Studies on Leucomycin. III. Isolation[1] *and Properties of Six Antibacterial Components in Leucomycin Complex*

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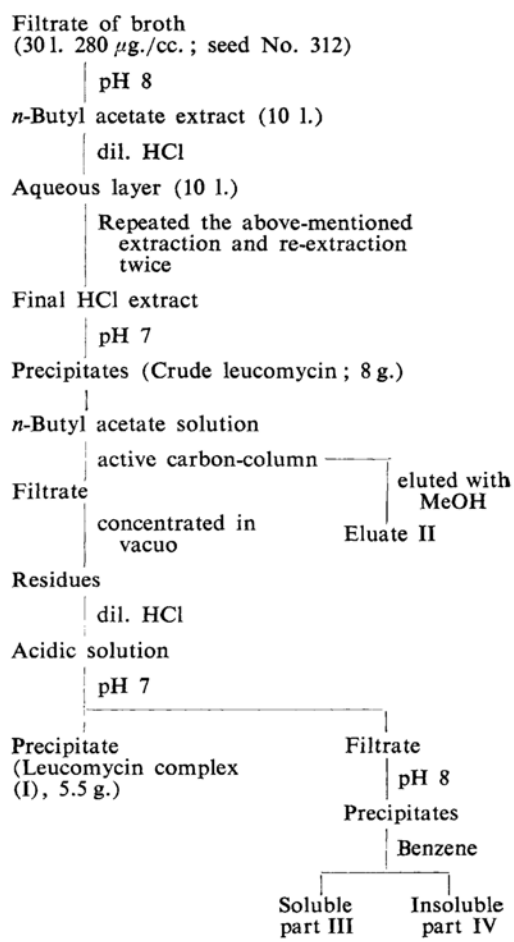
Leucomycin[2], a basic antibiotic obtained from the broth-filtrate of *Streptomyces kitasatoensis* Hata[3,4], has been presumed to be a macrolides on its physical, chemical[3,5] and antibacterial[3,6] properties. In the previous papers of this series[7,8] it was reported that the antibiotic was a mixture of six biologically active components, A_1, A_2, B_1, B_2, B_3 and B_4 and that the last four minor components, though in a minute amount, could be purified by fractional precipitation[8] of the mixture from aqueous and from benzene solutions (see Table I), followed by the further minute resolution of the resulting fractions by chromatography on a column of Amberlite IRC-50[8]. The main component A_1 which seemed to have the highest activity[8], however, had been scarcely separated from B_4 and another minor component A_2, by this method.

For the resolution of macrolides complex in preparative scale, counter current distribution has been reported to be effective[9-12]. Using this technique, leucomycin complex was also

TABLE I. FRACTIONATION OF LEUCOMYCIN



1) Japanese Pat., Application No. 13909 (1958).

2) Leucomycin used in this study, was obtained from the broth of *S. kitasatoensis* Hata No. 312, in 1956 and had a similar component composition to that of the original preparation by Hata[3,6]. Recent preparation from No. L-149-373, a mutant of the original strain, was almost free from B components.

3) T. Hata, Y. Sano, M. Ohki, Y. Yokohama A. Matsumae and S. Ito, *J. Antibiotics, Ser.* A6, (2), 87 (1953).

4) T. Hata, F. Koga and H. Kanamori, ibid., Ser, A, 6 (3), 109 (1953).

5) Y. Sano, ibid., Ser. A7 (3), 93 (1954).

6) T. Hata, Y. Sano, Y. Yokohama, S. Ito, S. Okazaki, K. Takeno, H. Ito, Y. Owada, Y. Saito and M. Soekawa, ibid., Ser. A6 (4), 163 (1953).

7) J. Abe, Y. Suzuki, T. Watanabe and K. Satake, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **81**, 969 (1960).

8) T. Watanabe et al., This Bulletin, **33**, 1100 (1960).

9) J. M. McGuire, L. Bunch, R. C. Anderson, H. E. Boaz, E. H. Flynn, H. M. Powell and T. W. Smith, *Antibiot. Chemoth.* 2, 281 (1952).

10) R. L. Sagner, F. A. Hochstein and K. Murai, *J. Am. Chem. Soc.*, 75, 4684 (1953).

11) S. P. Sindico, L. Ninet, S. Preud'homme and C. Coser, *Antibiotics Annual*, 724 (1955-1956).

