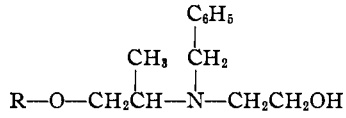
TABLE III

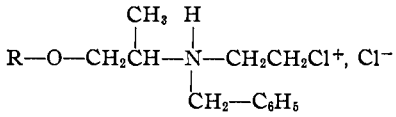
N-BENZYL-N-[2-(FLUORO- AND TRIFLUOROMETHYL-PHENOXY)-ISOPROPYL]-2-AMINOETHANOLS



R	Yield, %	B.p., °C.	Mm.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
o-Fluorophenyl	84	178–179	0.5	$C_{18}H_{22}O_2NF$	71.3	71.3	7.2	7.1	4.6	4.8
m-Fluorophenyl	79	174–175	.4	$C_{18}H_{22}O_2NF$	71.3	71.4	7.2	7.5	4.6	4.9
p-Fluorophenyl	79	180–181	.4	$C_{18}H_{22}O_2NF$	71.3	71.7	7.2	7.0	4.6	4.7
o-Trifluoromethylphenyl	45	168–169	.4	$C_{19}H_{22}O_2NF_3$	64.6	65.1	6.3	6.3	4.0	4.0
m-Trifluoromethylphenyl	82	173–174	.5	$C_{19}H_{22}O_2NF_3$	64.6	64.7	6.3	6.0	4.0	4.2

TABLE IV

N-BENZYL-N-(2-CHLOROETHYL)-1-(FLUORO- AND TRIFLUOROMETHYL-PHENOXY)-ISOPROPYLAMINE HYDROCHLORIDES,



R	Yield, %	M.p.,[a] °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found	Adenergic blocking action intravenous dose in mg./kg.
o-Fluorophenyl	69	158–159	$C_{18}H_{22}ONFCl_2$	60.3	60.3	6.2	5.9	3.9	4.0	5
m-Fluorophenyl	62	140–141	$C_{18}H_{22}ONFCl_2$	60.3	60.2	6.2	6.0	3.9	3.8	5
p-Fluorophenyl	69	136–137	$C_{18}H_{22}ONFCl_2$	60.3	60.1	6.2	6.3	3.9	4.1	1.0–2.5
o-Trifluoromethylphenyl[b]	52	141–142	$C_{19}H_{22}ONF_3Cl_2$	55.9	56.0	5.4	5.2	3.4	3.7	5
m-Trifluoromethylphenyl	74	146–147	$C_{19}H_{22}ONF_3Cl_2$	55.9	56.1	5.4	5.5	3.4	3.6	10

[a] Repeated recrystallization from absolute ethanol–ether required to give products showing melting points indicated. [b] Reaction carried out in chloroform solution.

phenoxy)-isopropyl]-2-aminoethanol boiling at 178–179° (0.5 mm.). Table III contains data on this series of compounds.

N-Benzyl-N-(2-chloroethyl)-1-(fluoro- and trifluoromethyl-phenoxy)-isopropylamine Hydrochlorides (I).—A typical procedure was: N-benzyl-N-[2-(2-fluorophenoxy)-isopropyl]-2-aminoethanol (9.2 g., 0.0304 mole) was added gradually to 25 ml. of redistilled thionyl chloride. After the initial vigorous reaction had subsided, the mixture was refluxed for 1 hour and the excess thionyl chloride was removed under reduced pressure. Several recrystallizations of the residue from absolute ethanol–ether gave 7.5 g. (69%) of N-benzyl-N-(2-chloroethyl)-1-(2-fluorophenoxy)-isopropylamine melting at 158–159°. Data on all of the final products are listed in Table IV.

Acknowledgment.—These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Kansas.

LAWRENCE, KANSAS RECEIVED MARCH 26, 1951

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

The Isolation and General Properties of Terramycin and Terramycin Salts

BY PETER P. REGNA, I. A. SOLOMONS, KOTARO MURAI, ALBERT E. TIMRECK, KARL J. BRUNINGS AND W. A. LAZIER

Methods for the isolation and purification of the broad spectrum antibiotic, terramycin, have been described. The high purity of the natural product obtained by these methods has been demonstrated by solubility measurements. The hydrated amphoteric base and the hydrohalides of terramycin have been prepared in a pure crystalline state and characterized with respect to molecular weight, composition, crystalline form, optical properties and solubility. The formation of water-insoluble mixed salts of terramycin has been described. The dissociation constants of the acid and base functions of terramycin have been determined by potentiometric titration. It has been observed that certain metal salts, such as calcium chloride, increase the acid strength of terramycin and the titration curves of these systems permit an interpretation as to the composition of the complexes formed.

