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SEPARATION OF THE HOMOLOGOUS COMPONENTS (A_1, A_3 AND B) OF PRIMYCIN BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Primycin is a natural complex, a mixture of homologous antibiotics. On thin-layer chromatography in neutral solvent systems the co-crystallizing components exhibit the phenomenon of secondary adsorption. The complex can be separated in *n*-butanol or chloroform solvent systems containing a high percentage of organic acids by prolonged chromatography. The bioautograms correspond to the composition of spots obtained by chemical detection. Detection by vanillin–sulphuric acid and by the Sakaguchi method was modified. The three main components of primycin (A_1, A_3 and B) are Sakaguchi-positive and their proportions are in good agreement with those of homogeneous permethylated products prepared from primycin sulphate for structural studies. Quantitative data for the base crystallized from methanol or dimethylformamide are different from those for the genuine sulphate. The genuine main components were separated for the first time by repeated preparative thin-layer chromatography. Their biological activities were different, the alkali labile A_3 component being the most active.

INTRODUCTION

The antibiotic primycin is produced by a strain isolated almost three decades ago[1–3]. The strain was first described as *Streptomyces primycini*, but was later identified as *Micromonospora galeriensis*[4]. Primycin is highly active against Gram-positive bacteria, mycobacteria, including polyresistant strains of the latter[2], protozoa and fungi[5]. No resistance developed to primycin has been recorded. The structure of the major component of primycin has been elucidated[6–9] and is shown below. It proved

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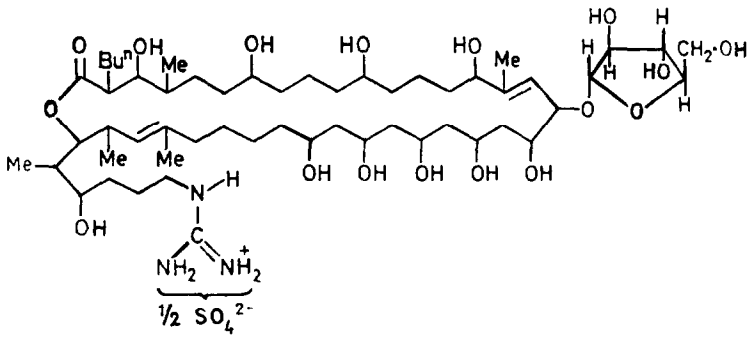

to be a polyhydroxylated, 36-membered, non-polyenic lactone with attached arabinose and guanidine moieties, a feature unprecedented among macrolide antibiotics.

Problems concerning the purity[3] and identity[10] of primycin are almost as old as the antibiotic itself. Initially various separation experiments led to the stabilization of biological, physical, chemical and analytical characteristics. Nevertheless, heterogeneity was indicated by the phenomenon of secondary adsorption[11], by the shape of solubility isotherms[3] and by the presence of five molecular ion peaks in the mass spectrum of an N-acetyl permethylated methyl ester of primycin amino acid[7]. The exact molecular formula ($C_{55}H_{104}N_3O_{17} \cdot 0.5SO_4$) could only be established with a knowledge of the full structure. It can be assumed that genuine primycin is a complex of homologous compounds, the separation of which was the aim of this investigation.

EXPERIMENTAL

Products

Four samples of different origin were investigated, namely:

(1) a sulphate of m.p. 192–195°C (decomp.) used for structural studies[6–9], recrystallized twice from methanol, biological activity against *B. subtilis* 0.03 μg/ml;

(2) a sample crystallized[5] from 83% dimethylformamide at 37°C over a period of 20 days, m.p. 202–206°C (decomp.), biological activity 0.03 μg/ml;

(3) primycin sulphate industry produced[12]: Nr. 781201, m.p. 178–180°C (decomp.), biological activity 0.05 μg/ml;

(4) a base prepared from the above material[13] by passing through a Dowex 1 × 2 (OH) ion-exchange resin and crystallization from methanol, m.p. 182–184°C, biological activity 0.03 μg/ml.