12) H. Schmitz, M. Misick, B. Heinemann and I. R. Hooper, *Antibiot. Chemoth.*, 7, 37 (1957).

analyzed[13]. The distribution diagram, though after only 30 transfers, seemed to suggest a partial resolution. These facts prompted us to study the counter current distribution of leucomycin complex (I) (see Table I) in more detail. In this paper we wish to report on these results. In addition, some properties of such purified antibacterial components as A_1, A_2, B_1, B_2, B_3 and B_4, will be described.

Solvent System for the Distribution of Leucomycin Complex. — The marked difference observed among the solubilities of each of the components of leucomycin complex in aqueous solution at pH 7~8 and in benzene[8], seemed to suggest the use of both solvents for the counter current distribution. Preliminary experiments showed that the addition of methanol to this binary system further improved the resolution. Fig. 1 presents the distribution diagram of leucomycin complex (I), after 150 transfers between the two layers which resulted from a mixture of five parts of benzene, two parts of M/15 sodium phosphate buffer at pH 6.5, and five parts of methanol (Solvent system 1); together with a composite paper chromatogram of leucomycin components in each distribution tube. The results suggested that system 1 could be used for the isolation of A_1, A_2 and B_1.

A solvent system which gave a more minute resolution of leucomycin complex, could be obtained by using much more acid citrate buffer and by adding a sufficient amount of choroform to the triple-system composed of benzene, citrate buffer and methanol. Differing from solvent system 1, the quartet-system separated into two layers very rapidly, though the volume-ratio of these two layers differed to some extent by a slight change of temperature. Fig. 2 illustrates the distribution diagram

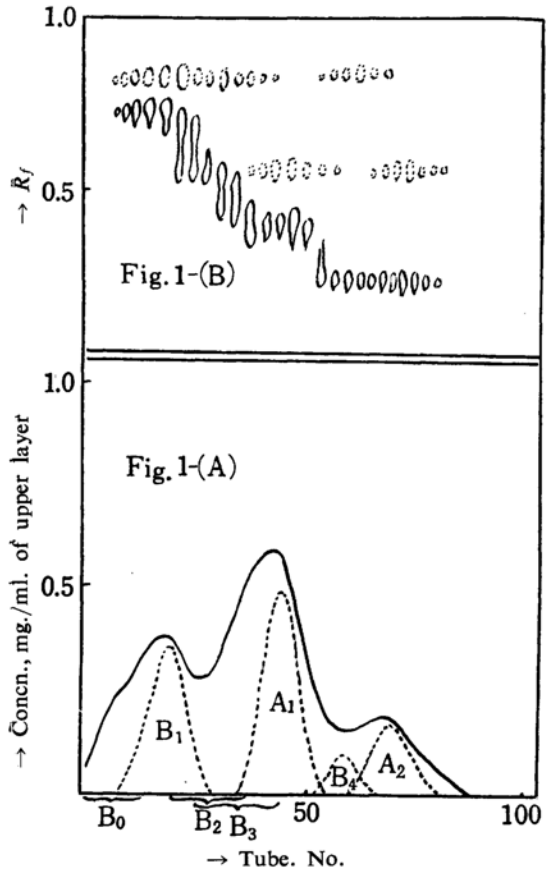


Fig. 1—(A). ---- Counter current distribution of luecomycin complex (I) using "Solvent system 1".

Fig. 1—(B). ---- Composite paper chromatogram of luecomycin components in each distribution tube. ○ developed with *n*-butyl acetate containing phosphate buffer (pH 6). ○ developed with phosphate buffer (pH 8) containing *n*-butyl acetate.

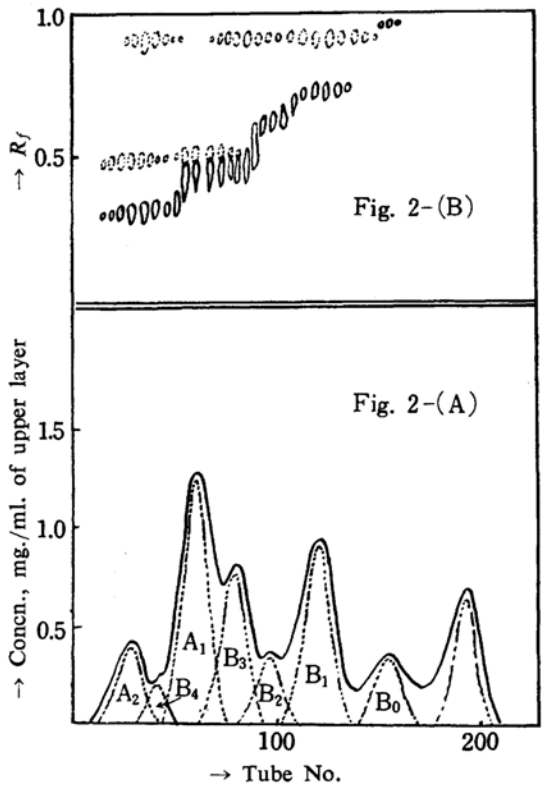


Fig. 2—(A). ---- Counter current distribution of leucomycin complex (I) using "Solvent system 2".