Terramycin, a new broad spectrum antibiotic elaborated by *Streptomyces rimosus*, has been described by Finlay, *et al.*[1,2] Preliminary information on isolation and data on some of the chemical and physical properties of this new natural product have been given by Regna and Solomons.[3] In the present paper, other methods of isolation and purification are described and more detailed information is given concerning the general properties of terramycin and some of its salts.

Terramycin is a pale yellow compound having a composition best represented by the formula $C_{22}H_{24-26}N_2O_9$. It crystallizes readily from water as the dihydrate which loses its water of crystallization on heating *in vacuo* at 100°. The anhydrous compound melts at 184.5–185.5° with decomposition. Anhydrous terramycin is a relatively stable antibiotic, losing only about 20% of its activity on

(1) A. C. Finlay, G. L. Hobby, S. Y. P'an, P. P. Regna, J. B. Routien, D. B. Seeley, G. M. Shull, B. A. Sobin, I. A. Solomons, J. W. Vinson and J. H. Kane, *Science*, **111**, 85 (1950).

(2) B. A. Sobin, A. C. Finlay and J. H. Kane, U. S. Patent 2,516,080 (July 18, 1950).

(3) P. P. Regna and I. A. Solomons, *Ann. N. Y. Acad. Sci.*, **53**, 229 (1950).

heating *in vacuo* for 140 hours at 105°. The optical rotation is sensitive to *p*H and strongly affected by the presence of boric acid.

Terramycin is amphoteric and forms well-defined salts with mineral acids and bases. Calculations, based on titration data (Fig. 1) of terramycin hydrochloride in aqueous solution at 28°, gave pK'_a 3.49, 7.55 and 9.24. Approximately the same values are obtained from titration data for solutions in methanol–water mixtures in which terramycin is more soluble than in water. Among the acid salts of terramycin, the hydrochloride and the hydrobromide are the best characterized thus far. These are bright yellow, beautifully crystalline compounds. The hydrohalides dissolve readily in water, but unless excess acid is added to a *p*H below 1.5 the crystalline free base separates on standing. The ultraviolet absorption spectra of terramycin and terramycin hydrochloride in methanol solutions are very similar (Fig. 2). In alkaline solutions, the absorption maximum shifts toward the visible and the solutions are noticeably more colored than neutral or acid solutions. It is also observed that the infrared spectra of terramycin dihydrate (Fig. 3) and terramycin hydrochloride (Fig. 4) in nujol mulls are not greatly different, although the absorption bands of terramycin dihydrate are somewhat better defined.

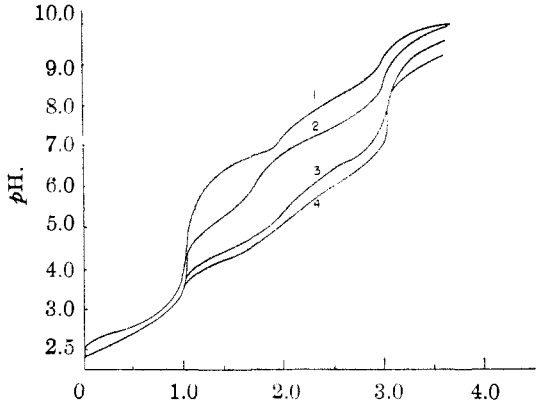


Fig. 1.—Titration curves of terramycin hydrochloride: (1) alone; (2) with 1/4 mole calcium chloride; (3) with 1/2 mole calcium chloride; (4) with 1–5 moles calcium chloride.

The di-sodium and di-potassium salts of terramycin are yellow crystalline hydrates which are readily soluble in water and insoluble in alcohol. Freshly prepared aqueous solutions are bright yellow, turning darker on standing. They lose very little biological potency over a period of four days when held at temperatures below 15°. The calcium and magnesium salts of terramycin are only slightly soluble in water. Terramycin readily forms mixed salts with a number of pairs of bivalent metal ions. The barium–calcium and barium–magnesium salts are very insoluble in water and precipitate readily from aqueous solutions at *p*H 8.5 to 9.5. Reproducible formulas for the mixed salts have thus far not been obtained.

