Journal of Chromatography, 131 (1977) 191–203

CHROM. 9440

SEPARATION OF GENTAMICIN C-COMPLEX INTO FIVE COMPONENTS BY CRAIG DISTRIBUTION

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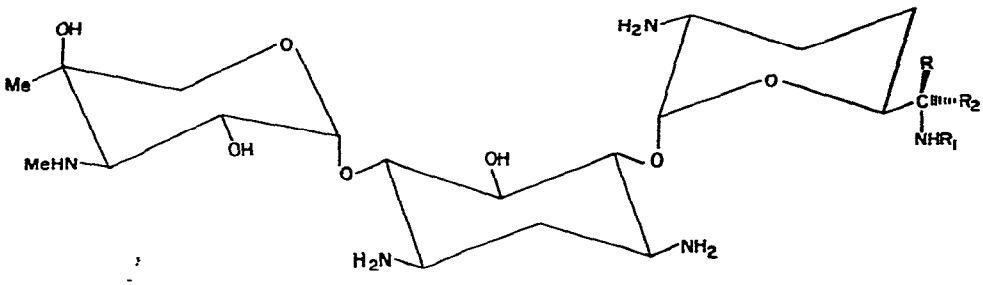
(Received May 10th, 1976)

SUMMARY

A gentamicin C-complex preparation was separated preparatively into five components by Craig distribution. Gentamicins C_1, C_2, and C_{1a} were the major components, whereas components C_{2a} and C_{2b} represented only 4% of the total C-complex mixture. Paper chromatographic analysis showed that the gentamicin C_{2b} separated by Craig distribution was identical with a gentamicin isolated from *Micromonospora purpurea* var. JI-33 fermentation broth and identified as 6′-N-methylgentamicin C_{1a}. Similarly, component C_{2a} was identical with a previously separated gentamicin component tentatively identified as the 6′-C epimer of gentamicin C_2.

INTRODUCTION

Fermentation of *Micromonospora* species *purpurea* and *echinospora* produces a family of aminocyclitol antibiotics called gentamicin. Gentamicin can be divided into two groups: (a) gentamicin C-complex, the major antibiotic product of the fermentation and (b) the co-produced polar gentamicin components. The C-complex can be differentiated from the polar components chromatographically[1–3] and chemically[4–9]. Gentamicin C-complex can be separated by thin-layer, column, and paper chromatography[10] into three major components, *viz.* gentamicins C_1, C_2, and C_{1a}. These components differ chemically only in the extent of methylation at the 6′-C position. Each of these components exhibits comparable biological activity against most susceptible organisms[11,12].



Gentamicin C_1	$R = R_1 = Me$; $R_2 = H$
Gentamicin C_2	$R = Me$; $R_1 = R_2 = H$
Gentamicin C_{2a}	$R = R_1 = H$; $R_2 = Me$
Gentamicin C_{2b}	$R = R_2 = H$; $R_1 = Me$
Gentamicin C_{1a}	$R = R_2 = R_1 = H$

Evidence that the C-complex may contain additional components was first suggested by Maehr[13]. Using Craig distribution and paper chromatography, Maehr showed that the C-complex may contain as many as seven components. Kershner[14] subsequently isolated a C-complex component that appeared between components C_1 and C_2 after a Craig distribution of 5000 transfers. This component, initially referred to as gentamicin C_{2a} but later changed to gentamicin C_{2b}, was identified as 6′-N-methylgentamicin C_{1a} by mass spectrometry[15]. Later, Thomas and Tappin[16] reported the separation of gentamicin into seven components and suggested that the three components eluted before C_{1a} might be the components described by Wagman *et al.*[17] as the polar, minor components. The remaining four components consisted of gentamicins C_1, C_2, and C_{1a} and an unidentified component eluted between components C_1 and C_2.

Antibiotic XK-62-2, very similar in its physical, chemical, and biological characteristics to the components of the C-complex, was recently isolated from a fermentation of *Micromonospora sagamiensis* var. *nonreducans* XK 62[18] and was shown to be 6′-N-methylgentamicin C_{1a} (gentamicin C_{2b})[19]. Daniels *et al.*[20] reported the isolation of gentamicin C_{2b} from *Micromonospora purpurea* var. JI-33 and demonstrated by NMR and chromatographic studies that gentamicin C_{2b} and antibiotic XK-62-2 were identical.

This paper presents in detail the separation of gentamicin C-complex and [Me-^{14}C]gentamicin C-complex into five biologically active C-complex components by Craig distribution. Each component is identified by chromatographic comparison with authentic gentamicins.

EXPERIMENTAL

Preparation of gentamicin free base

Gentamicin free base was prepared from gentamicin sulfate according to the procedure of Rosselet *et al.*[21].

Preparation of [Me-^{14}C]gentamicin C-complex

[Me-^{14}C]Gentamicin C-complex sulfate was prepared as described by Lee *et al.*[22].

Craig distribution

Separations were carried out in a 1020-cell automatic Craig distribution apparatus (H. O. Post, Spectrum Medical Industries) of 10 ml fixed lower phase volume. A chloroform–methanol–17% ammonia (2:1:1) solvent system was used in all experiments. Lower phase levels were maintained using a 0.2 ml co-current. Each transfer cycle consisted of a 15-stroke equilibration phase and a 3-min settling period.