Fig. 2—(B). ---- Composite paper chromatogram of leucomycin components in each distribution tube; ○ developed with *n*-butyl acetate containing phosphate buffer (pH 8) containing *n*-butyl acetate.

13) Y. Sano. T. Hoshi and T. Hata, *J. Antibiotics*, Ser. A7, (3) 88 (1954).

of leucomycin complex (I), after 210 transfers between the two layers which resulted from a mixture of ten parts of benzene, nine parts of M/15 sodium citrate buffer of pH 4.9, eight parts of chloroform and twenty parts of methanol (Solvent system 2); together with a composite paper chromatogram of leucomycin components in each distribution tube. From the appropriate tubes after this distribution, five kinds of antibacterial component could be isolated completely free from other components though the yields of B_2 and of B_3 were not so excellent as that of A_1 A_2 or B_1 due to the partial resolution of the former two.

For the resolution of B components, especially of B_2 and B_4 which were only slightly soluble in benzene, another system composed of chloroform and acid buffer seemed to be promising, as B components showed a moderate solubility in both solvents. In this case, too, the resolution was improved by adding an appropriate amount of a third solvent which could be mixed with upper and lower layers at any ratio, and the effect of acetone seemed to be superior to that of methanol in this solvent system. Fig. 3 shows the distribution diagram of antibiotic IV (see Table I) present in the filtrate fraction from leucomycin complex (I) precipitated at pH 7, after 250 transfers between the two layers which resulted from a mixture of one part of chloroform, two parts of M/15 sodium acetate buffer of pH 4.5, and two parts of acetone (Solvent system 3), together with a composite paper chromatogram of leucomycin components in each distribution tube. From the appropriate fractions after this distribution, all B components, B_1, B_2, B_3 and B_4, could be isolated in a good yield, respectively. The results suggested that the counter current distribution with "Solvent system 3" was the most suitable method for the isolation of four B components; if the most part of A_1 which was the main component of leucomycin complex, had been previously removed.

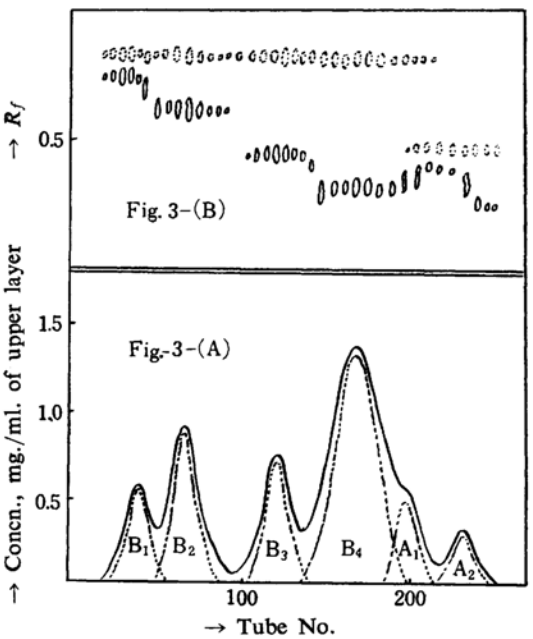


Fig. 3—(A). ---- Counter current distribution of fraction IV (See Table I, B components rich fraction) using "Solvent system 3".
Fig. 3—(B). ---- Composite paper chromatogram in each distribution tube; ○ developed with *n*-butyl acetate containing phosphate buffer (pH 6). ○ developed with phosphate buffer (pH 8) containing *n*-butyl acetate.