Terramycin has a marked tendency to form complexes with certain inorganic salts. Evidence for this characteristic are (1) the increase in solubility of the base in aqueous and alcoholic solutions in the presence of calcium chloride, (2) the enhanced acidity induced by alkaline earth and heavy metal salts in aqueous solutions of terramycin, and (3) the marked change in rotation in the presence of these salts. A titration curve of an aqueous solution of terramycin containing one mole of calcium chloride is shown in Fig. 1, and it is observed that the pK'_a values of the acidic groups are shifted to the acid side by almost 2 *p*K units. It can be seen that the marked acidifying effect of calcium chloride does not increase further after one mole of calcium chloride has been added, and the maximum effect is already observed upon addition of one-half mole. In all probability, several complexes exist in these solutions, such as (terramycin)$_4$CaCl$_2$, (terramycin)$_2$ CaCl$_2$, and, less likely, terramycin·CaCl$_2$. Under one set of conditions, a crystalline complex has been isolated having a composition in fairly good agreement with the formula (terramycin)$_4$·CaCl$_2$.[4]

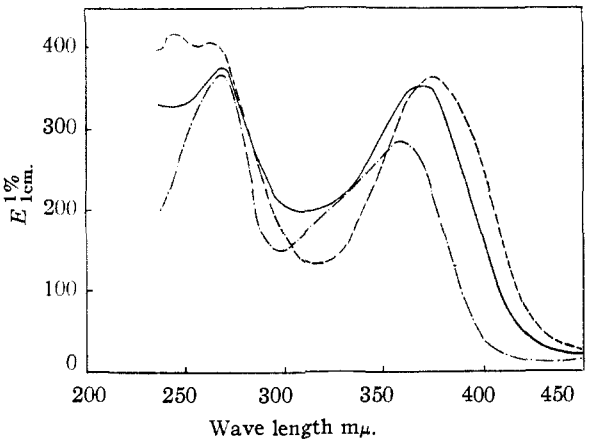


Fig. 2.—Absorption spectrum of terramycin: —, terramycin in methanol $E^{1\%}_{1cm.}$ = 361 (270 mμ), 343 (370 mμ); —.—, terramycin (0.001%) in 0.01 *N* methanolic hydrochloric acid $E^{1\%}_{1cm.}$ = 353 (270 mμ), 267 (359 mμ); – – – –, terramycin in 0.01 *N* sodium hydroxide methanol; $E^{1\%}_{1cm.}$ = 415 (245 mμ), 405 (264 mμ), 352 (375 mμ).

The solubility properties of terramycin and its salts set the pattern for isolation and purification procedures. Terramycin may be extracted with butanol from alkaline solutions, or it may be precipitated from fermentation broth by certain pairs of multi-valent metal ions. A convenient method of isolation involves (1) precipitation of the mixed barium–magnesium salt from the filtered culture broth, (2) liberation of the antibiotic from the impure mixed salt with excess sulfuric acid, followed by filtration, (3) precipitation of crude terramycin by neutralization, (4) solubilization in alcohol containing calcium chloride, and (5) crystallization of the hydrochloride by addition of excess hydrochlo-

(4) We have observed that the dissociation constants of certain 3-hydroxy-γ-pyrones, such as kojic acid and maltol, are increased by calcium chloride to about the same degree as in the case of terramycin. The titration curves of kojic acid in 50% methanol–water solutions of calcium chloride indicate formulas of (kojic acid)$_3$CaCl$_2$ and kojic acid ·CaCl$_2$. A number of other compounds, such as polyphenols and phenol carboxylic acids, have been studied for this effect in an attempt to determine the nature of this type of complex formation. A detailed paper on this subject is now in preparation.