In the first counter-current distribution separation 7.0 g gentamicin C-complex base was dissolved in 70 ml upper phase and distributed in the first seven tubes. In the second separation 1.58 g of [Me-^{14}C]gentamicin C-complex sulfate was converted to free base, dissolved in 20 ml upper phase and placed in the first two tubes. In the final separation 4.25 g of [Me-^{14}C]gentamicin, rich in the polar components, was converted to free base, dissolved in 40 ml upper phase, and distributed in the first four tubes.

Thin-layer chromatography

Silica gel G (250 μm and cellulose MN 300 (250 μm) TLC plates from Analtech were developed in the lower phase of chloroform–methanol–28% ammonia (1:1:1). Gentamicin components were detected by ninhydrin spray reagent (1.0 g ninhydrin, 100 ml water, 100 ml 1-butanol, 200 ml pyridine).

Paper chromatography

Paper chromatograms were developed descendingly on Whatman No. 1 paper in a system consisting of the lower phase of chloroform–methanol–17% ammonia (2:1:1) for 4 h in a tank saturated with upper and lower phases.

Disc diffusion assay

For qualitative disc diffusion assay of Craig distribution fractions, 100 μl upper phase of every fifth tube was pipetted onto 12.7-mm antibiotic assay discs (Schleicher and Schuell). The discs were allowed to air dry and then placed on agar seeded with *Staphylococcus aureus* ATCC 6538P. Diameters of growth-inhibition zones were measured after an 18-h incubation period.

Quantitative disc diffusion assays were performed according to the procedure of Oden *et al.*[23].

Bioautography

Following separation of gentamicin components by paper chromatography, the dried chromatograms were placed on agar plates seeded with *Staphylococcus aureus* ATCC 6538P. After 30 min the chromatograms were removed and the plates incubated at 37° for 16 h.

Assay for radioactivity

For radioactivity assay of Craig distribution fractions, 100 μl upper phase of every fifth tube was counted in an Intertechnique liquid scintillation spectrometer (Model SL30). Radioactivity scans were done using a Nuclear Chicago scanner (Model 10002).

Antibiotic standards

All gentamicin standards were supplied by Schering (Bloomfield, N.J., U.S.A.). Gentamicin C-complex standard was in the sulfate form, possessing a biological activity of 612 μg/mg. Gentamicins C_1, C_2, C_{2b}, and C_{1a} were supplied as aqueous solutions of the corresponding free base. Each sample was assayed at 1000 μg/ml. The sample of gentamicin C_{2a} supplied for thin-layer and paper chromatographic comparison purposes was an aqueous solution assaying at 1000 μg/ml. This sample was prepared from material isolated from a Craig distribution separation of gentamicin C-complex by Marquez[24].

Mathematical treatment of data

The mobilities of gentamicin components in Craig distribution experiments are expressed in terms of their partition ratios (C)

$$C = \frac{r^*}{n - r^*}$$

where r^* is the number of the peak tube and n is the number of transfers.

The fitting of theoretical curves to the radio activity curves of the Craig distribution separations was performed by application of the following equation

$$Y_x = \frac{Y_{r^*}}{\text{antilog} \dfrac{0.434\, x^2 (C+1)^2}{2nC}}$$

where n and C have the same significance as above, Y_x is the cpm in a tube x tubes away from the peak tube, and Y_r* is the cpm in the peak tube.

The fitting of Gaussian curves to radioactivity scans was carried out graphically by application of the following relationship

$$y_x = 0.39894 e^{\frac{-x^2}{2}}$$

where y_x is the height at any distance x from the mean.

RESULTS AND DISCUSSION

Craig distribution of gentamicin C-complex

The first study was designed to compare the separation of gentamicin C-complex in a 2000-transfer experiment, using a 1020-tube Craig apparatus and employing the qualitative disc diffusion assay, to that obtained by Maehr[13] in a 200-tube Craig apparatus employing weight analysis. A comparison of the separations obtained by Maehr (Fig. 1) with the separation achieved after 2000 transfers (Fig. 2) revealed an enhanced separation of the components in the latter case. This was the result of several improvements in the Craig distribution procedure. Refinements in the processes of recycling and removal of fractions containing separated components followed by replacement of the solvent system were made possible through the use