Apparatus

A Varioperpex LKB 12000 peristaltic pump from LKB (Bromma, Sweden) and a Shimadzu high-speed thin-layer chromatographic (TLC) scanner (CS-920) from Desaga (Heidelberg, F.R.G.) were used.

Plates

The TLC plates used were DC-Plastikfolien Kieselgel 60 F_{254}, 0.2 mm (Merck, Darmstadt, F.R.G.), DC-Alufolien Kieselgel 60 (without indicator), 0.2 mm (Merck) and PSC-Fertigplatten, Kieselgel 60 F_{254}, 2 mm (Merck).

Solvent systems

The following solvent systems were used:

(I) chloroform–methanol–acetone–water (28:42:14:16), (II) chloroform–methanol–water (45:45:10), (IIIa) chloroform–methanol–formic acid–water (50:35:14:1), metastable, (IIIb) chloroform–methanol–acetic acid–water [(45:30:15:20), lower phase with 1% methanol]: selectivity is like IIIa, but stable till one week, (IV) *n*-propanol–acetic acid–water (70:10:20), (V) *n*-Butanol–acetic acid–water (40:10:50, upper phase) and (VI) *n*-butanol–methanol–acetic acid–water (75:2:8:15).

Chromatographic procedure

Primycin sulphate was dissolved in *n*-butanol–ethanol–water (25:25:50) (BEV), whereas the more soluble base was dissolved in warm (50°C) methanol with shaking and then dried with a stream of cold air on the starting line of the plates. Development was carried out in tanks lined with filter-paper and saturated overnight with the solvent. Development was repeated after the plates had been dried for at least 30 min in a stream of air until odourless. Occasionally overdevelopment for a period double the normal development time was applied once, without intermediate drying.

Qualitative TLC. With the aid of a capillary tube, 10–15-μl samples of 0.5% primycin solutions were applied in the form of 10-mm streaks. The length of development, *e.g.* on a 10 × 10 cm plate, was 2 × 8 cm.

Two-dimensional TLC. Using square plates, the reference substances were applied on the edge of the plate separated by the front line, whereas the sample to be tested was dried on the corner of the plate. Development was carried out in the extended mode in both directions using the same or different solvent systems.

Quantitative TLC. A 10-μl volume (50 μg) of a 0.5% solution was dried on the plate as a 6 mm band, or 10 μl (100 μg) of a 1.0% solution as a 10 mm band. The length of development on, e.g., a 20 × 20 cm plate was 2 × 16 cm. After the first and second developments the carefully dried chromatograms were treated with vanillin–sulphuric acid and after standing for 1 day were scanned with a Shimadzu densitometer at 590 nm. R_F values were calculated from "distance" readings and percentage proportions of the chromatogram from "area" readings.

Preparative separation. On to PSC glass plates with 2-mm layers 2 ml and on to 0.2 mm layers on plastic sheets with 0.2-mm layers 0.2–0.3 ml of a 1% primycin base solution in methanol were applied as bands of length 18 cm using a peristaltic pump set to constant delivery. Plates with the thicker layer were developed at least three times and those with the thinner layer twice. For detection the dried and odourless PSC glass plates were immersed to a depth of 3 cm in pre-cooled Sakaguchi reagents (I and II), whereupon the bands turned red at room temperature. With the DC plastic sheets, 1-cm wide strips were cut from both sides and developed with vanillin–sulphuric acid at 105°C.

Zones corresponding to those revealed on the edges were scraped off and eluted in a micro-percolator with 10% acetic acid–methanol until the vanillin–sulphuric acid test became negative. The residue obtained after evaporating the eluate *in vacuo* was dissolved in the upper phase of solvent system V. After shaking with water, primycin concentrated in the upper phase, whereas contamination by solubilized adsorbent was removed in the lower phase. On evaporating the upper phase 90% of the pri-

mycin transferred into the layer could be recovered. Components that were first separated on thick layers and subsequently by multiple chromatography on thin layers were obtained on a miligram scale.

