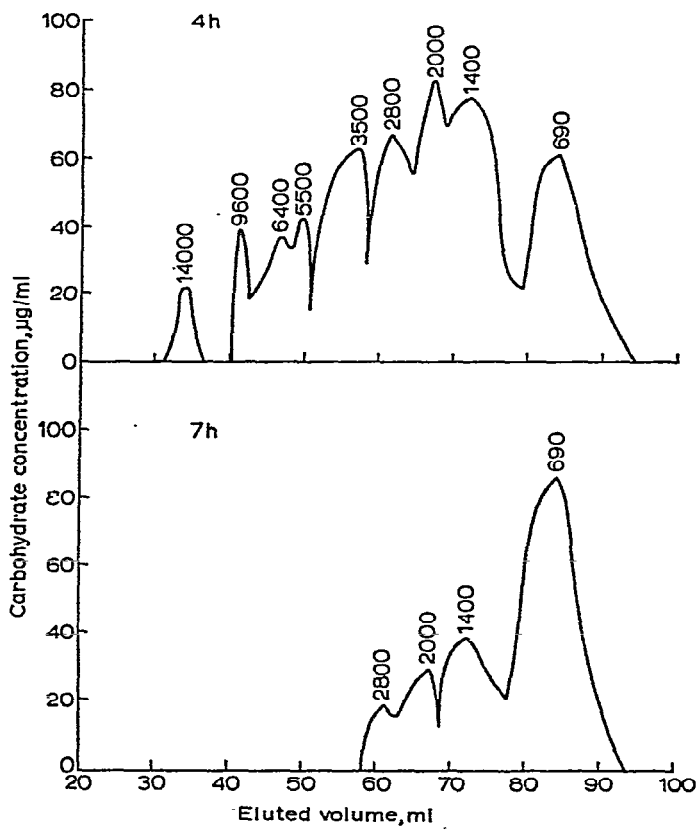



Fig. 1. Bio-Gel P-10 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 4–7 h) of *Klebsiella* K54 exopolysaccharide.

obtained on gel-permeation chromatography of the appropriate hydrolysates (Figs. 1 and 2).

A striking feature of these results is the very rapid and drastic decrease in $\overline{M}_w$ observed, even in the early stages of mild, acid hydrolysis (see Table I and Fig. 1). This may be ascribed to the high susceptibility to acid of the fucosyl-glucose linkages in the polysaccharide chain, noted by Conrad *et al.*[6]. Close agreement between the observed $\overline{M}_w$ values and those predicted from the corresponding values of $x$ on the assumption of even distribution of these acid-labile linkages throughout the molecule (*e.g.*, when $x$ is 0.12, the predicted $\overline{M}_w$ is 1,300, and the observed value is 1,100)

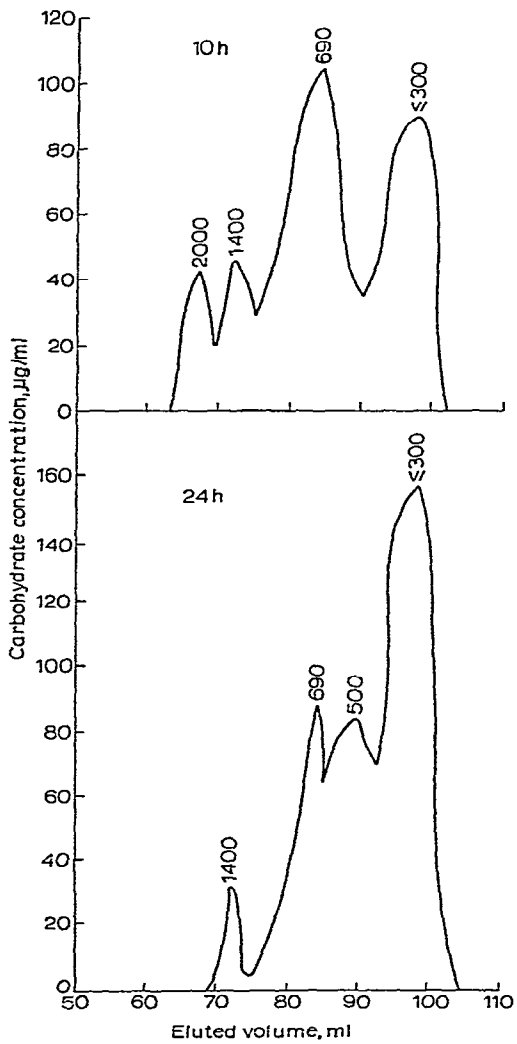



Fig. 2. Bio-Gel P-10 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 10–24 h) of *Klebsiella* K54 exopolysaccharide.