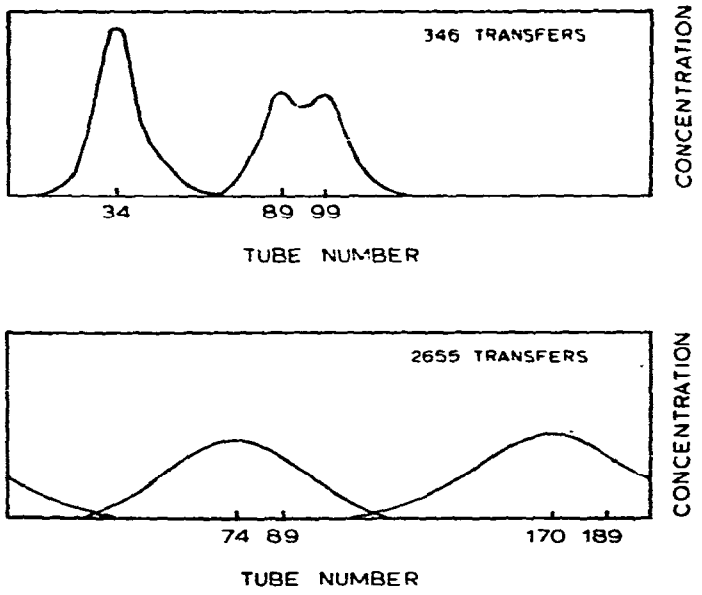


Fig. 1. Distribution of gentamicin C-complex (3.5 g) in a 200-tube Craig apparatus after 346 transfers. Antibiotic concentrations were determined by weight analyses. The solvent phases containing band C_1 were removed and the solvent system replaced. The distribution after 2655 transfers followed the removal of band C_1.

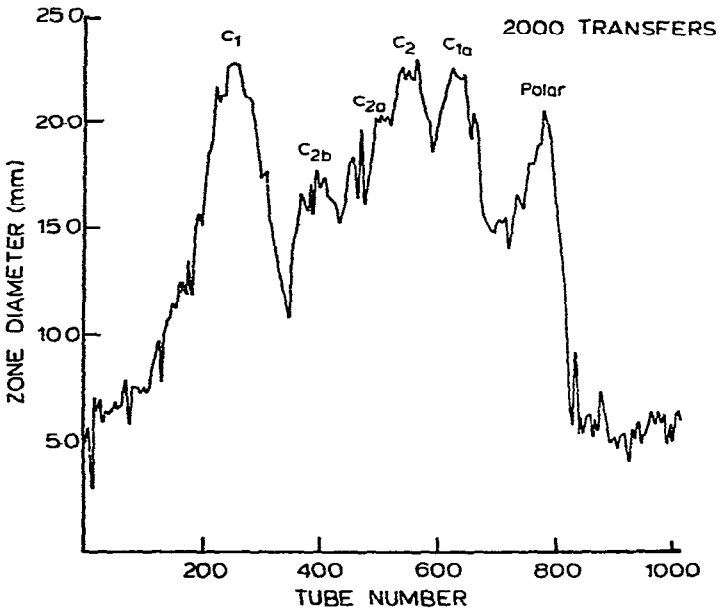


Fig. 2. Distribution of gentamicin C-complex (7.0 g) in a 1020-tube Craig apparatus after 2000 transfers. The antibiotic charge was distributed in the first seven tubes. Antibiotic concentrations were determined by disc diffusion assay.

TABLE I

CHROMATOGRAPHIC IDENTIFICATION OF SAMPLES TAKEN FROM PEAK TUBES AFTER 2000 TRANSFERS

Band from which sample was taken	*Peak tube (r*) of band*	*C*	*R_F values on silica gel G**
C_1	250	1.74	0.40
C_{2b}	390	2.39	0.36
C_{2a}	500	3.17	0.36
C_2	550	3.65	0.36
C_{1a}	620	4.56	0.31
Polar**	780	9.00	<0.31

* Plates were developed in the lower phase of chloroform–methanol–28% ammonia (1:1:1).

** The polar components are a group of biologically active aminoglycoside antibiotics that are co-produced with gentamicin C-complex in submerged fermentations of *Micromonospora purpurea* and *Micromonospora echinospora*. They consist of the gentamicin A-complex[25–27], B[28], B_1[9], and X-complex[9].

of the larger Craig apparatus. In addition, the greater sensitivity of the disc diffusion assay revealed the presence of two additional components in the C-complex. Table I presents the chromatographic characteristics of samples obtained from peak tubes (*r**). It is clear from these data that what was previously designated gentamicin C_2 is actually a complex of three components. Thin-layer chromatographic analysis indicated that the final band in the Craig distribution graph ($C = 9.00$) was a mixture of the polar components present in the gentamicin C-complex starting material.

Quantitation of the gentamicin components based upon the disc diffusion assay procedure employed in the above experiment was not feasible. This was indi-

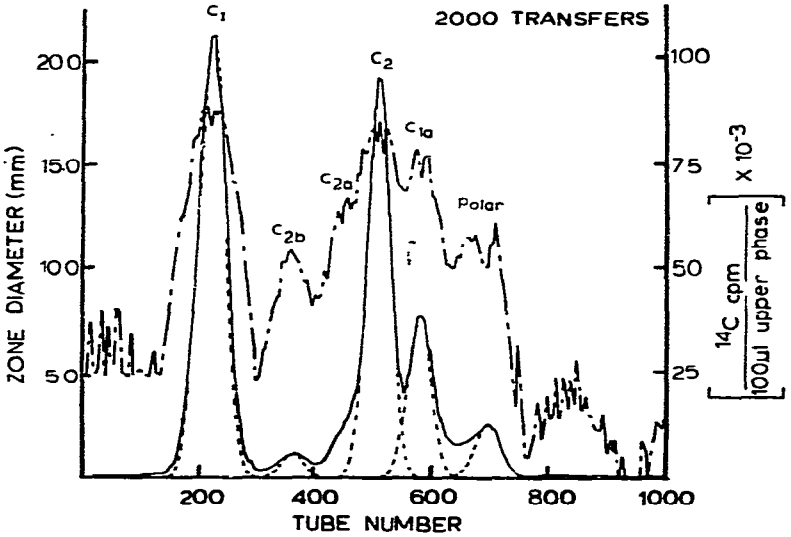


Fig. 3. Distribution of [Me-^{14}C] gentamicin C-complex in a 1020-tube Craig apparatus after 2000 transfers. The labeled gentamicin (1.58 g sulfate) was charged as the free base to the first two tubes. Antibiotic concentrations were determined by disc diffusion assay (—·—) and radioactivity assay (———). Theoretical curves (– – – –) were fitted to the radioactivity curve.

