*Studies on Leucomycin. II. Preparative Chromatography of Macrolides on Amberlite IRC-50**

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As shown in the previous paper[1], leucomycin, a basic antibiotic produced by *Streptomyces kitasatoensis* Hata[2-4], was proved by the paper-chromatographic and -electrophoretic analysis, to be a mixture composed of at least six biologically active components, A_1, A_2, B_1, B_2, B_3 and B_4. Preliminary experiments on the chromatographic separation of these components in a preparative scale showed, that both leucomycin groups, A and B, were able to be absorbed on a column of alumina or silisic acid from the benzene solution and to be separately eluted with the same solvent containing methanol in this order. Using this or similar chromatographic systems, however, recoveries of the antibiotics was markedly low (50~70 per cent in weight, less than 40 per cent in biological activity; presumably accompanied with decomposition) and a more minute resolution into each component, was unsuccessful.

In course of our studies, it was found that leucomycin absorbed on a column of carboxylic resin, such as Amberlite IRC-50 in H-cycle, could be eluted quantitatively with sodium citrate buffer, and that all six components in the leucomycin complex were separately eluted with a mixture of citrate buffer and ethanol, in such a high concentration as the chromatographic system could be used for a preparative separation of these components. Using this method, not only leucomycin complex, but also some derivatives of leucomycin A_1 and other macrolides such as erythromycin[5], magnamycin[6] and spiramycin[7], having dimethylamino sugar as the basic moiety[8], could be purified from the contaminated minor components, which had been separated only with difficulties by counter current distribution. In this paper, we propose to report these results. In addition some properties of purified leucomycin components B_1, B_2, B_3 and B_4 and the composition of these components in leucomycin complex will be reported.

Experimental

Materials.—Leucomycin complex (I)** and the fractionated antibiotics (II, III, IV) were kindly prepared for this study by J. Abe et al., at the Research Institute, Toyo Jozo Co. Ltd., according to the methods summarized in Table I. Erythromycin, magnamycin and spiramycin were the samples furnished as "standard antibiotics" through the National Institute for Preventive Medicine, Tokyo. Diacetylleucomycin A_1 ($C_{50}H_{85}O_{19}N$, m. p. 127~129°C)[9], propionylleucomycin A_1 ($C_{49}H_{85}O_{18}N$, m. p. 123~126°C)[10], tetrahydroleucomycin A_1 ($C_{46}H_{85}O_{17}N$, m. p. 162~164°C)[11], and a basic moiety derived from leucomycin A_1 with the methanolysis ($C_{34}H_{59}O_{13}N$, m. p. 154~155°C; corresponding to carimbose fragment in magnamycin)[12], were prepared from leucomycin A_1 by the present author, according to the methods which will be published soon.

* Pat., Application No. 13908 (1959).

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3) T. Hata, F. Koga and H. Kanamori, *J. Antibiotics*, Ser. **A6** No. 3, 109 (1953).

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11) T. Watanabe, will be published.

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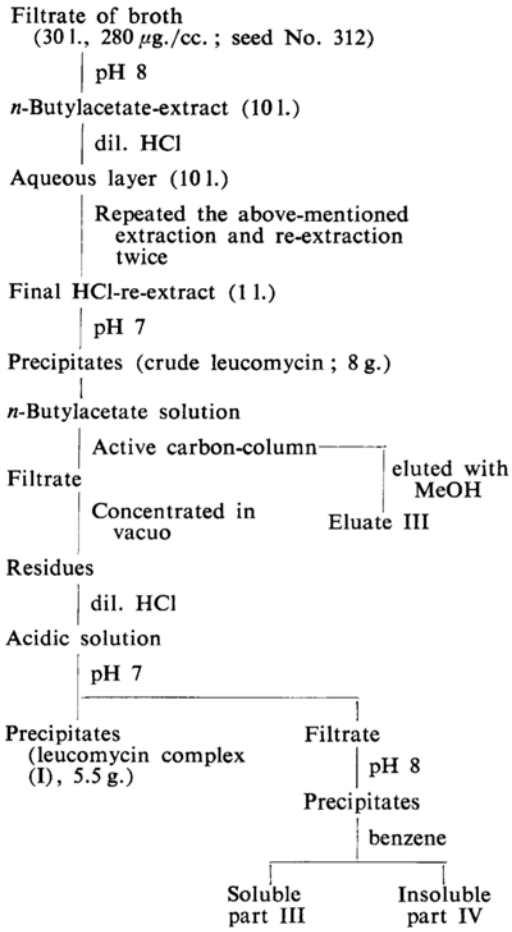
TABLE I. FRACTIONATION OF LEUCOMYCIN