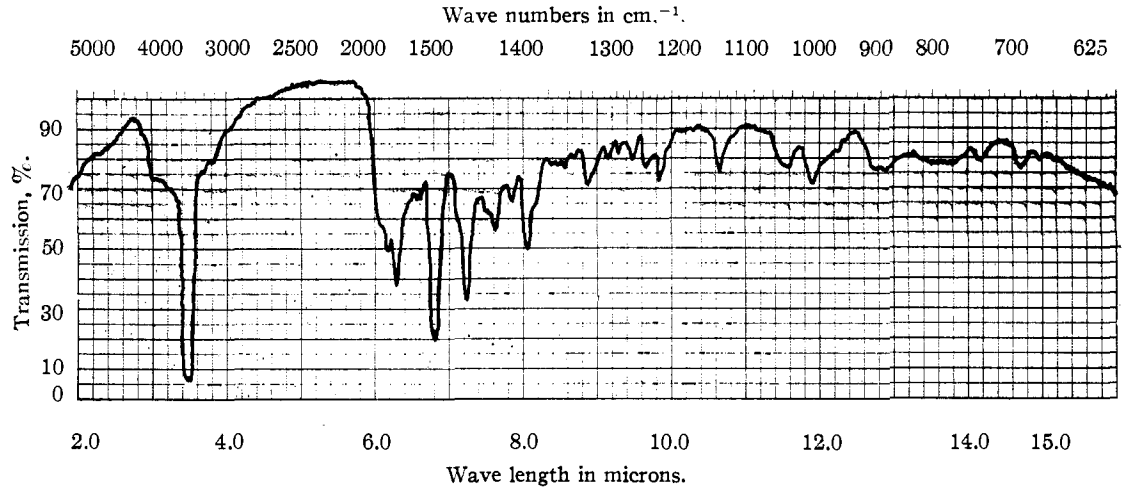


Fig. 3.—Terramycin dihydrate in Nujol mull.

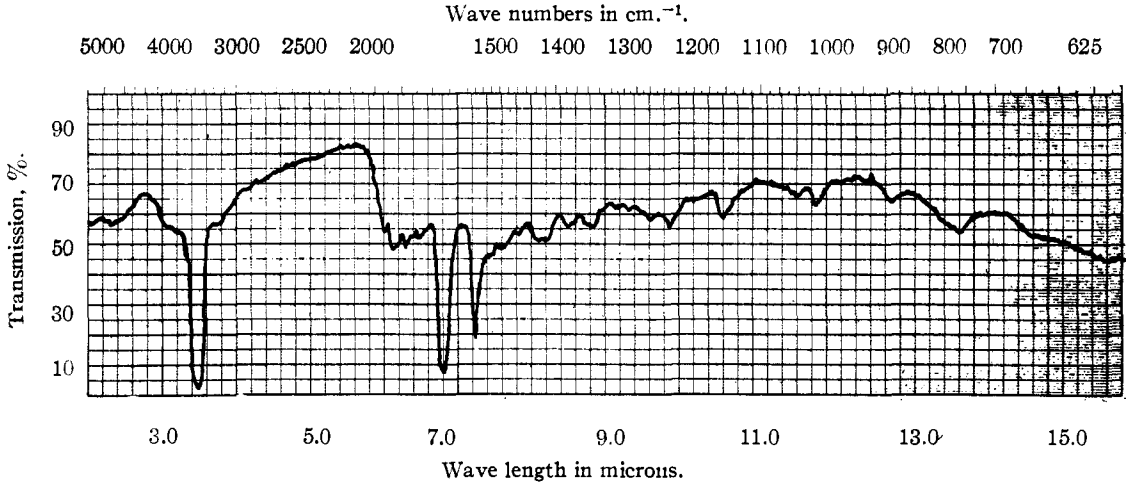


Fig. 4.—Terramycin hydrochloride in Nujol mull.

ric acid to the methanol solution. Terramycin hydrochloride can be purified further by conversion to the base and repeating steps (4) and (5).

Terramycin and its hydrohalide salts are readily obtained in a high state of purity by the method outlined above. The purity of terramycin hydrochloride obtained in Step 5 above and a more highly purified hydrochloride is illustrated by the solubility curves shown in Fig. 5. The points on these curves represent equilibrium solubility which requires approximately 18–20 hours for attainment. The stability of terramycin hydrochloride in methanol at 35° was found to be satisfactory for these conditions. The points on the solubility curve are precise within at least ±0.5%, and thus the purity of the better sample shown is not less than 99.5%. The homogeneity of terramycin has also been established by countercurrent distribution between butanol and a *p*H 2.5 buffer, and by paper chromatography.[3]