Leucomycin A_1 and A_2.—As shown in Fig. 2, the observed distribution curves of A_1 and of A_2, showed no serious disagreement with the theoretical ones calculated from the maximum peak position[14], nor with the distribution of A_1 and A_2 estimated by paper chromatography. The facts seemed to indicate the homogeneity of both the components resolved by counter current distribution. The presumption was verified by the identity of the observed and the calculated re-distribution

TABLE II. PROPERTIES OF LEUCOMYCIN A_1 AND A_2

	A_1	A_2
Partition coefficient K		
Solvent system 1	0.92	2.33
Solvent system 2	0.33	0.14
Solvent system 3	1.86	3.00
$\lambda_{max}^{N/1000\ HCl}(\varepsilon_{max})$	233 mμ (2.45×10^4)	231 mμ (1.98×10^4)
pK'_a	7.1	7.1
Equivalent as base		
Found.	920~950	1220~1250
(Calcd.)	(918)[a]	(1265)[b]
C%; Found. (Calcd.)	60.99 (60.13)	62.13 (61.66)
H%: Found. (Calcd.)	8.72 (8.75)	8.61 (8.70)
N%; Found. (Calcd.)	1.52 (1.54)	1.10 (1.11)
Antibacterial activity (unit/mg.)	1000	910
Content (%) in I	33	19
II	—	—
III	8	4
IV	3	1

a) as $C_{46}H_{81}NO_{17}$
b) as $C_{65}H_{111}NO_{22}$

14) L. C. Craig and O. Cost, *Anal. Chem.*, 21, 508 (1949).

curves (using "Solvent system 1 or 2" for the samples isolated with "Solvent system 2 or 1", respectively).

In Table II, are summarized the analytical data and some properties of leucomycin A_1 and A_2. There have been reported several macrolides, from picromycin ($C_{25}H_{43}NO_7$)[15] up to magnamycin ($C_{42}H_{67}NO_{16}$)[16]. Minor components A_2 ($C_{65}H_{111}NO_{22}$) seemed to be a macrolide of highest molecular weight, as far as we know. Main component A_1 ($C_{46}H_{81}NO_{17}$), on the other hand, was similar to magnamycin in the molecular weight and pK'_a, though the former had an antibacterial activity higher than that of the latter. In Table II, are also presented the contents of A_1 and A_2, in leucomycin complex and in several fractions derived during the preparation of fraction I. These values calculated from the distribution diagram with "Solvent system 2" were not inconsistent with the A_1- and A_2-content estimated from the elution diagram from a column of Amberlite IRC-50 with a mixture of ethanol and M/15 sodium citrate buffer at pH 5[8]. The results also indicated that both the components were less soluble in aqueous solution at pH 7, less absorbed on a column of active carbon, and more soluble in benzene than B components. Fig. 4 represents infrared spectra of A_1. The presence of OH, C=O (ester) and C=C was presumed from the characteristic absorption at 3450 cm^{-1}, 1750 cm^{-1} and 1680 cm^{-1}, respectively. A shoulder at 1710 cm^{-1} seemed to be due to the presence of the second C=O (lactone).

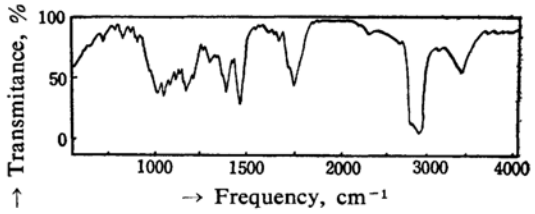


Fig. 4. Infrared spectrum of leucomycin A_1. (in Nujol)

Infrared spectrum of A_2 was extremely close to that of A_1.

Leucomycin B_1, B_2, B_3 and B_4. — Four components, B_1, B_2, B_3 and B_4, found in leucomycin complex as minor components, could be isolated by counter current distribution using "Solvent system 3" from the filtrates III and IV of leucomycin complex (I) precipitated at pH 7[8], from the mother liquid of the recrystallization of leucomycin complex (I); and from the methanol-eluate II of the carbon-column previously treated with *n*-butyl acetate solution of leucomycin[7,8]. As shown in Fig. 3, the distribution curves of these components agreed with the corresponding theoretical curve. The homogeneity of the component so isolated was further verified by analytical chromatography on a column of Amberlite IRC-50[8].