Chromatography on Amberlite IRC-50. — One hundred grams of the resin (XE-64) of approximately 150 mesh, was suspended in 500 cc. of 1 N sodium hydroxide and the suspensions were heated on a water bath at 60°C for 3 hours under occasional stirring. After the decantation of brown colored supernatant, the resin of the Na-cycle was washed with distilled water thoroughly, then suspended in 1 N hydrochloric acid at room temperature. After 30 minutes, the resin was washed with distilled water until the washing became free from acid. Forty grams of so purified resin of H-cycle, was added as thick suspension in water to a column of 1 cm. in the diameter, to a hight of 45 cm. To the column, 10 mg. of sample to be analyzed was added as 1 per cent solution in N/100 hydrochloric acid. As soon as the applied sample solution had been allowed to flow into the column, washing with 150 ml. of distilled water, then elution with a mixture of 96 per cent ethanol and M/5 sodium citrate buffer (Na^+ 1.6 g. ion per liter, pH 5.0) in an equal volume, was initiated at a flow rate of 9 cc. per cm^2 per hour at 15°C. The effluent was collected in 4.5 cc. fraction by means of automatic fraction collector. For the chromatography in a preparative scale (15 g. of sample), a column of 3×180 cm. was used.

Chemical Assay of Leucomycin in the Effluent. —The concentration of leucomycin in each effluent fraction was determined photocolorimetrically with sulfuric acid as follows. An aliquot of sample solution was mixed with an equal volume of concentrated sulfuric acid under adequate shaking, and the resulting red brown color was measured between 10 and 30 minutes after the addition of sulfuric acid, with Kotaki photometer Model D2 using filter 470. Experiments showed, that the readings were strictly proportional to leucomycin concentration up to an optical density of about 1.1 equivalent to approximately 500 μg per cc. of leucomycin complex (I) and they were not affected by the presence or absence of M/5 citrate and of 50 per cent ethanol. As the color developed per unit weight of leucomycin components, were not just the same, the correction was made for the calculation of weight composition of each component. This method could be also applied with success for the assay of leucomycin derivatives and other macrolides, except erythromycin which with sulfuric acid colored only slightly. The fact, however, did not prevent the determination of an approximate elution position of erythromycin with this coloration.

Paper Chromatography of Leucomycin.—Leucomycin components contained in each fraction of chromatographic effluent, were identified from the chromatographic R_f values, A and B groups could be easily distinguished from the R_f developed with *n*-butyl acetate containing M/15 sodium phosphate (pH 6); while A_1 and A_2; B_1, B_2, B_3 and B_4 from the R_f developed with M/15 sodium phosphate containing *n*-butyl acetate (pH 8) on a liquid paraffin-treated filter paper. The position of the leucomycin component migrated on the filter paper, was detected as a growth-inhibited zone of *Bacillus subtilis* on the bioautogram[1].

Isolation of Leucomycin Components.—Fractions corresponding to each peak on the elution diagram, were combined and cocentrated to about one third volume under reduced pressure. The basic antibiotics in the aqueous concentrates, after adjusting the pH to 8, were extracted with *n*-butyl acetate. From the acid re-extract of the ester layer, leucomycin was crystallized by adjusting the pH to 7. For the recrystallization benzene-cyclohexane solvent for A group and hot benzene for the B group were used.

Assay of Antibacterial Activity.—The assay of the antibacterial activity of the so purified components, was kindly done by the Bioassay Division, Toyo Jozo Co. Ltd., according to the usual cup method against *Bacillus subtilis* No. 209 as the test organism.