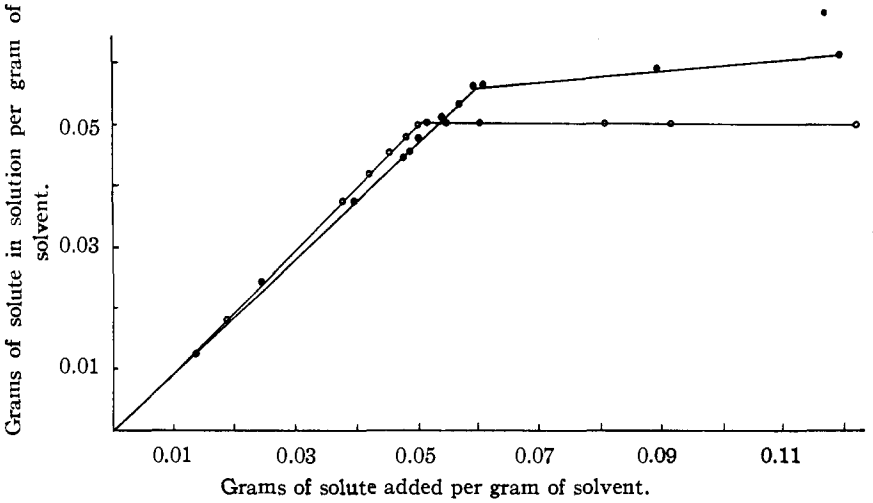


Fig. 5.—Solubility curves: terramycin hydrochloride in methanol. at 35°: O, purified; ●, crude.

Experimental

Isolation of Crude Terramycin.—Terramycin broth (990 γ/ml.) was acidified to *p*H 2.5 with concentrated hydrochloric acid and stirred for 2 hours with 40 g. per liter of Super-cel. After filtering on a büchner funnel, 11.2 liters of the clarified broth, containing 11.08 g. of terramycin, was treated during one-half hour with a solution of 171 g. of barium chloride dihydrate and 22.8 g. of magnesium chloride hexahydrate in 475 ml. of water. The solution was then adjusted to *p*H 8.5 by the slow addition of 10% sodium hydroxide. Two hundred grams of Super-cel was added and the precipitate filtered and washed with one liter of water. An assay on the combined filtrates showed that about 7% of the terramycin had not been precipitated. The washed filter cake was suspended in one liter of water, and the mixture was acidified to *p*H 1.5 with 50% sulfuric acid. After stirring vigorously for 30 minutes, the Super-cel and the precipitated barium sulfate were separated by filtration. The precipitate was re-extracted with dilute sulfuric acid, and the combined filtrates were adjusted to *p*H 3.0 with 10% sodium hydroxide and seeded with crystalline terramycin. The solution was adjusted to *p*H 5.0 and after stirring for 20 minutes was readjusted to *p*H 7.0 and stirred for an additional hour. The crude terramycin was filtered on a büchner funnel and dried *in vacuo* at 50°; yield 13.4 g.; microbiological potency, 655 U./mg. The microbiological assays were carried out by a turbidimetric method[5] using *Klebsiella pneumoniae* PCI 602 and crystalline anhydrous terramycin as a standard.

Terramycin Hydrochloride.—The dried mixture (13.4 g.) containing 8.8 g. of terramycin was finely ground and suspended in 85 ml. of methanol. There was added with stirring 20 ml. of a saturated solution of calcium chloride in methanol. After one-half hour, one gram of Darco G-60 was added and the stirring continued for 20 minutes. The solution was filtered, and the Super-cel carbon cake was washed with 25 ml. of a saturated methanolic-calcium chloride solution, then with 25 ml. of 1.5 *N* methanolic hydrochloric acid and finally with 25 ml. of anhydrous methanol. To the combined filtrate and washes, 10.3 ml. of concentrated hydrochloric acid was added slowly (10 minutes), and the solution seeded with terramycin hydrochloride. Slow stirring was continued for one hour, after which time the crystals were collected on a büchner funnel and washed with 25 ml. of 1.5 *N* methanolic hydrochloric acid, followed by 25 ml. of methanol. The terramycin hydrochloride was air-dried overnight at 50°. The yield was 7.1 g. having a potency of 740 γ/mg.

The impure crystalline material (7.1 g.) was suspended in 20 ml. of methanol and treated with 1.6 g. of triethylamine in order to convert the hydrochloride to the free base. Ten ml. of methanol, containing 3.5 g. of calcium chloride, was added to produce a clear solution, and to this there was added 1.2 g. of Darco G-60. After filtering, the filtrate was treated with 11.5 ml. of concentrated hydrochloric acid and after stirring for one-half hour the terramycin hydrochloride was collected on a filter and dried at 50° *in vacuo*. The anhydrous terramycin hydrochloride weighed 3.0 g.; potency, 920 γ/mg.