cated by integration of the individual bands represented in Fig. 2. It is known that the polar components are minor contaminants in gentamicin C-complex material[17,22], yet in Fig. 2 they account for nearly 10% of the combined peak areas. This discrepancy appeared to result from the assay technique used. The disc diffusion assay can only be used quantitatively over a limited antibiotic concentration range[25]. That the component concentrations in many of the Craig distribution tubes exceeded this limitation is shown in a later experiment.

To overcome the limitations of the disc diffusion assay, a second study was performed using [Me-^{14}C]gentamicin C-complex and employing a radioactivity assay. An initial Craig distribution experiment of 2000 transfers was performed. Fig. 3 reveals the superiority of the radioactivity assay over the biological assay as demonstrated by the close similarity between the experimental radioactivity curve and the theoretical curves. Gentamicin bands C_{1a}, C_2, and C_1 appear as the major bands, while band C_{2b}, the barely discernable band C_{2a}, and the band of the co-produced polar components represent gentamicin components present in minor concentrations. The non-theoretical shape of the polar component band is confirmation that this band represents more than one labeled component.

The contents of tubes 170 through 270, corresponding to band C_1, were pooled separately. The contents of the remaining tubes were combined together, evaporated to dryness, and desalted. The resulting gentamicin material was converted to free base form and a 2000-transfer Craig distribution was carried out. Following this separation the solvent regions containing the polar components and residual material from band C_1 were removed, replaced with fresh solvent, and the Craig apparatus programmed for an additional 3000 transfers (Fig. 4). After a total of 5000

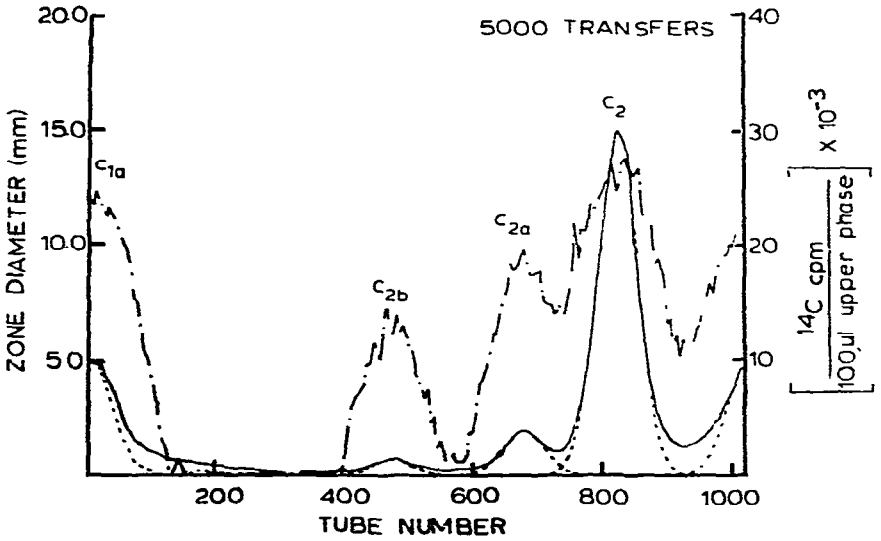


Fig. 4. Distribution of [Me-^{14}C] gentamicin C-complex after 5000 transfers following removal of band C_1 and the band of the polar components at 2000 transfers. Antibiotic concentrations were determined by disc diffusion assay (— · —) and radioactivity assay (———). Theoretical curves (——) were fitted to the radioactivity curve.

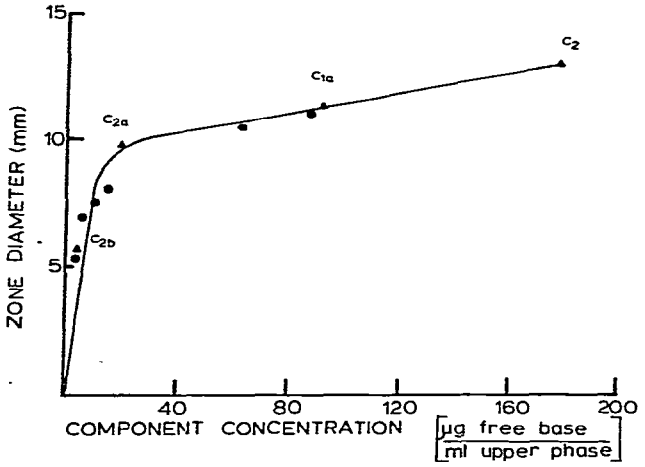


Fig. 5. Correlation between diameters of growth-inhibition zones of the disc diffusion assay, as employed in Craig distribution experiments, and gentamicin component concentration. Concentrations at several locations along each band were determined by quantitative disc diffusion assay. Samples representing band C_{1a} were assayed in reference to gentamicin C_{1a}, samples representing bands C_{2b}, C_{2a}, and C_2 were assayed in reference to gentamicin C_2. Component concentrations of peak tubes (r^*) are indicated by a triangle, ▲.

transfers band C_{2b} was extensively separated from band C_{2a}, whereas band C_{2a} was only partially separated from band C_2.