Detection reagents and methods

Bioautography. The medium consisted of beef extract (Difco) 3 g, Bactr. peptone (Difco) 5 g, NaCl 5 g, Na_2HPO_4 10 g, KH_2PO_4 1 g, Bactr. agar (Difco) 18 g and distilled water to 1000 ml. Sterilization was carried out at 121°C and the pH was adjusted to 8.0. At 53°C the medium was spread out on 18 × 25 cm sterilized glass trays (130 ml on each) and then mixed with a suspension (1:2000) of *Bacillus subtilis* spores (ATCC 6633). From the chromatograms run on plastic sheets or aluminium foil, 5 mm strips were cut out, placed on top of agar plates and incubated at 37°C for 18–24 h.

Vanillin–sulphuric acid. To a solution of analytical-reagent grade vanillin (0.25 g) in a mixture of *n*-propanol and carbon tetrachloride (1:1) cooled at −15°C, concentrated sulphuric acid (2.5 ml) was added in portions with stirring. The reagent is colourless and can be stored in a deep-freeze for 2 weeks.

The carefully dried layers were submerged in the reagent by tilting a tank containing the reagent, the excess was allowed to drip off, the solvent was allowed to evaporate with the plates in a horizontal position and drying was completed with a stream of air. These operations were repeated and finally the spots were developed by heating at 105°C for 2–3 min. Components of primycin appear as grey-violet spots against a pink background. The chromatograms can be stored for several years. The sensitivity of the reagent is 0.1 μg per spot.

Sakaguchi reaction[14]. (a) Solution I. Sodium hydroxide (5 g) is dissolved in 10 ml of distilled water with cooling, diluted with 90 ml of methanol, filtered and 0.1 g of 8-hydroxyquinoline is dissolved in it. When stored in a refrigerator the reagent is stable for 1 week.

(b) Solution II. In pre-cooled 10% ethanol (100 ml) is suspended N-bromosuccinimide (NBS) (0.5 g), which dissolves with the formation of bromine. When stored in a refrigerator the reagent is stable for 5 days.

(c) Reaction. The layers immersed in cooled solution I are dried in a stream of air until lemon yellow, then submerged in solution II, whereupon bright red spots of primycin appear. The dried chromatograms can be stored for several weeks. The sensitivity of the reagent is 1 μg per spot.

RESULTS AND DISCUSSION

TLC methods and solvents effective with macrolide antibiotics[15] are not applicable to the separation of primycins. Primycin sulphate is hardly soluble even in *n*-butanol–ethanol–water (25:25:50) at 50°C, whereas the more soluble base can dissolved in methanol, but only to a maximum extent of 0.5–1%. Depending on the circumstances, on one thin layer, 50–200-μg spots can be applied, preferably as streaks.

Of the reagents generally used to reveal TLC spots, *e.g.*, antimony trichloride, Molisch reagent, thymol–, anisaldehyde– and vanillin–sulphuric acid, Dragendorf reagent chlorotoluidine and Sakaguchi reagent, the Sakaguchi method as modified