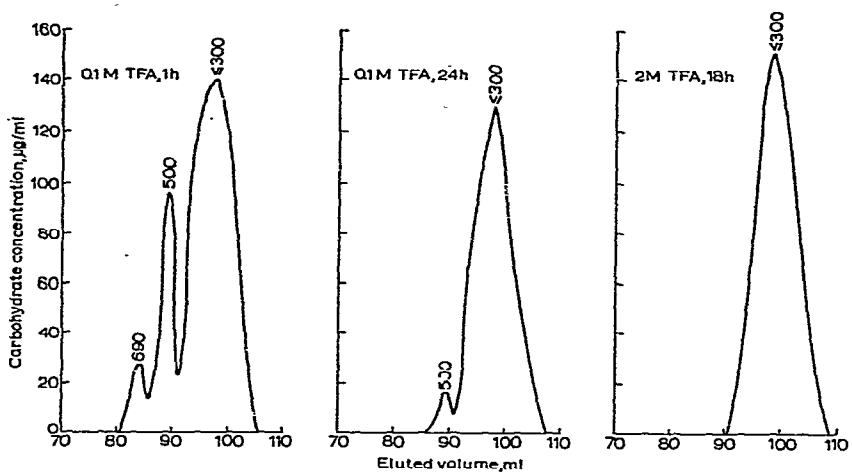



Fig. 3. Bio-Gel P-10 elution patterns on further hydrolysis of *Klebsiella* K54 exopolysaccharide.

affords additional evidence for the regular structure postulated for the polysaccharide.

A component having a molecular weight of 690 (according to gel-permeation chromatography) occurs in high proportion (60% by weight) in the hydrolysate obtained after treatment of the polysaccharide with 0.01M trifluoroacetic acid for 7 h (Fig. 1); this is believed to correspond to the structurally significant aldotetraouronic acid isolated by others[6,7,19] (mol. wt. 664). The fact that the multiple peaks in the elution patterns shown in Fig. 1 occur at molecular weights that, to a good approximation, are all multiples of 700 is a further indication of the presence in the molecule of a repeating unit of this size.

No lower oligosaccharides or monosaccharides were detected in the hydrolysate by either paper chromatography (solvents *a* and *b*) or gel-permeation chromatography until hydrolysis in 0.01M trifluoroacetic acid had proceeded for 10 h. After hydrolysis for 24 h, a major peak at molecular weight 500 was seen in the gel-permeation chromatogram (Fig. 2); this would appear to be the aldotriouronic acid, *O*-β-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyluronic acid-(1→3)-L-fucose, that was isolated by Conrad *et al.*[6]. This component persists on further hydrolysis in 0.1M trifluoroacetic acid for 24 h, disappearing only after the additional treatment with 2M acid for 18 h (see Fig. 3). A high proportion of the aldobiouronic acid produced, which was characterised[6] as 3-*O*-α-D-glucopyranosyluronic acid-L-fucose, survives even this last hydrolysis step. The great stability to acid hydrolysis of this aldobiouronic acid linkage is reflected in the very small amount of fucose released from the polysaccharide under the conditions used in the present work; for example, the molar ratio of glucose to fucose in the hydrolysate was found (by AutoAnalyzer) to be 2:0.5, instead of the theoretical 2:1.

No pyruvate was detected in this sample of the polysaccharide, but p.m.r. spectroscopy indicates the presence of acetate groups; the characteristic signal[15] at

$\tau'$ 7.8 shifts upfield, by ~0.3 p.p.m., to that of the acetate ion after saponification of the *O*-acetyl groups by treatment of the polysaccharide with 0.75M sodium hydroxide at 40° for 5 min. Integration suggests the presence of one *O*-acetyl group to every two fucosyl residues in the molecule, that is, one to every two of the repeating tetrasaccharide units in the chain; this is in accordance with the conclusions drawn by Sutherland[7,19] from enzymic hydrolysis studies of this polysaccharide. The value of the equivalent weight of the polysaccharide determined in the present study (680, corresponding to a uronic acid content of 26%) is also compatible with this postulated molecular structure (theoretical equivalent weight, 667).

This work on the exopolysaccharide of *Klebsiella* K54 has afforded both extension and corroboration of the results obtained by others, which suggests that the techniques used may be applied with confidence to *Klebsiella* polysaccharides in general.