After 5000 transfers had been completed, an experiment was performed to delineate the apparent discrepancy in the size of band areas between the disc diffusion assay and the radioactivity assay. One-milliliter samples of upper phase were taken from peak tubes (r^*) of each band and the concentration of gentamicin determined using quantitative disc diffusion assay[23].

Fig. 5 reveals the relationship found between the zone diameters obtained in the disc diffusion assay and component concentration. Gentamicin concentrations of samples from r^* tubes of bands C_{2a} and C_{2b} fall in the linear portion of the curve, while gentamicin concentrations of samples from r^* tubes of bands C_2 and C_{1a} lie in the asymptotic portion of the curve.

Band C_{2b} was removed from the Craig machine after 5000 transfers. The emptied region was replaced with fresh solvent and 2000 additional transfers were carried out (Fig. 6). After 7000 transfers band C_{2a} was extensively separated from band C_2, and band C_2 was sufficiently separated from band C_{1a} to permit the isolation of the gentamicin components represented by these two bands without substantial cross-contamination. Fig. 7 presents a summary of the separation achieved throughout the course of the 7000 transfers.

To investigate the reproducibility of the separation obtained above, a second Craig distribution experiment involving 7000 transfers was performed. The separation achieved in this experiment was comparable to that given above.

Identification of gentamicin C-complex components

The materials defined by bands C_1, C_2, and C_{1a} were examined by thin-layer

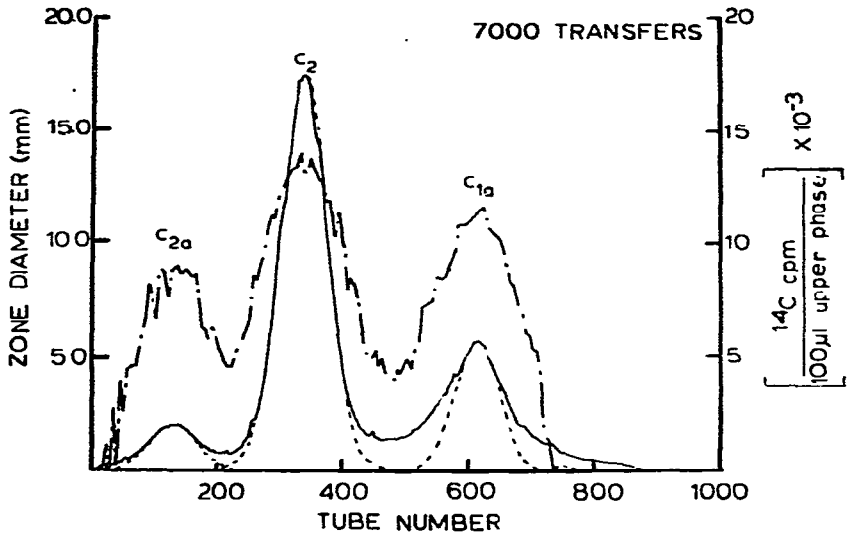


Fig. 6. Distribution of [Me-^{14}C]gentamicin C-complex after 7000 transfers. Band C_1 and the band of the polar components were removed at 2000 transfers, and band C_{2b} at 5000 transfers. Antibiotic concentrations were determined by disc diffusion assay (— · —) and radioactivity assay (———). Theoretical curves (——) were fitted to the radioactivity curve.

and paper chromatography. Each band was compared with reference samples of gentamicin components C_1, C_2, and C_{1a}, employing bioautographic and ninhydrin detection techniques. The materials isolated from bands C_1, C_2, and C_{1a} were found to correspond to gentamicins C_1, C_2, and C_{1a}, respectively. Gentamicins C_1, C_2, and