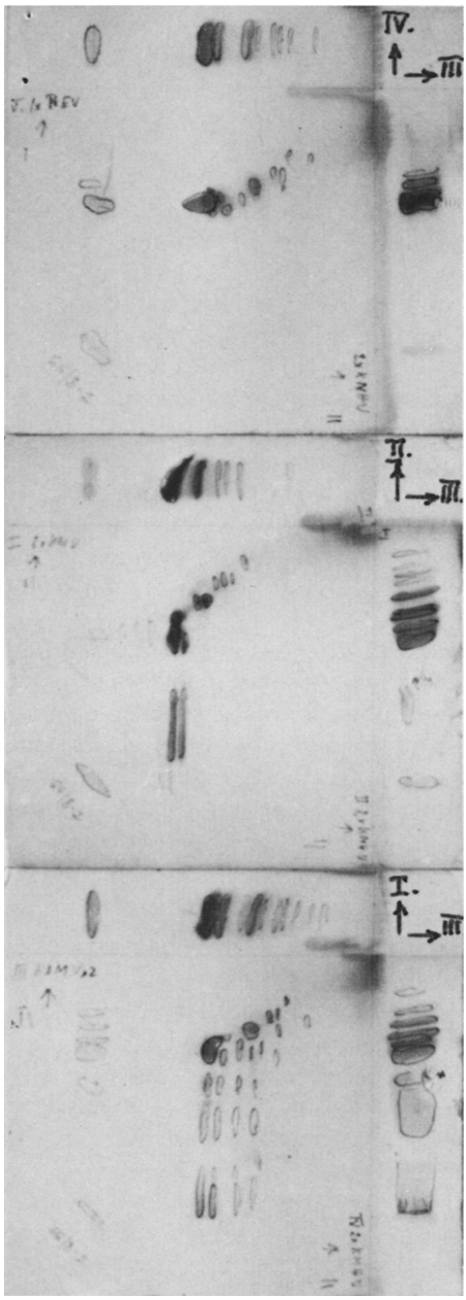

Fig. 1. Two-dimensional thin-layer chromatograms demonstrating the secondary adsorption of primycins Solvent systems: vertically, I, II and IV as indicated; horizontally, IIIa on all three layers.

by us[14] (1 μg) and the vanillin–sulphuric acid reagent (0.1 μg) gave characteristic and sensitive colour reactions with primycin. Instead of spraying, the reagents were preferably applied by immersion and subsequent drying.

More than 230 solvent systems have been tried for the separation of primycins, on both untreated and formamide-impregnated TLC plates. The reproducibility is better on the former. On ready-made Kieselgel 60 layers (Merck) primycin components give well defined spots. One or two phase systems containing *n*-butanol have a greater elution capacity but lower selectivity than chloroform–methanol systems. The phenomenon of secondary adsorption known in the paper chromatography of primycin[10] was also observed in TLC. This is manifested by mutual association of the components, causing their partial or total retention followed by their separation on repeated elution. In order to demonstrate this, the primycin complex was subjected to two-dimensional TLC in two solvent systems using repeated extended elution (Fig. 1). In one dimension with increasing polarity of the solvent system (vertically from bottom to top I, II and IV) the complex is separated to four, two and one adsorbed spots. In the second dimension (horizontally), when eluted with the most selective system (III), the same spots disproportionate. An residual primycin spot can also be observed on the "second start spot". The poor separation can be ascribed to the structural features of primycins, inasmuch as NH and OH groups in the molecules establish stable hydrogen bridges[3].

The role of different solvent components (chloroform, acetone, alcohols, water, formamide and organic acids) in the separation of primycin components was studied in detail. In has long been known that tailing of the spots, characteristic also of other macrolides[15], can be eliminated by the addition of a small amount of acetic acid, formic acid or ammonia. As primycin is very sensitive to alkalis, high percentage of an organic acid in the eluant proved to be essential. The best solvent for the complex

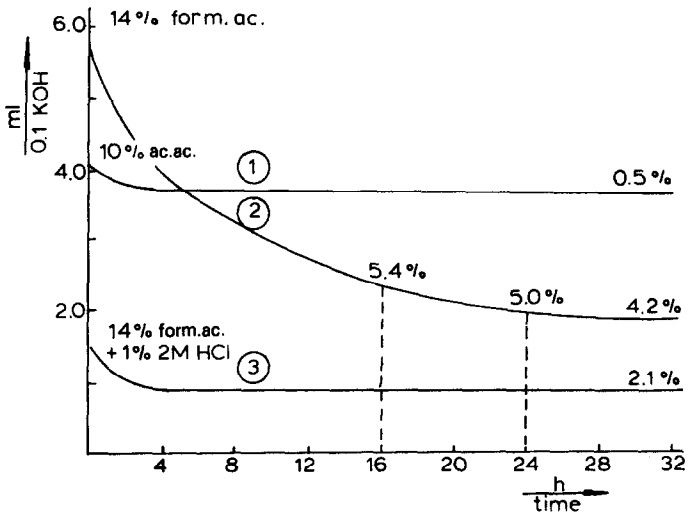

Fig. 2. Stability isotherms as a function of time. Samples of 0.2 ml were diluted with dilute (1:1) ethanol (15 ml) and titrated with 0.1 *M* potassium hydroxide solution in ethanol. Solvent systems: (1) V; (2) IIIa; (3) IIIa + 1% 2 *M* HCl. Form. ac. = formic acid; ac. ac. = acetic acid.