Anal. Calcd. for $C_{22}H_{24}N_2O_9 \cdot HCl$: C, 53.10; H, 5.06; N, 5.64; Cl, 7.15. Found: C, 52.80; H, 5.14; N, 5.80; Cl, 7.16. Tests for phosphorus, sulfur and non-ionic halogen were negative.

Terramycin hydrochloride crystallizes from methanol in the form of needles. Recrystallization from water at 50° results in platelets.

Crystallographic Data.[6]—Terramycin hydrochloride crystallizes from aqueous solution in the form of yellow elongated plates. These are biaxial negative, with parallel extinction and have the refractive indices[3] $\alpha = 1.639$, $\beta = 1.686$, $\gamma = 1.721$, ± 0.003. The highest refractive index is shown when the electric vector vibrates parallel to the direction of elongation (the *a* axis). The crystals are orthorhombic, with space group $P2_12_12_1$, and unit cell dimensions, $a = 11.19$, $b = 12.49$, $c = 15.68$ (cm. $\times 10^{-8}$). The density, found from experiment to be 1.51, gives the value 499 $\pm$ 5 for the molecular weight of the asymmetric unit. As it is probable that this unit consists of one molecule of the terramycin hydrochloride without water of crystallization, the molecular weight of the anhydrous base, as given by the X-ray data, is 462.5 $\pm$ 5. This is in agreement with the molecular weight found approximately by titration methods,[3] and with the formula $C_{22}H_{24}N_2O_8 \cdot HCl$.

Terramycin.—Crystalline terramycin was readily obtained from the pure hydrochloride by solution in water, neutralization to *p*H 6 and filtration after one hour. The crystals of terramycin dihydrate so obtained are biaxial and show parallel extinction. Refractive indices are: $\alpha = 1.634 \pm 0.004$, $\beta = 1.646 \pm 0.004$ and $\gamma > 1.70$. Terramycin dihydrate, prepared in this way, was dried to constant weight *in vacuo* over calcium chloride at 25°. Drying at 60° *in vacuo* gave anhydrous terramycin; the compound sintered at 182° and melted at 184.5–185.5°, with decomposition, when placed in a bath at 175° and heated at the rate of 2° per minute.

Anal. Calcd. for $C_{22}H_{24}N_2O_9 \cdot 2H_2O$: C, 53.22; H, 5.68; N, 5.64; (H_2O, 7.26); mol. wt., 496.46. Found: C, 53.05; H, 5.91; N, 5.64; (H_2O, 7.40 Karl Fischer determination; 7.31 volatile, 56° at 100 microns). The molecular weight of anhydrous terramycin, determined ebullioscopically in methanol, is 440 $\pm$ 30.

Terramycin gives positive ferric chloride, Pauly, Friedel-Crafts, Fehling and Molisch tests. The dihydrate melts with decomposition at 181–182°; at equilibrium $[\alpha]^{25}D$ −196.6° (*c* 1%, 0.1 *N* hydrachloric acid); $[\alpha]^{25}D$ −2.1° (*c* 1%, 0.1 *N* sodium hydroxide). The optical rotation of terramycin is affected by the presence of boric acid and calcium chloride: $[\alpha]^{25}D$ 0° (*c*, 1% methanol containing 1% boric acid); $[\alpha]^{25}D$ −234.2° (*c*, 0.5% methanol containing 1% saturated calcium chloride–methanol solution). The optical rotation of terramycin dihydrate in methanol decreases from an initial value of $[\alpha]^{25}D$ +26° (*c*, 0.5%) to $[\alpha]^{25}D$ +11.3° after standing 16 hours.

Terramycin Sodium Salt.—Terramycin (10 g.) was suspended in 200 ml. of water and treated with two moles of sodium hydroxide. The solution was filtered and vacuum freeze-dried (10.2 g.). The amorphous sodium salt was dissolved in 125 ml. of methanol, heated to 40° and seeded. After standing for three hours, the sodium salt was filtered, washed with methanol, re-suspended in 100 ml. of methanol and warmed to 35°. After cooling, the lemon-yellow crystals were filtered, washed with methanol, finally with ether and dried (7.5 g.); potency 900 γ/mg.

