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Tetracycline-Urea Compound

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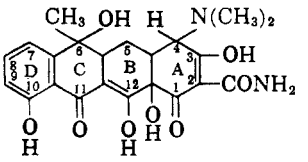
The formation and properties of an equimolecular compound between tetracycline and urea are described. Neither 7-chlorotetracycline nor 5-hydroxytetracycline form insoluble, equimolecular compounds with urea, and tetracycline does not form such compounds with other related amides.

Published methods for isolation and purification of the broad-spectrum antibiotic tetracycline[1] (I) from *Streptomyces aureofaciens* fermentations or from catalytic reduction of 7-chlorotetracycline[1]



I

have generally involved precipitation of the antibiotic as an insoluble form from aqueous or organic solvent solutions. Thus tetracycline has been isolated from concentrated aqueous solutions obtained from fermentation sources or from catalytic reduction of 7-chlorotetracycline by precipitation as the isoelectric form.[2,3] The isoelectric form has also been isolated from organic solvent extracts prepared from fermentation sources.[4] Isolation of tetracycline as the hydrochloride has also been used in situations where concentrated organic solvent solutions of the antibiotic can be caused to crystallize or where the salt can be precipitated by addition of a non-polar solvent.[4] Precipitations of tetracycline as a complex with calcium chloride and other metal salts, with long-chain alkyltrimethylammonium halides, alkyl sulfate esters, and alkyl sulfonic acids, have been variously reported as refining methods for tetracycline.

These techniques can be favorably extended to include precipitation of tetracycline as an insoluble crystalline compound with urea in the molecular proportions of 1:1. The tetracycline-urea compound may be precipitated from aqueous solutions or from organic solvent extracts of the antibiotic. Thus tetracycline-urea may be used in many cases where tetracycline has been precipitated as the isoelectric form.

(1) The trademarks of the American Cyanamid Co. for tetracycline and 7-chlorotetracycline are Achromycin and Aureomycin, respectively.

(2) P. P. Minieri, H. Sokol, and M. C. Firman, U. S. Patent No. 2,734,018, Feb. 7, 1956.

(3) L. H. Conover, U. S. Patent No. 2,699,054, Jan. 11, 1955.

(4) J. Lein and A. Gourevitch, U. S. Patent No. 2,739,924, Mar. 27, 1956.

Tetracycline-urea is most easily prepared from purified tetracycline by slurrying neutral tetracycline in saturated aqueous urea solution or by addition of solid urea to aqueous solutions of tetracycline hydrochloride and appropriate *p*H adjustment. Neutral tetracycline is dissolved by the urea solution and tetracycline-urea is precipitated almost immediately. Under these conditions the tetracycline-urea compound precipitated is a trihydrate with about the same solubility in water as neutral tetracycline prepared at the isoelectric point(*ca.* 300 μg./ml.). When concentrated aqueous (acid) extracts prepared from initial organic solvent extracts of fermentation mashes containing tetracycline are treated with solid urea similarly the tetracycline-urea trihydrate is formed.

Organic solvent extracts of *S. aureofaciens* fermentation mashes prepared as described by Minieri, *et al.*,[2] precipitate tetracycline-urea as a monohydrate when treated with aqueous urea solutions. Thus a methyl isobutyl ketone extract prepared using a quarternary ammonium salt as a "carrier" deposits tetracycline-urea monohydrate when stirred with saturated aqueous urea solution with *p*H adjustment to about *p*H 3.5. Similar treatment of organic solvent solutions of tetracycline pre-

TABLE I

COMPARATIVE SOLUBILITIES OF TETRACYCLINE-UREA MONOHYDRATE AND NEUTRAL TETRACYCLINE

Solvent	Tetracycline-Urea, $1H_2O$, μg./Ml.	Neutral Tetracycline, $3H_2O$, μg./Ml.
Acetone	21,000	45,000
Amyl acetate	545	3,400
Benzene	43	1,400
Butanol	18,200	29,500
Carbon tetrachloride	19	424
Cellosolve	103,000	204,000
Chloroform	520	840
Dimethylformamide	52,000	62,000
Dioxan	24,200	83,000
Ethanol	45,000	43,000
Ethyl acetate	8,300	12,400
Methanol	13,000	26,400
Methyl isobutyl ketone	2,900	57
2-Propanol	11,000	9,200
Tetrahydrofuran	9,300	57,800
Water	325–500	310–1100

pared by catalytic reduction of 7-chlorotetracycline[3,5,6] yields tetracycline-urea.