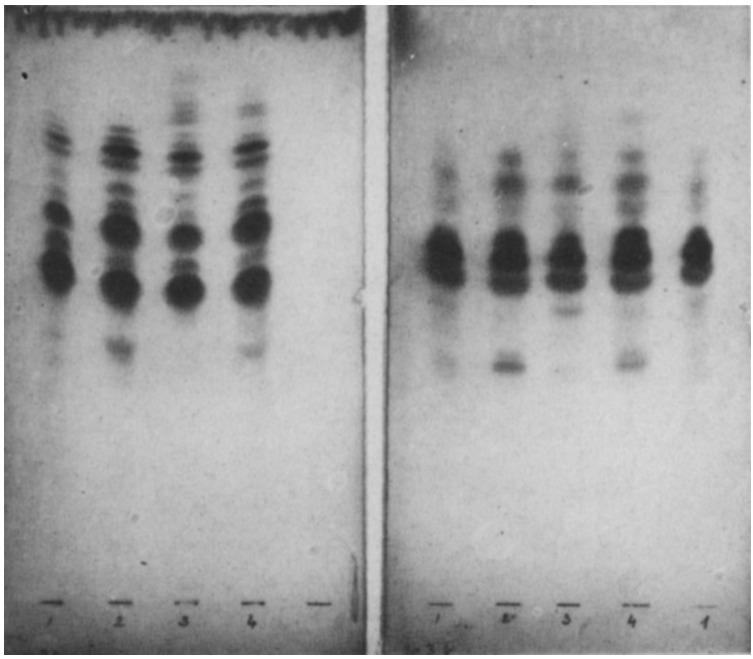

Fig. 3. Thin-layer chromatograms of various primycin preparations: (1) genuine sulphate; (2) crystallized from dimethylformamide; (3) genuine sulphate (Chinoin); (4) sulphate-free base. Solvent system: left, IIIa; right, (V), extended development twice.

proved to be anhydrous formic acid, in which it is soluble to the extent of more than 20% by the formation of formates not yet characterized. The complex is less soluble in acetic acid, but no esterification takes place (solvent system IIIb). The content of free acid as a function of time with the selected solvent systems, *i.e.* of the acetic acid-containing Partridge mixture (V) and of the mixture specially developed by us for primycin (IIIa), was followed by alkalimetric titration (Fig. 2). The upper phase of system V is stable for several days, apart from a small change in the first 3 h. On the other hand, in system IIIa esterification of methanol by formic acid takes place, relatively fast in the first 16 h, then slower, when the curve asymptotically approaches the abscissa. This esterification can be accelerated by 0.02 *M* hydrochloric acid. The resulting solvent system contains only a small amount of free acid and is characterized by a reduction in the R_F value of primycin. It is advantageous to leave mixture IIIa standing for 16 h and to use it until 24 h after its preparation, by which time its content of formic acid has decreased from 14% to about 5%. In this solvent system esterification or decomposition of the genuine primycin components by concurrent reactions has not been observed.

Another condition for the selective separation of primycin homologues is that the R_F values of fractions A_1–A_3 should be around 0.5, which can be achieved by repeated chromatography and careful drying in between. With solvent systems having an eluotropic effect, or when chromatography is carried out only once for double the length of time, the selectivity deteriorates.

A visual comparison of the chromatograms of primycin samples of different

TABLE I

QUANTITATIVE DATA (%) FOR THE TLC-SEPARATED MAIN COMPONENTS OF THE PRIMYCIN COMPLEX COMPARED WITH THE YIELDS OF PERMETHYLATED PRIMYCIN