*Capsular polysaccharide of* Klebsiella *K-type 4.* — This polysaccharide, like that from *Klebsiella* K54, appears to have a relatively simple structure. The constituent sugars are glucose, mannose, and glucuronic acid; the presence of a small proportion of galactose, noted both here and by others[16,22], is believed to be due to contamination, either from the agar culture medium or from some intracellular polysaccharide (this was observed also in the case of the K54 polysaccharide[17]). The proportion of galactose in hydrolysates from different preparations of this polysaccharide varied considerably (from 3 to 20 mole % of the neutral sugar fraction), whereas the molar ratio of glucose to mannose remained constant at ~2:0.5.

The results of the present study of the acid hydrolysis of the *Klebsiella* K4 exopolysaccharide are shown in Tables III and IV, and Figs. 4 and 5. It is apparent (Table III) that this polysaccharide has a much lower value of $\overline{M}_w$ than has that from K-type 54; the relative decrease accompanying the early stages of hydrolysis in 0.01M trifluoroacetic acid is, however, less than that observed for the K54 polysaccharide, so that after hydrolysis for 3 h most of the product is within the molecular-weight range fractionated by Bio-Gel P-300 rather than P-10 (*cf.* Figs. 1 and 4). Once again, the elution patterns show multiple peaks and, as with the K54 polysaccharide, most

TABLE III

PARTIAL HYDROLYSIS OF *Klebsiella* K4 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

| *Time* (*h*) | $\overline{M}_w$ | $[\alpha]_D^a$ (*degrees*) | *Degree of scission* (x) |
|---|---|---|---|
| 0 | 210,000[b] | +90 | |
| 1 | 140,000 | +87 | 0.01 |
| 3 | 20,500 | +80 | 0.03 |
| 6 | 4,800 | +72 | 0.07 |
| 10 | 1,600 | +63 | 0.31 |
| 24 | 1,200 | +55 | 0.40 |
| 48 | 640 | +47 | 0.48 |

[a]Concentration (*c*), 0·42. [b]Single peak (Sagavac 6F).

TABLE IV

FURTHER HYDROLYSIS OF DEGRADED *Klebsiella* K4 POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

| *Time* (*h*) | $[\alpha]_D^a$ (*degrees*) | *Degree of scission* (x) |
|---|---|---|
| 0 | +47 | 0.48 |
| 2 | +41 | 0.65 |
| 7 | +34 | 0.90 |
| 24 | +32 | 0.92 |

[a]Concentration (*c*), 0·35.

of these occur at molecular weights approximating closely to integral multiples of 700. This observation, taken in conjunction with the appearance of a major peak at molecular weight 720 in the gel-permeation chromatogram obtained after hydrolysis of the polysaccharide in 0.01M trifluoroacetic acid for 10 h (Fig. 5), strongly suggests the presence of a repeating tetrasaccharide sequence.

Paper chromatography (solvents *a* and *c*) of the hydrolysate after treatment of the polysaccharide with 0.01M trifluoroacetic acid for only 3 h showed the presence of some glucose and traces of oligosaccharides, both neutral and acidic, having the mobilities associated with di- and tri-saccharides. The glycosidic linkages within the repeating sequence of sugars in this case are evidently more susceptible to acid hydrolysis than are those between the sugars comprising the tetrasaccharide repeating unit in the K54 polysaccharide. The gel-permeation chromatograms in Fig. 5 suggest that isolation of the component having molecular weight 720, in any appreciable quantity, may prove difficult, since degradation thereof to lower oligosaccharides and monosaccharides is well advanced even when higher oligomers are still present in the hydrolysate in fairly large proportion. Gel-permeation and paper chromatography (solvent *c*) indicate that the breakdown pattern on further hydrolysis of the degraded polysaccharide in 0.1M, and thereafter in 2M, trifluoroacetic acid is similar to that noted in the case of the K54 polysaccharide. The aldobiouronic acid produced, which persists even after the additional treatment with 2M trifluoroacetic acid, is believed to contain mannose, in view of the slow release of this sugar on hydrolysis of the polysaccharide. For this reason, the actual molar ratio of glucose to mannose in the repeating unit may well be 2:1, rather than the observed 2:0.5.