The tetracycline-urea hydrates have low water solubility and also less solubility in several organic solvents than do neutral tetracycline hydrates. In methanol, butanol, Cellosolve, and acetone, tetracycline-urea hydrate is about half as soluble as neutral tetracycline.

The stability of the tetracycline-urea monohydrate in terms of accelerated stability testing is good. Heating at 60° for eight days does not alter the antibiotic potency or its physical appearance.

TABLE II

ACCELERATED STABILITY TESTS ON TETRACYCLINE-UREA MONOHYDRATE (60° HEATING IN CLOSED CONTAINERS)

Time, Days	Spectrophotometric Assay, μg./Mg.	Microbiological (Turbidimetric) Assay, μg./Mg.	Color Value[a]
0	850	870	0.203
1	850	845	0.257
8	848	843	0.269

[a] An arbitrary value corresponding to $E^{1\%}_{1\,cm.}$ at 450 mμ, measured on a 0.5% solution of 0.1*N* sulfuric acid within 5 minutes after dissolving.

Tetracycline-urea is dissociated into its components by dissolving in any solvent. When dissolved in warm methanol or dilute aqueous acids the solutions have the properties of tetracycline in solution. Ultraviolet spectra in either dilute acid or dilute alkali are identical with tetracycline hydrochloride reference material. Paper chromatographic mobility in the *p*H 3 buffered paper/butanol system[7] or in a 3% aqueous sodium arsenite system[8] is indistinguishable from that of neutral tetracycline or its hydrochloride. Optical rotation of dilute solutions of tetracycline-urea is essentially that of tetracycline neutral. The distribution coefficients of tetracycline and tetracycline-urea between *p*H 2.5 phosphate buffer and 80%–20% (v./v.) chloroform-butanol are the same, 0.13 and 0.14, respectively. The rate of epimerization to 4-*epi*-tetracycline is slightly greater for tetracycline-urea than for neutral tetracycline, the half-epimerization time for tetracycline-urea being 8.4 hr. in 1*M* sodium dihydrogen phosphate–methanol, for neutral tetracycline, 10 hr.

(5) J. H. Boothe, J. Morton, J. P. Petisi, R. G. Wilkinson, and J. H. Williams, *J. Am. Chem. Soc.*, **75**, 4621 (1953).

(6) L. H. Conover, W. T. Moreland, A. R. English, C. R. Stephens, and F. J. Pilgrim, *J. Am. Chem. Soc.*, **75**, 4622 (1953).

(7) N. Bohonos, A. C. Dornbush, L. I. Feldman, J. H. Martin, E. Pelcak, and J. H. Williams, *Antibiotics Annual*, **1953/1954**, Medical Encyclopedia Inc., New York, 1953, p. 49.

(8) T. Berti and L. Cima, *Boll. soc. ital. biol. sper.*, **30**, 1123 (1954); *Boll. ist. sieroterap. milan.*, **33**, 643 (1954).

Tetracycline-urea cannot be recrystallized from organic solvents as dissociation to free tetracycline occurs. Recrystallization from saturated aqueous urea solution is possible, the tetracycline-urea dissolving in the saturated urea solution and being precipitated immediately from solution. Neutral tetracycline is precipitated from dilute acid solutions of tetracycline-urea on raising the *p*H, and the neutral material is also recovered from methanol solutions of tetracycline-urea on dilution with water. The facile dissociation of the addition compound in solution is similar to the reported dissociation of the 1:1 urea compounds of the enolic forms of certain β-ketoesters. Ethyl phenylketoparaconate and ethyl oxalacetate both form urea compounds in the molecular ratio of 1:1 and these compounds are easily dissociated on solution.[9] In contrast the 1:1 urea compounds of 2-amino-4,6-dimethylpyrimidine and 2-hydroxy-4,6-dimethylpyrimidine can be recrystallized and require base for their dissociation.[10]

Indeed tetracycline-urea is known as such only in the solid state, as evidenced by the characteristic melting point and by infrared spectrum. In potassium bromide disks tetracycline-urea has an infrared spectrum characteristic of neutral tetracycline but with additional bands at 5.75–5.80 μ and 6.55–6.60 μ.