Anal. Calcd. for $C_{22}H_{22}N_2O_9 \cdot Na_2 \cdot 2H_2O$: C, 48.80; H, 4.48; N, 5.18; Na, 8.51; (H_2O, 6.66). Found: C, 48.12; H, 5.07; N, 5.23; Na, 8.82; (H_2O, 7.01).

Terramycin Calcium Chloride Complex.—Twenty grams of crude terramycin–calcium magnesium salt (precipitated from broth) was dissolved in 400 ml. of water and concentrated hydrochloric acid added to *p*H 2.5. The solution was filtered and then stirred with 300 ml. of *n*-butanol in order to eliminate the undesirable colored products from solution. Fifty grams of sodium chloride was added and the stirring continued for one-half hour. The two phase mixture, containing solid in suspension, was filtered on a büchner funnel and the precipitate washed with 100 ml. of wet *n*-butanol. The wet precipitate was then extracted with 150 ml. of methanol and the insoluble material separated by filtration. Twenty-five ml. of water was added, and the solution was allowed to stand overnight at 0°; 5.8 g. of light yellow crystals was obtained (potency 870 γ/mg.). Two grams of the crystals was dissolved in methanol and, after filtering, 5 ml. of water was added. After standing overnight at 0°, the light yellow crystals were collected on a büchner funnel. After drying *in vacuo* 1.5 hours at room temperature, the crystals weighed 1.2 g. and were found to contain 14.6% volatile matter as determined by drying at 100° for two hours *in vacuo*.

Anal. Calcd. for $(C_{22}H_{24}N_2O_9)_4 \cdot CaCl_2$: $CaSO_4$, 6.97; Ca, 2.05; Cl, 3.64. Found: $CaSO_4$, 6.38; Ca, 1.81; Cl, 3.49.

Purity by Solubility Measurement.—The purity of terramycin hydrochloride has been determined using the solubility method.[7,8] Methanol was selected as the solvent for these studies after a variety of solvents had been tested. At 35°, solutions of terramycin hydrochloride at a concentration of the order of 10 mg./ml. were found to be stable

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(6) J. H. Robertson, I. Robertson, P. F. Eiland and R. Pepinsky, THIS JOURNAL, in press.

(7) T. J. Webb, *Anal. Chem.*, **20**, 100 (1948).

(8) J. H. Northrop and M. Kunitz, *J. Gen. Physiol.*, **13**, 781 (1930).

for a period of at least six days. The solubility analysis was carried out in a thermostatically controlled constant temperature bath at 35 ± 0.1°. The procedure used was essentially that of Dalton and Schmidt.[9]

Titration of Terramycin Hydrochloride.—Since aqueous solutions of terramycin tend to precipitate terramycin base in the range *p*H 5 to 7.5, the potentiometric titrations were carried out at less than 0.002 molar concentration. The solution was titrated at 28 ± 0.2° under nitrogen using 0.2 *N* sodium hydroxide. pK'_a values were calculated from the titration data plotted in Fig. 1 in the regions of half equivalents using the equation[10]

$$pK_{a'} = P_{aH} - \log \frac{(Na^+) - (OH^-)}{(acid) - (Na^+) + (OH^-)}$$

where (Na^+) and (OH^-) are the molar concentrations of the respective ions and (acid) is the initial molar concentration of terramycin hydrochloride being titrated. pK'_a values of 3.49, 7.55 and 9.24 were obtained for terramycin hydrochloride. The precision of the measurements was of the order of ±0.05 *pK* unit. Values obtained from titration curves for methanol–water mixtures were found to be in good agreement with those obtained from titration of aqueous solutions. The titration data for the terramycin–calcium chloride solutions were obtained similarly.

Acknowledgments.—The authors are indebted to Dr. B. A. Sobin for the early work on the isolation and the preliminary characterization of terramycin. We wish to express our appreciation to Dr. J. A. Means for the microanalyses and to Mr. R. C. Kersey for the biological assays contained in this paper. We are also indebted to Mr. G. B. Hess for the infrared and ultraviolet absorption measurements.

BROOKLYN, N. Y. RECEIVED MARCH 15, 1951

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(10) P. L. Kirk and C. L. A. Schmidt, *ibid.*, **81**, 237 (1929).