The exopolysaccharide of *Klebsiella* K4 further resembles that from K54 in that both contain acetate groups, but not pyruvate. The acetate signal in the p.m.r. spectrum is shifted 0.35 p.p.m. upfield after saponification of the polysaccharide as described. Integration suggests the presence of one *O*-acetyl group to every four sugar residues. The value found for the equivalent weight of this polysaccharide (710) is, together with the other evidence presented, compatible with a repeating unit containing one glucuronic acid residue, 3 hexose residues, and an acetate group (calculated equivalent weight, 704).

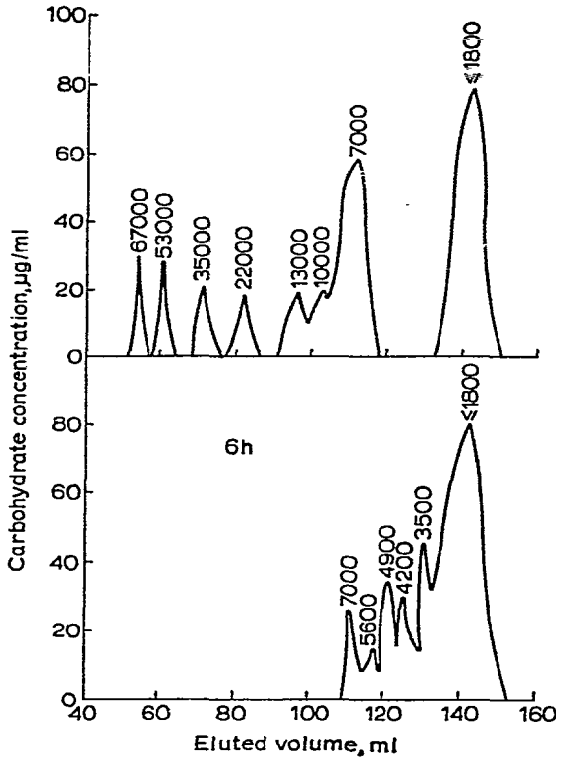



Fig. 4. Bio-Gel P-300 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 3–6 h) of *Klebsiella* K4 exopolysaccharide.

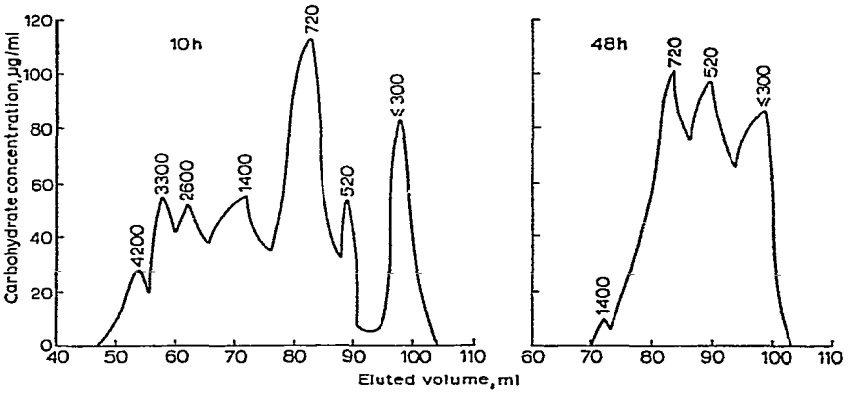



Fig. 5. Bio-Gel P-10 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 10–48 h) of *Klebsiella* K4 exopolysaccharide.

*Capsular polysaccharide of* Klebsiella *K-type 64.* — The capsular polysaccharide of *Klebsiella* K64, which contains residues of glucose, mannose, rhamnose, and glucuronic acid, was the subject of a note[23] published in 1958. The results of the present study are shown in Tables V and VI, and Figs. 6 and 7. The $\overline{M}_w$ value is similar to that of the *Klebsiella* K54 exopolysaccharide, but the relative decrease accompanying hydrolysis in 0.01M trifluoroacetic acid is comparable with that observed for the K4 polysaccharide, so that $\overline{M}_w$ remains relatively high throughout this hydrolysis step, which proceeds more slowly than does similar hydrolysis of the other *Klebsiella* polysaccharides examined. The molecular weights of the hydrolysis products lie largely within the fractionation range of the agarose gel, even after treatment of the polysaccharide with 0.01M trifluoroacetic acid for 4 h, and thereafter fractionation on Bio-Gel P-300 remains necessary throughout the rest of this phase of the hydrolysis (Fig. 6).