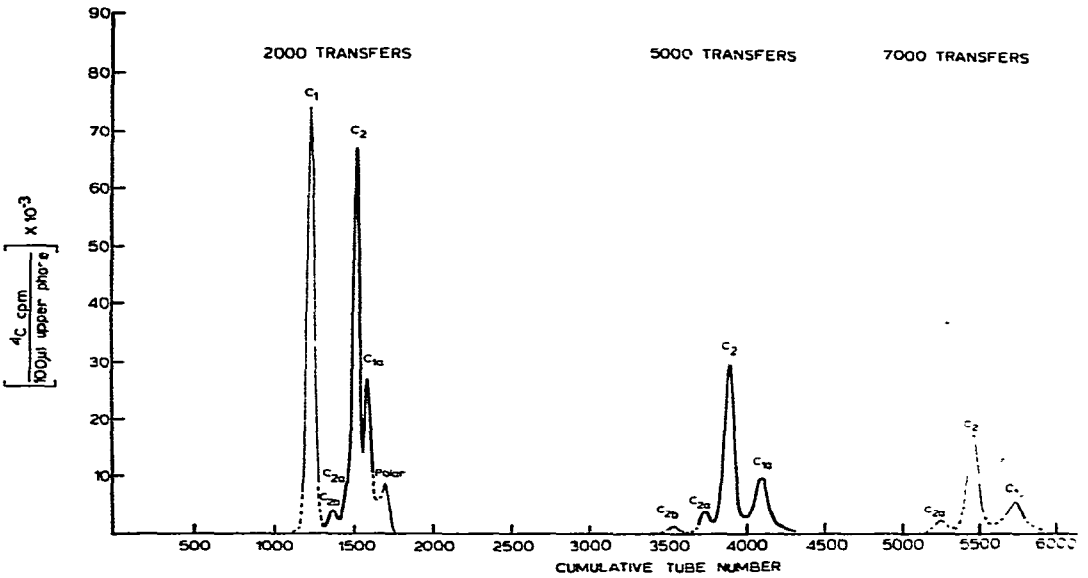


Fig. 7. Summary of Craig distributions of [Me-^{14}C]gentamicin C-complex. The bands that were removed are designated by a thin line, the bands that remained are presented as a broad line, and the areas that were discarded are represented by a broken line.

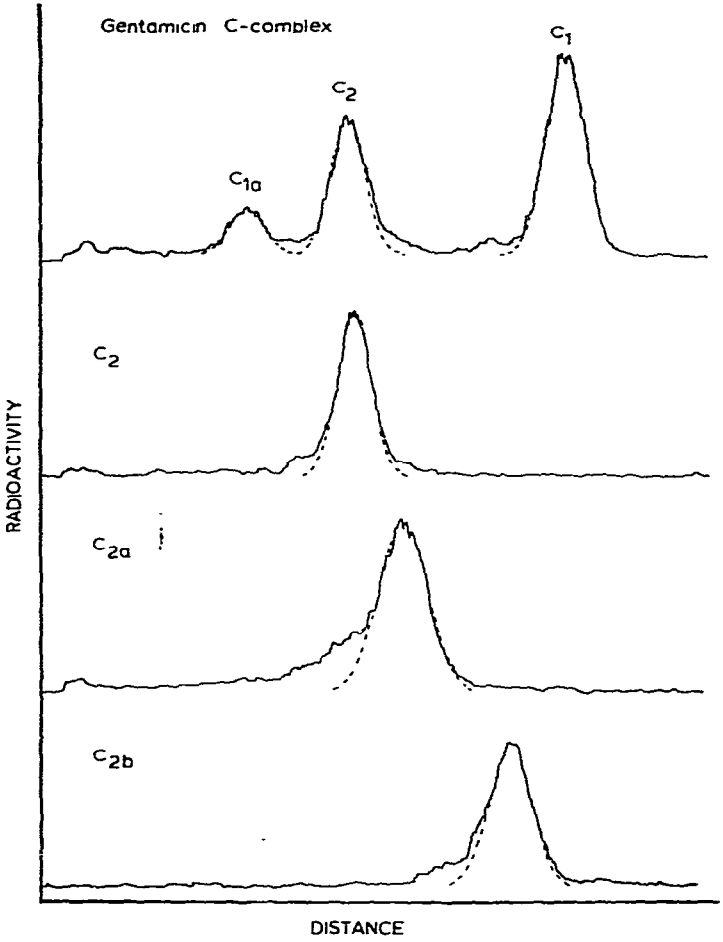


Fig. 8. Radioactivity scan (———) of materials isolated from bands C_2, C_{2a}, and C_{2b} in reference to gentamicin C-complex. The isolated materials were chromatographed on paper. Gaussian curves (– – –) were fitted to the radioactivity scans.

C_{1a} separated by Craig distribution were of comparable degrees of purity to the corresponding reference samples.

Particular attention was paid to the chromatographic characteristics of the materials isolated from bands C_{2a} and C_{2b}. Fig. 8 presents a radioactivity scan of a paper chromatogram comparing the materials isolated from bands C_2, C_{2a}, and C_{2b} with gentamicin C-complex standard. The R_{C_1} (1.00) values were 0.58, 0.69, and 0.90, respectively. Each of the radioactivity curves of the materials isolated from bands C_2, C_{2a}, and C_{2b} in Fig. 8 contained a shoulder region on the left side. It was impossible to determine if this shoulder area was attributable to chromatographic tailing or if it represented contamination by other C-complex components. Maximum contamination values of materials isolated from bands C_2, C_{2a}, and C_{2b} were determined by assuming that tailing was negligable. These values are presented in Table II.