Permethylated primycins[9]	*Yield (%)*	*TLC-separated main components as determined by densitometry (%)*							
		Symbol	*Relative R_F*		*Genuine sulphate*	*± Δ (%)*	*Crystalline*[5]	*Genuine sulphate*[12]	*Base*[13]
			III	*V*					
Equiv. wt. 1467, p*K* 11.9	12.9	B	1.47	1.20	12.5	−4	17.6	20.3	14.2
Trimethylurea	18.3	A_3 A_2	1.20 1.10	0.95 1.07	16.8	−1.5	30.2	20.2	27.6
Trimethylguanidine	49.9	A_1	1.0	1.0	51.7	+1.8	33.3	40.2	30.3
Minor components [100−(A+B)%]	18.8	–	–	–	19.0	+0.2	18.9	19.3	27.9

origin revealed that there are no significant differences in their qualitative composition. The chromatogram in Fig. 3 shows three major components (A_1, A_3 and B), all giving a positive reaction with vanillin–sulphuric acid or the Sakaguchi reagents, corresponding to the biologically active major components, and also, depending on the sensitivity of detection, 10–12 minor components. The bioautograms correspond to the composition of the chemically detected chromatograms. With the Partridge mixture (V) the order of spots changed (A_2, A_1, A_3 and B).

In order to assess the quantitative relationship between the components, the more selective solvent system IIIa, containing chloroform, was applied and the optimal conditions were strictly adhered to. The following samples were chromatographed (Fig. 3):(1) primycin sulphate used for structural elucidation[6–9], (2) a sample crystallized from 83% dimethylformamide[5], (3) industrially produced primycin sulphate[12] and (4) primycin base obtained from the former by ion exchanger[13]. One day after chromatography and detection the plates were evaluated with a Shimadzu microcomputer-based densitometer. R_F values relative to spot A_1 (1.0) were calculated from "distance" readings. The percentage proportions of the components were calculated from "area" readings and based on the total intensity (100%) of the spots.

Quantitative data for the major components A_1, A_2, A_3 and B, as TLC-separated in mixture IIIa, by six parallel determinations on samples 1 and 3, representing genuine primycin sulphate (Table I), are in good agreement with the yields of homogeneous trimethylguanidine (49.9%), trimethylurea (19.3%) and of a yet unidentified product (equivalent weight *ca.* 1467, 12.9%) prepared in the course of structural elucidation[9]. The difference between the proportions of the permethylation product (100%) and of the total of its main components (18.8%) agreed with analogous data for the analysed minor components. Based on the agreement of the prepared per-

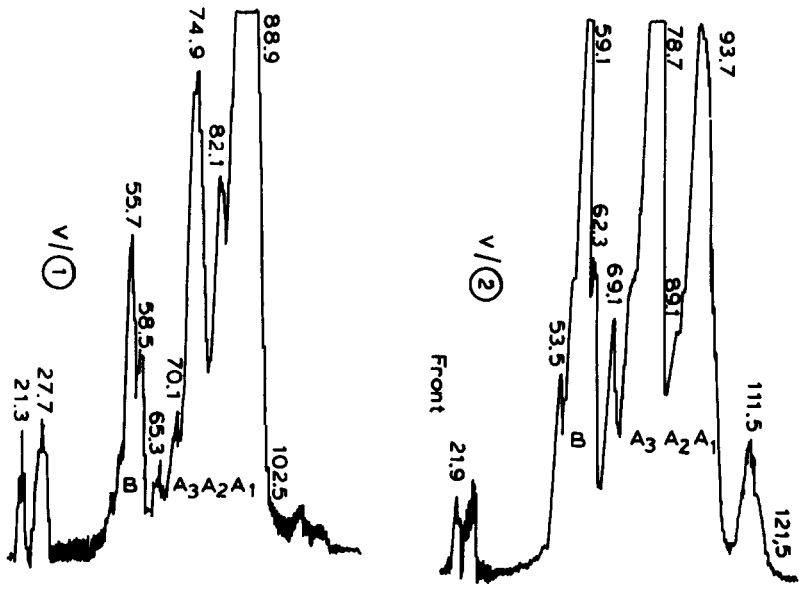

Fig. 4. Recordings of chromatograms in IIIa of primycin complexes of two types: V/1, genuine sulphate[6–9] crystallized from methanol; V/2, sample crystallized from 85% dimethylformamide[5].

methylated[9] and quantitative analysed genuine primycin data, the identity of the main components can be confirmed. We observed, however, that three main components of genuine primycin sulphate are Sakaguchi-positive and hence are derivatives of guanidine. A trimethylurea derivative cannot give a positive Sakaguchi reaction and its formation can be explained by the following mechanism[9]: "the trimethylated urea derivative is obviously formed by partial hydrolysis of the guanidine unit by mildly basic silver oxide during the course of methylation". We have observed the extreme alkali lability of the genuine A_3 component of as yet unknown structure, inasmuch as it decomposed even in an indifferent solvent within 10 min under the action of an anion-exchange resin of pH 10.