No product of any obvious significance is apparent until the hydrolysis is continued in 0.1M trifluoroacetic acid; after further hydrolysis under these conditions for 2 h, the preponderance (53% by weight) of a product having molecular weight 1100 is evident from the gel-permeation chromatogram (Fig. 7). Although a large amount

TABLE V

PARTIAL HYDROLYSIS OF *Klebsiella* K64 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

| *Time* (*h*) | $\overline{M}_w$ | $[\alpha]_D$[a] (*degrees*) | *Degree of scission* (x) |
|---|---|---|---|
| 0 | 1,700,000[b] | +28 | |
| 1 | 520,000 | +29 | 0.01 |
| 4 | 140,000 | +30 | 0.04 |
| 7 | 29,000 | +32 | 0.06 |
| 10 | 17,000 | +35 | 0.07 |
| 24 | 6,900 | +36 | 0.14 |
| 48 | 4,100 | +38 | 0.17 |

[a]Concentration (*c*), 0·40. [b]Single peak (Sagavac 6F).

TABLE VI

FURTHER HYDROLYSIS OF DEGRADED *Klebsiella* K64 CAPSULAR POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

| *Time* (*h*) | $[\alpha]_D$[a] (*degrees*) | *Degree of scission* (x) |
|---|---|---|
| 0 | +38 | 0.17 |
| 2 | +48 | 0.39 |
| 7 | +49 | 0.77 |
| 24 | +50 | 0.86 |

[a]Concentration (*c*), 0·36.

of material of lower molecular weight is also present, it should be possible to isolate this component, which must constitute a major portion of the repeating unit of the polysaccharide, by gel-permeation chromatography on a preparative scale.

Paper chromatography (solvents *a* and *b*) shows that rhamnose is the only sugar released in appreciable amount during hydrolysis of this polysaccharide in 0.01M trifluoroacetic acid. Glucose, some mannose, and several oligosaccharides, both neutral and acidic, appear when the hydrolysis is continued in 0.1M acid. The gradual breakdown of the structurally significant component having molecular weight 1100 to lower oligosaccharides, as this hydrolysis step proceeds, is demonstrated both by the elution patterns on Bio-Gel P-10 (Fig. 7) and by paper chromatography. Like the other *Klebsiella* polysaccharides examined, the K64 polysaccharide yields, on hydrolysis in 0.1M trifluoroacetic acid, an aldotriouronic acid showing some resistance

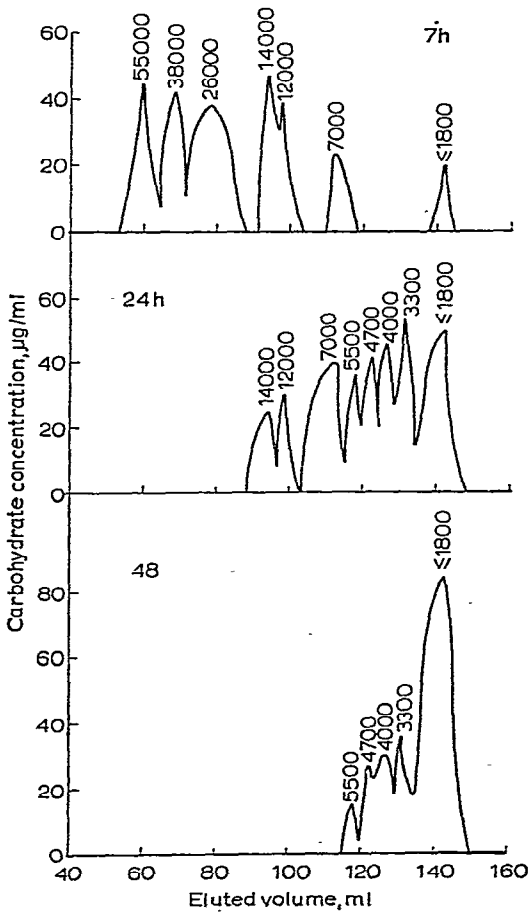



Fig. 6. Bio-Gel P-300 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 7–48 h) of *Klebsiella* K64 exopolysaccharide.