Results

Chromatography of Leucomycin.—Fig. 1-A represents a typical elution diagram of a leucomycin complex (I) from a column of Amberlite XE-64 (H-cycle) with a mixture of ethanol and M/5 sodium citrate buffer (pH 5.0) in an equal volume. Fig. 1-B illustrates the paper-chromatograms of the above-mentioned effluent fractions detected by the growth-inhibition of

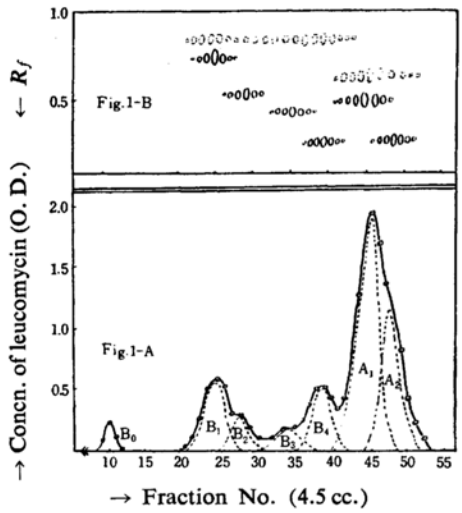


Fig. 1-A. Chromatography of leucomycin complex (I) on 1×40 cm. column of Amberlite XE-64, eluted with a mixture of 96 per cent ethanol and M/5 citrate buffer in an equal valume, at a flow rate of 9 cc. per cm² per hour at 15°C. -⊙- concentration (uncorr.) assayed photocolorimetrically. ---- concentration of each component presumed from paper chromatographic analysis (Fig. 1-B).

Fig. 1-B. Composite paper chromatograms of each effluent fraction. ○ developed with *n*-butyl acetate containing phosphate buffer (pH 6). ○ developed with phosphate buffer (pH 8) containing *n*-butyl acetate.

Bacillus sabtilis. These results indicated that leucomycin components, B_1, B_2, B_3, B_4, A_1 and A_2 were eluted from the column in this order and these components could be separated from each other under the conditions used, except the last two components A_1 and A_2, which could be only partially separated***. A minor component B_0 eluted at the hold-up volume, colored with concentrated sulfuric acid but showed no antibacterial activity (less than 10 unit per mg.). Some discrepancies occasionally observed between both of the amounts of leucomycin complexes, assayed photocolorimetrically and biologically, might be due in part to the presence of this component. The recovery of leucomycin from the column assayed both photocolorimetrically and biologically, was more than 90 per cent and the rechromatography of each component showed an elution of only one component at the same position, indicating that no appreciable degradation occurred for this antibiotic.

*** Recently, however, both of the components were found to be completely separated on a longer column (1.5~2.0 m.), if the amount of the sample added to the column was limited.

The reproducibility of this chromatography was fairly good and the position of the elution of each component varied only within 5 per cent, so far as the chromatographic conditions mentioned rather in detail in the experimental part were exactly maintained. The flow rate of 9 cc./cm²/hr. seemed to be an upper limit for the elution without any broadening of each peak. The pH of buffer should be selected between 4 and 8, as the more acidic or alkaline pH caused slight degradation of leucomycin even at room temperature. In this region, the higher the pH, the less the spreading of the elution peak but also lesser than the separation of the components among each other. Most important in this chromatographic method is the temperature. At higher temperature than 25°C, all components were eluted very rapidly, while at a lower than 10°C very slowly, and both of the extremes resulted into a poor resolution. For a precise analysis, a device to maintain the temperature of the column at a constant (15~20°C) might be profitable.

Leucomycin Components.—From the elution diagram as shown in Fig. 1, the composition of the leucomycin complex (I) was estimated to be composed of 11 per cent of B_1, 7 per cent of B_2, 8 per cent of B_3, 13 per cent of B_4, 32 per cent of A_1 and 24 per cent of A_2, in weight respectively. These values were by no means inconsistent with those estimated from the counter current distribution diagram of the same sample[13].

Using the same chromatographic technique, another sample of leucomycin complex (I) produced by a new strain (seed No. L 149-373) and some fractionated leucomycin samples (II, III and IV) from the original strain, were analyzed. The results indicated, that a new strain of *Streptmyces kitasatoensis* produced mainly A components; B components produced by the original strain were much more absorbed on a column of active carbon from the *n*-butyl acetate solution and were precipitated at higher pH from the aqueous solution than A components did; and the crystallization of B components from benzene was effective for the separation of B_2 and B_4 from less active components B_1 and B_3. Four leucomycin components, B_1, B_2, B_3 and B_4 and partially purified A_1 (contaminated small amount of A_2) were actually isolated from the preparations which had been previously fractionated (see Table I) according to the above mentioned method by the use of preparative column (3×180 cm.).

The antibacterial activity of A_1, though still contaminated with a small amount of A_2, was as much as 1000 unit per mg. This value was

13) T. Watanabe, H. Nishida, J. Abe and K. Satake, This Bulletin, 33, 347 (1960).