The data in Table I also reveal that the proportion of component A_1 was 40–50% in genuine primycin sulphate (samples 1 and 3) compared with 30–35% in the samples recrystallized from dimethylformamide (2) and in the base recrystallized from methanol (4), having the same composition. In the same samples, the proportions of the less soluble components A_2 and A_3 were 17–20% compared with 28–30%. This difference in component ratios with the composition remaining constant can be explained by assuming that in the mildly alkaline medium at 37°C less soluble components (A_1, A_3 and B) are enriched, while more of the more polar main component remains in the mother liquor. The layer chromatograms in Fig. 4 represent two types of primycin complexes, samples one and two having the same biological activity (0.03 μg/ml). The product crystallized from the highly polar dimethylformamide (V/2, m.p. 202–206°C) is less soluble and higher melting than that crystallized several times from methanol (V/1, m.p. 192–195°C). Primycin is a natural complex, both of its types forming stable molecular associates by hydrogen bonds[3] and crystallizing together.

In this work the major components of primycin homologues were separated by repeated thick- and thin-layer chromatography. Elution was carried out in highly polar solvent systems containing a high percentage of organic acids (IIIa and V) and, owing to the small R_F values, extended development was applied two or three times. Drying of the thick plates was lengthy (1 day). Detection of the zones was carried

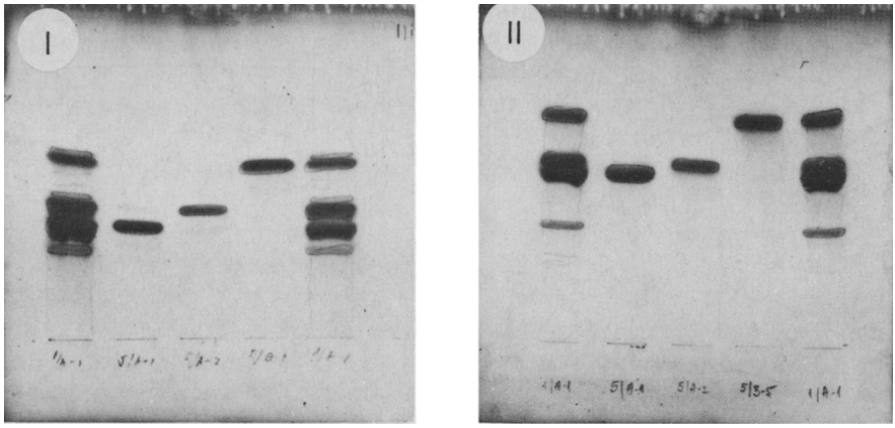

Fig. 5. Isolated main components of primycin: in the direction of development, A_1, A_3 and B. Solvent systems: plate I, IIIa; plate II, V.

out carefully on the edges of the glass plates by means of the Sakaguchi reaction. With the thin-layer sheets the zones were localized by cutting out three parallel strips and detected with vanillin–sulphuric acid. The material scraped off, containing more than 100 times the weight of the material to be separated as ballast, was eluted with methanolic acetic acid. The layer ballast material was separated from primycin fractions by distribution between the phases of Partridge mixture (V). Primycins were concentrated in the upper layer and layer material in the lower phase. By this method 90% of the original primycin could be recovered.

When the fractions isolated by layer chromatography were tested for their biological activity against *Bacillus subtilis* using successive dilutions, the following data were obtained (Fig. 5): A_1 0.05, A_3 0.03 and B 0.2 μg/ml. The double component A_1, A_3 proved to be more active (0.02 μg/ml).

Studies of the synergism of genuine primycins, their separation by column chromatography and their characterization will be reported elsewhere.

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