To identify the gentamicin components isolated from bands C_{2a} and C_{2b}, each

TABLE II

RADIOACTIVE CONTAMINATION OF MATERIALS ISOLATED FROM BANDS C_{2a}, C_{2b}, AND C_2

Band from which material was isolated	*Band from which contaminant was derived*	*Maximum per cent of radioactive contamination**
C_{2a}	C_2	30
C_{2b}	C_{2a}	15
C_2	C_{1a}	2

* The contamination value was determined by comparing the area of the radioactivity curve with the corresponding Gaussian curve (Fig. 8) and applying the following relationship

$$\frac{\text{actual counts} - \text{theoretical counts}}{\text{actual counts}} \times 100 = \text{maximum \% contamination}$$

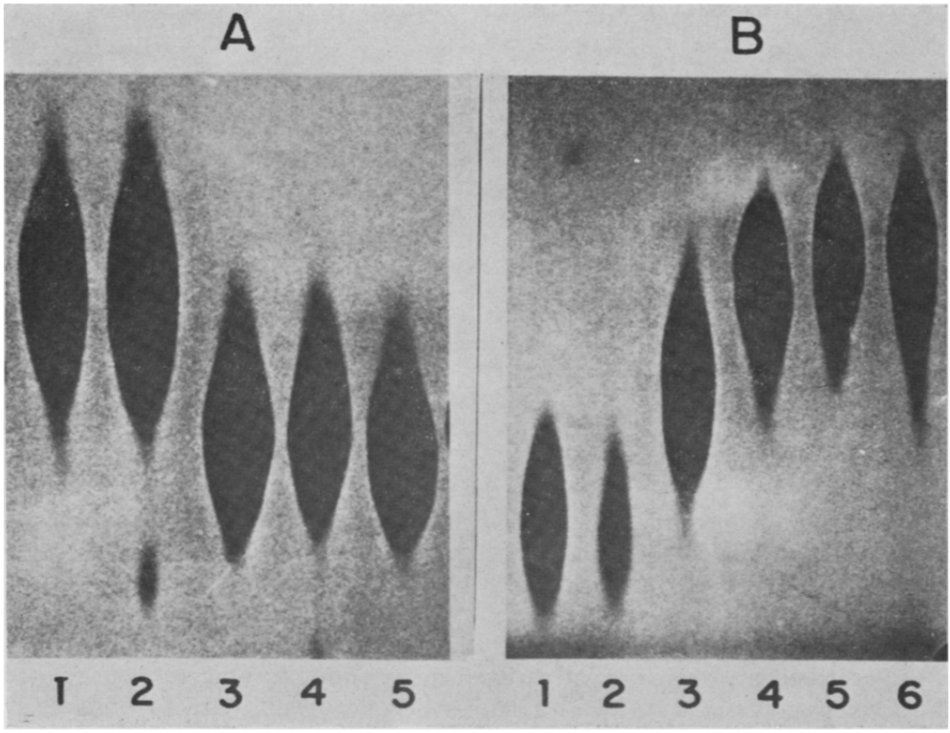


Fig. 9. Bioautograms of materials isolated following Craig distribution of [Me-^{14}C]gentamicin C-complex employing 7000 transfers. The materials were chromatographed on paper. (A) 1 = Material isolated from band C_{2b} (0.26 μg); 2 = gentamicin C_{2b} standard (0.50 μg); 3 = material isolated from band C_{2a} (0.26 μg); 4 = gentamicin C_{2a} previously isolated by Marquez[24] (0.50 μg); 5 = material isolated from band C_2 (0.26 μg). (B) 1 = Gentamicin C_2 standard (0.50 μg); 2 = material isolated from band C_2 (0.26 μg); 3 = material isolated from band C_{2a} (0.26 μg); 4 = material isolated from band C_{2b} (0.26 μg); 5 = material isolated from band C_1 (0.13 μg); 6 = gentamicin C_1 standard (0.50 μg).

isolated material was compared by bioautography with a sample of gentamicin C_{2b} produced by *Micromonospora purpurea* var. JI-33, and with a sample of gentamicin C_{2a} previously separated by Craig distribution and assigned a preliminary structure based on proton NMR studies[29,30] corresponding to the 6′-C epimer of gentamicin C_2. Fig. 9 shows that the material isolated from band C_{2b} corresponds chromatographically with gentamicin C_{2b}, while the material isolated from band C_{2a} corresponds with gentamicin C_{2a}.

The composition by weight of each of the components in gentamicin group C_2 (*i.e.*, components C_2, C_{2a}, and C_{2b}) is presented in Table III. Components C_{2a} and C_{2b} collectively comprise 12% of the total weight of gentamicin group C_2. Based on specific activities of 0.92 and 1.38 μCi/mg for components C_{1a} and C_1, respectively, integration of all gentamicin C-complex bands (excluding the polar component band) in Fig. 3 indicated that components C_{2a} and C_{2b} collectively accounted for 4% of gentamicin C-complex.

TABLE III

RELATIVE COMPOSITION AND SPECIFIC ACTIVITY OF THE GROUP C_2 COMPONENTS

Component	*Specific activity (μCi/mg free base)*	*% total weight of group C_2**
C_{2b}	1.38	4.2
C_{2a}	1.92	7.7
C_2	1.37	88.1

* These values were determined by integration of each of the group C_2 bands presented in Fig. 9. The area values thus obtained were divided by the corresponding specific activities and each result expressed as a percentage of the total weight of gentamicin group C_2.

CONCLUSION

Gentamicin C-complex consists of a mixture of five biologically active components, *viz.* gentamicins C_{1a}, C_2, C_{2a}, C_{2b}, and C_1. Components C_{2a} and C_{2b} are minor in concentration, comprising only 4% of the C-complex component mixture. All five gentamicin C-complex components differ from each other in the degree of methylation and stereochemistry at the 6′-C position. Component C_{2b} is 6′-N-methylgentamicin C_{1a} while component C_{2a} is tentatively identified as the 6′-C epimer of gentamicin C_2.