Leucomycin B components (C_{34}—C_{38}) had a molecular weight similar to that of erythromycin A[18], but the pK'_a value (6.8) was similar to those of leucomycin A and magnamycin. The antibacterial activity was considerably lower than that of A_1. In Table III,

Table III. Yields, properties and antibacterial activity of leucomycin components

Component	*K* (Solvent system 1)	*K* (Solvent system 2)	*K* (Solvent system 3)	m. p. °C	C % Found (Calcd.)	H % Found (Calcd.)	N % Found (Calcd.)
B_0	0.1	2.3	—	—	—	—	—
B_1	0.19	1.3	0.15	214.5~216.5	60.30(60.00)	8.22(8.20)	1.99(2.00)[a]
B_2	0.37	0.91	0.28	214.0~216.0	56.85(56.50)	8.14(8.05)	1.66(1.63)[b]
B_3	0.54	0.50	0.67	216.0~217.0	59.39(59.73)	7.65(7.72)	2.05(2.05)[c]
B_4	1.50	0.19	1.22	221.0~223.8	56.27(56.81)	7.59(7.64)	1.81(1.78)[d]

	Antibacterial activity (unit/mg.)*	$\lambda_{max}^{N/1000\ HCl}$ (ε_{max})	Yield (%) from I*	II**	III**	IV***
B_0	<10	—	2	8	0	0
B_1	100	234 mμ(2.24×10⁴)	11	23	50	24
B_2	215	234 mμ(2.47×10⁴)	6	12	20	29
B_3	570	234 mμ(2.32×10⁴)	19	38	27	13
B_4	700	233 mμ(2.45×10⁴)	8	20	9	30

* Leucomycin complex (I); 560 unit/mg. ** Using "Solvent system 2".
*** Using "Solvent system 3".
a) as $C_{35}H_{59}NO_{13}$ b) as $C_{38}H_{65}NO_{16}$ c) as $C_{34}H_{53}NO_{13}$ d) as $C_{38}H_{59}NO_{16}$

15) H. Brockmann et al., *Ber*, 84, 284 (1951).
16) R. L. Wagner, F. A. Hochstein and K. Murai, *J. Am. Chem. Soc.*, 75, 4684 (1953).
17) E. H. Flynn, M. V. Sigal and F. R. Wiley, ibid., 76, 3121 (1954).

are summarized the analytical data, some properties and the content in leucomycin complex (I) and in several fractions II, III and IV. The last data indicated that B components could be fractionated, and that the fractions thus obtained could be further separated into two groups, B_1 and B_3 rich III, and B_2 and B_4 rich group IV. It seems to be interesting that the original strain of *Streptomyces kitasatoensis* produced as many as six kinds of macrolide, while the mutant (strain No. L-149-373) gave only A_1 and A_2 without any decrease of the weight of total antibiotics. The elucidation and comparison of the structure of these components might give valuable information on the biosynthesis of macrolides.

Experimental

Materials.—Leucomycin complex (I) and the fractionated antibiotics II, III, IV were prepared by the methods summarized in Table I.

Counter Current Distribution. — The procedure was achieved at 15~20°C in an automatic "Craig-type" apparatus having 300 transfer tubes No. 555 F, Mitamura Shoten, Tokyo). The sample (1~10 g.) was added as a concentrated solution in the lower solvent layer (5~10%) into the first few tubes. The volume of the upper and the lower layer in each distribution tube was 10 cc. each. The partition coefficient of distributed components was calculated from the peak position on the distribution diagram and the total transfer-number. The theoretical distribution curve was calculated from the partition coefficient so obtained, according to Craig's method[14].

Determination of the Concentration of Leucomycin. — The concentration was assayed by the photometry of red brown color which was produced by mixing 0.5 cc. of the sample (aqueous layer in the distribution tube) with 2.0 cc. of concentrated sulfuric acid[8]. The concentration in organic-solvent rich layer, was calculated from the above-mentioned concentration in aqueous layer and the calculated partition coefficient.

Paper Chromatography of Leucomycin Components.—Paper chromatography of leucomycin in each distribution tube was achieved by the method already reported[7], using 0.02 cc. of the organic-solvent rich phase as the sample.

Crystallization of Each Component.—The leucomycin components separated by counter current distribution, was crystallized from the aqueous solution by adjusting the pH to 8 according to the method reported already[8].

Antibacterial Activity. — The assay of the antibacterial activity of the components so purified, was done by the Bio-assay Division, Toyo Jozo Co. Ltd., according to the usual cup assay method with the use of *Baccillus subtilis* No. 209 as the test organism.

Absorption Specta. — Absorption spectra were measured by Hitachi Spectrophotometer Model DUP-2.

pK'_a.—pK'_a was measured on the sample solution in a dimethylformamide (60%) at a concentration of 0.05 per cent, by titrating with 1 N hydrochloric acid with the use of Toa-denpa pH meter HM-5.

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