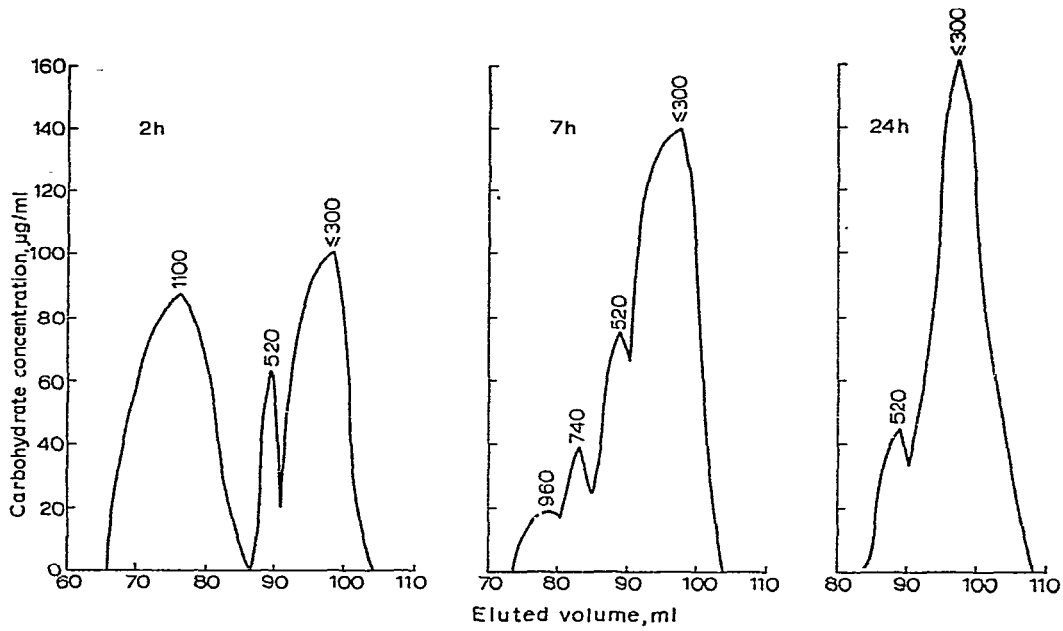



Fig. 7. Bio-Gel P-10 elution patterns on further hydrolysis (0.1M trifluoroacetic acid, 100°, 2–24 h) of *Klebsiella* K64 exopolysaccharide.

to further hydrolysis, and an aldobiouronic acid (containing mannose[23]) that survives even the treatment with 2M trifluoroacetic acid.

A further resemblance between the K64 polysaccharide and the others studied is seen in the presence of acetate groups in the molecule; the characteristic signal in the p.m.r. spectrum is shifted 0.35 p.p.m. upfield following saponification of these groups. However, the p.m.r. spectrum also contains a signal at $\tau'$ 8.5 which is indicative of the presence of pyruvate[14,15]; further evidence for this substituent is afforded by the detection of pyruvic acid on paper chromatograms of the hydrolysates (by use of the *o*-phenylenediamine spray reagent[24]), and by colorimetric analysis[13], from which the proportion by weight of pyruvate in the polysaccharide is estimated at ~7%. A ratio of one *O*-acetyl group and one pyruvic acid acetal to each rhamnose residue in the molecule is indicated by integration of the appropriate signals in the p.m.r. spectrum.

A value of 570 was found for the equivalent weight of this polysaccharide; any corresponding estimate of the uronic acid content must, of course, take into account the presence of pyruvic acid in the molecule. The molar ratio of the neutral sugar components, rhamnose, glucose, and mannose, in an hydrolysate was given by the AutoAnalyzer as 1.0:2.0:1.5. As with the K4 polysaccharide, the proportion of mannose found under these conditions will inevitably be low as a consequence of incomplete hydrolysis of aldobiouronic acid linkages involving residues of this sugar; the true ratio of glucose to mannose in the polysaccharide may be 2:3 or even 2:4.

All the evidence presented here seems to indicate that the chemical repeating unit in the molecule of the exopolysaccharide of *Klebsiella* K64 contains a comparatively long sequence of sugars. At the stage of hydrolysis where the fragment having molecular weight 1100 is produced in high proportion, the rhamnose residues, which are believed to be present in side chains[23,25], will have been completely removed, together with the acetate and pyruvate groups. The molecular weight of the whole repeating unit is thus probably nearer 1400 than 1100. It is of interest to note that the capsular polysaccharide of *Klebsiella* K7, which has also been found to contain pyruvate[14], appears to have a chemical repeating unit of similar size; gel-permeation chromatograms of hydrolysates from this polysaccharide[5,26] show the presence of a major product having molecular weight 1300. The presence of pyruvic acid acetal-linked to a sugar residue may in some way stabilize contiguous glycosidic linkages, resulting in longer sequences of linkages that are relatively resistant to acid hydrolysis; this possibility will be investigated.

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