[CONTRIBUTION FROM DEPARTMENT OF CHEMISTRY AND SCHOOL OF MEDICINE, STANFORD UNIVERSITY]

The Isolation in Crystalline Form and Characterization of the Two Isomeric Cytidylic Acids Derived from Yeast Nucleic Acid[1]

BY HUBERT S. LORING AND NYDIA G. LUTHY

The problem of the variation in rotation shown by different samples of cytidylic acid, $[\alpha]_D$ +23° to +49°, has been resolved by the isolation of a new isomer with an optical rotation of $[\alpha]_D$ +20.7° as well as of that formerly obtained with $[\alpha]_D$ +49°. The latter was prepared by a new procedure involving fractionation of dibrucine uridylate and dibrucine cytidylate with pyridine. Crystals of the two isomers differ in melting point behavior, in solubility, in crystalline habit and in indices of refraction. The $[\alpha]_D$ +49° compound could be readily deaminated with the formation of a product which gave a highly insoluble dibrucine salt with similar solubility properties and optical activity as the dibrucine uridylate usually isolated from ribonucleic acid hydrolysates. On deamination and treatment with brucine, the new isomer gave a relatively soluble dibrucine salt, which was not definitely characterized.

In the course of a search for an isomeric dibrucine uridylate different from that usually isolated from yeast nucleic acid, the specific rotations of the fractionated dibrucine salts of the pyrimidine nucleotides were determined in pyridine. It was found that the dibrucine salts corresponding, in general, to the cytidylate fraction, in contrast to dibrucine uridylate, failed to dissolve in this solvent. Examination of the properties of the pyridine-insoluble residues proved them to consist of free cytidylic acid as well as of the brucine salt. Since a sample of dibrucine cytidylate prepared from crystalline cytidylic acid[2] behaved similarly, it became evident that dibrucine cytidylate could be largely decomposed by pyridine with the formation of free cytidylic acid.

The above mentioned method was applied on a preparative scale to the dibrucine salts of the pyrimidine nucleotide fraction present after acid hydrolysis of yeast ribonucleic acid. After prolonged extraction of the mixture of dibrucine salts with pyridine, the residue, recrystallized from aqueous alcohol, showed the typical behavior of free cytidylic acid and gave a specific rotation of $[\alpha]_D$ +50.3°. Recrystallization did not significantly affect either the optical activity or decomposition point. The cytidylic acid fractions obtained from the mother liquors of the $[\alpha]_D$ +50° gave specific rotations between $[\alpha]_D$ +31° and +49°, and evidently were comparable to those prepared by alkaline hydrolysis,[2] $[\alpha]_D$ +39° to +42° or to those prepared by acid or alkaline hydrolysis,[3] $[\alpha]_D$ +37°. Although an optical rotation of $[\alpha]_D$ +49° has been reported previously for cytidylic acid,[4] a value of $[\alpha]_D$ +23.4° was also found on one occasion by Thannhauser and Dorfmuller.[5] No explanation has been offered, heretofore, for the seeming discrepancies in optical activity. It occurred to us, in view of the optical rotation of $[\alpha]_D$ +21.4° for the reported synthetic cytidine-2-phosphate,[6] that the above results might be explained by the presence of varying amounts of an isomeric product with a lower rotation than $[\alpha]_D$ +49°. Recrystallization of the samples of lower specific rotation from aqueous alcohol resulted in some increases in the rotation of the products, but in general, did not lead readily to material either of $[\alpha]_D$ +49° or of less than $[\alpha]_D$ +31°.

Attention was next turned to the possibility of fractionating the cytidylic acid mixture as the relatively insoluble crystalline phosphotungstate.[2] The latter was isolated from the pyrimidine nucleotide fraction and was recrystallized from 1 *N* sulfuric acid. The cytidylic acid phosphotungstate which first separated was converted to free cytidylic acid, and in contrast to the sample of highest specific rotation mentioned above, gave $[\alpha]_D$ +20.7°. Repeated recrystallization of this material from aque-

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(2) Loring, Roll and Pierce, *J. Biol. Chem.*, **174**, 729 (1948).

(3) Barker, Gulland, Smith and Thomas, *J. Chem. Soc.*, 904 (1949).

(4) Levene, *J. Biol. Chem.*, **41**, 484 (1920); Bredereck and Richter, *Ber.*, **71**, 718 (1938).

(5) Thannhauser and Dorfmuller, *Z. physiol. Chem.*, **104**, 65 (1919).

(6) Gulland and Smith, *J. Chem. Soc.*, 1527 (1948).