TABLE II. YIELDS, PROPERTIES AND ANTIBACTERIAL ACTIVITY OF LEUCOMYCIN COMPONENTS

Component	Yield (%) from I	II	III	IV	m. p. °C	Molecular formula	Antibacterial activity (unit/mg.)*
A_1	32	12	10	0	132.0~137.0	—	1000
A_2	24	6	3	0	—	—	—
B_0	5	1	0	0	—	—	10
B_1	11	16	53	25	214.5~216.5	$C_{35}H_{59}NO_{13}$	100
B_2	7	28	1	31	214.0~216.0	$C_{38}H_{65}NO_{16}$	215
B_3	8	29	28	12	216.0~217.0	$C_{34}H_{53}NO_{13}$	570
B_4	13	8	5	32	221.0~223.8	$C_{38}H_{59}NO_{16}$	700

* Leucomycin complex (I) ; 560 unit/mg.

approximately 1.8~1.9 times as much as that of leucomycin complex (I) produced by the original strain, and was the highest one as far as known on leucomycin samples. These facts will be explained by the contamination of less active B components. It may be of interest that the activity of B components varied from 700 units to 100 units. According to J. Abe slight undesirable properties observed in the leucomycin complex (produced by the original strain) was mainly due to the presence of B components and their action increased in the following order; B_4, B_3, B_2 and B_1, thus with the decrease of the antibacterial activity. These results are summarized in Table II, together with some analytical data of purified B components.

Chromatography of Leucomycin Derivatives.—Under the same condition as used for the separation of leucomycin complex, some leucomycin derivatives, acetyl-[9], propionyl-[10], tetrahydroleucomycin A_1[11], and a basic moiety derived from A_1 by methanolysis[12], were also eluted quantitatively, but at a different position, which are summarized in Table III. The results indicated that this chromatography was effective for the purification of these derivatives from the unreacted leucomycin A_1. Less-retardation of the basic moiety derived from A_1 by methanolysis and more retardation of the two acyl derivatives on a column of carboxylic acid resin, will be related to their different pK'_a values (pK'_a 5.0 for acyl derivatives; pK'_a 8.1 for basic moiety derived from A_1 by methanolysis) from the original leucomycin A_1 (pK'_a 7.1)****, but the different chromatographic behaviors of A_1 and of the tetrahydro-derivative and of other leucomycin components (pK'_a 6.8~7.1) indicated that " Weiss's effect " would play a predominant rôle in these separations.

Chromatography of Other Macrolides.—Table III lists the elution position of several macrolides, including erythromycin, magnamycin, and spiramycin, from a column of Amberlite XE-64 (H-cycle) with an equi-volume mixture of ethanol and M/5 sodium citrate buffer (pH 5.0). These results, together with the results of the paper-chromatographic and -electrophoretic analysis[1], indicated that all six antibiotics contained in leucomycin complex different from one another well-known macrolides, namely: erythromycin, magnamycin and spiramycin. As reported already by the other authors, antibiotics belonging to macrolides were usually produced as a complex composed of a main and a few minor components, the latter of which could be fairly separated with some difficulties. In these experiments,

TABLE III. THE ELUTION POSITION OF SOME LEUCOMYCIN DERIVATIVES AND MACROLIDES UNDER THE SAME CONDITIONS AS USED FOR THE SEPARATION OF LEUCOMYCIN COMPLEX (I)

Antibiotics			Volume cc.
Leucomycin	Main component	A_1	180~225
	Minor component	B_1	90~120
		B_2	112~144
		B_3	144~166
		B_4	157~189
		A_2	198~238
	Derivatives of A_1	Des-sugar	50~ 76
		Tetrahydro-	71~126
		Acetyl-	201~255
		Propionyl-	197~259
Erythromycin	Main component		126~193
	Minor component		99~144
			189~250
Magnamycin	Main component		76~126
	Minor component		67~ 90
			98~148
Spiramycin	Main component		234~292
	Minor component		126~157
			211~270

**** Basicity of leucomycin is due to the presence of 3-dimethylamino sugar, so the pK'_a was fairly affected by the 2,4-*O*-substitution.

too, some commercial samples of the above-mentioned antibiotics, though considered to be fairly purified, showed the presence of these minor antibiotics. Such minor components of usual macrolides, however, had been limited to only two or three kinds. The presence of many minor components, as much as five in leucomycin seemed to belong to the first case.

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