Craig distribution is shown to be an excellent method for the separation of all five C-complex components. Its use as an analytical and preparative chromatographic technique, as described in this paper, might well be extended to other closely related aminocyclitol compounds of natural and semi-synthetic origin. The advantages of Craig distribution for the separation of gentamicin C-complex lie in its ability to handle large quantities of material, to separate the components without prior derivatization of the starting material, and the ease of isolation of the separated antibiotic substances.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Olwen van Es for her assistance in performing the Craig distribution experiments and Rush Slivjanovski for his help in preparing a computer program for determining the theoretical curves.

REFERENCES

1 H. Maehr and C. P. Schaffner, *J. Chromatogr.*, 30 (1967) 572.
2 G. H. Wagman, J. A. Marquez and M. J. Weinstein, *J. Chromatogr.*, 34 (1968) 210.
3 G. H. Wagman and J. V. Bailey, *J. Chromatogr.*, 41 (1969) 263.
4 H. Maehr and C. P. Schaffner, *J. Amer. Chem. Soc.*, 89 (1967) 6787.
5 D. J. Cooper, M. D. Yudis, R. D. Guthrie and A. M. Prior, *J. Chem. Soc., C*, (1971) 960.
6 D. J. Cooper, M. D. Yudis, H. M. Marigliano and T. Traubel, *J. Chem. Soc., C*, (1971) 2876.
7 D. J. Cooper, P. J. L. Daniels, M. P. Yudis, H. M. Marigliano, R. D. Guthrie and S. T. K. Bukhari, *J. Chem. Soc., C*, (1971) 3126.
8 D. J. Cooper, J. A. Waitz, M. Counelis and J. Weinstein, *Belg. Pat.*, 768,796 (1972).
9 P. J. L. Daniels, in S. Mitsuhashi (Editor), *Drug Action and Drug Resistance in Bacteria. 2. Aminoglycoside Antibiotics*, Tokyo University Press, Tokyo, 1975, p. 77.
10 E. M. Oden, G. H. Wagman and M. J. Weinstein, in F. Kavanaugh (Editor), *Analytical Microbiology*, Vol. II, Academic Press, New York, 1972, p. 271.
11 M. J. Weinstein, G. H. Wagman, E. M. Oden and J. A. Marquez, *J. Bacteriol.*, 94 (1967) 789.
12 J. A. Waitz and M. J. Weinstein, *J. Infect. Dis.*, 119 (1968) 355.
13 H. Maehr, *Ph.D. Thesis*, Rutgers, The State University, New Brunswick, N.J., 1964.
14 A. S. Kershner, *Ph.D. Thesis*, Rutgers, The State University, New Brunswick, N.J., 1971.
15 K. L. Rinehart, Jr., P. Schaefer, J. C. Cook, C. P. Schaffner and A. S. Kershner, *Ann. Conf. Mass Spectrom. Allied Topics, 19th, Atlanta, Ga., May 2–7, 1971.*
16 A. H. Thomas and S. D. Tappin, *J. Chromatogr.*, 97 (1974) 280.
17 G. H. Wagman, J. A. Marquez, J. V. Bailey, D. Cooper, J. Weinstein, R. Tkach and P. Daniels, *J. Chromatogr.*, 70 (1972) 171.
18 R. Ikachi, I. Kawamoto, S. Takasawa, M. Yamamoto, S. Sato, T. Sato and T. Nara, *J. Antibiot.*, 27 (1975) 793.
19 R. S. Egan, R. L. Devault, S. L. Muellar, M. I. Levenberg, A. C. Sinclair and R. S. Stanaszek, *J. Antibiot.*, 28 (1975) 29.
20 P. J. L. Daniels, C. Luce and T. L. Nagabushan, *J. Antibiot.*, 28 (1975) 35.
21 J. P. Rosselet, J. Marquez, E. Meseck, A. Murawski, A. Hamden, C. Joyner, R. Schmidt, D. Migliore and H. L. Herzog, *Antimicrob. Agents Chemother.*, (1963) 14.
22 B. C. Lee, R. G. Condon, G. H. Wagman, K. Byrne and C. P. Schaffner, *J. Antibiot.*, 27 (1974) 822.
23 E. M. Oden, H. Stander and M. J. Weinstein, *Antimicrob. Agents Chemother.*, (1963) 8.
24 J. A. Marquez, unpublished data.
25 W. W. Wright, in A. Balows (Editor), *Current Techniques in Antibiotic Susceptibility Testing*, Charles C. Thomas, Springfield, Ill., 1974, p. 26.
26 T. L. Nagabushan, W. N. Turner, P. J. L. Daniels and J. Morton, *J. Org. Chem.*, 40 (1975) 2836.
27 T. L. Nagabhushar, P. J. L. Daniels, R. S. Jaret and J. B. Morton, *J. Org. Chem.*, 50 (1975) 2835.
28 H. Maehr and C. P. Schaffner, *J. Amer. Chem. Soc.*, 92 (1970) 1697.
29 J. Weinstein, O. J. Cooper and P. J. L. Daniels, *Abstr. Interscience Conf. Antimicrob. Agents Chemother., 12th*, 1972, p. 9.
30 P. J. L. Daniels, personal communication.