The specificity of formation of the tetracycline-urea compound is of interest. Whereas tetracycline dissolves in saturated aqueous urea solutions and immediately crystallizes as the urea compound, 7-chlorotetracycline, 5-hydroxytetracycline,[11] 4-*epi*-tetracycline (quatrimycin),[12] 7-chloro-4-*epi*-tetracycline (7-chloroquatrimycin),[12] anhydrotetracy-

TABLE III

SOLUBILITIES OF SEVERAL TETRACYCLINES IN UREA SOLUTIONS AT 25°

Tetracycline	Solubility in Saturated Urea, μg./Ml.	Solubility in Water, μg./Ml.
Tetracycline neutral	1,770	1,000
7-Chlorotetracycline neutral	24,400	500
5-Hydroxytetracycline neutral	9,200	450
4-*epi*-Tetracycline neutral	47,900	18,300
7-Chloro-4-*epi*-tetracycline neutral	41,000	870
Anhydrotetracycline hydrochloride	396,000	52,000
Iso-7-chlorotetracycline neutral	10,300	200

(9) H. Gault and M. Suquet, *Bull. soc. chim. France*, 598 (1950).

(10) S. Birtwell, *J. Chem. Soc.*, 1725 (1953).

(11) The trademark of Chas. Pfizer & Co. for 5-hydroxytetracycline is Terramycin.

(12) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom, and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79**, 2849 (1957).

cline, and *iso*-7-chlorotetracycline dissolve but do not precipitate as insoluble compounds. Their solubility in urea solutions is greater than in water. Further, aqueous solutions of thiourea, formamide, acetamide, guanidine, *N*-methylurea, *N,N'*-diethylurea, *N,N*-diethylurea, and *N*-isopropylurea, while generally dissolving tetracycline, do not form insoluble amide compounds.

This high degree of selectivity in combination with the high degree of solubility of 7-chlorotetracycline base in saturated urea solution provides a means of separation of tetracycline from mixtures of tetracycline and 7-chlorotetracycline. 7-Chlorotetracycline is coprecipitated with the tetracycline-urea compound from aqueous solutions; however, the 7-chlorotetracycline is in the isoelectric form, not as a urea compound. The 7-chlorotetracycline content of mixtures is reduced by half on one precipitation of tetracycline as the urea compound.

TABLE IV

FRACTIONATION OF 7-CHLOROTETRACYCLINE (CTC) FROM TETRACYCLINE (TC) BY FORMATION OF TETRACYCLINE-UREA COMPOUND

Composition of Synthetic Mixture		Composition of Insoluble Product		Recovery of Activity	
CTC, μg./mg.	TC, μg./mg.	CTC, μg./mg.	TC, μg./mg.	CTC, %	TC, %
1,000	0	892	—	53.5	—
800	200	650	327	47.5	91.0
600	400	363	576	40.8	92.7
500	500	263	662	39.4	93.5
400	600	215	705	45.3	94.3
200	800	105	794	53.0	95.2
0	1,000	—	905	—	96.2

EXPERIMENTAL

All spectrophotometric assays were performed in 0.1*N* sulfuric acid solutions, compared against a pure standard reference antibiotic (tetracycline hydrochloride = 1000 μg./mg.), and determined on either a Beckman Model DU Quartz spectrophotometer or on a Cary Recording Spectrophotometer, Model 11S. Microbiological assays were turbidimetric assays using *Staphylococcus aureus*. A differential alkaline destruction of 7-chlorotetracycline followed by acid destruction and assay using the long wave length maxima was used for analysis of mixtures of 7-chlorotetracycline and tetracycline.

Tetracycline-urea trihydrate. A. *From tetracycline hydrochloride.* Five grams of pure tetracycline hydrochloride was agitated with 25 ml. of saturated aqueous urea solution until solution was complete. The solution was diluted with 25 ml. of water and filtered immediately. After five hours at room temperature the precipitated crystals were filtered and washed with five 10-ml. portions of water. The crystals were dried *in vacuo* over phosphorus pentoxide, weighing 3.34 g., melting with decomposition at 143–146° (Fisher-Johns block), $[\alpha]_D^{25}$ −222° (0.5%, MeOH), −229° (0.5%, 0.03*N* HCl), assaying 840 μg./mg. (spectrophotometric) and 810 μg./mg. (microbiological). An additional 0.78 g. was recovered from the chilled filtrate.

Anal. Calcd. for $C_{23}H_{34}N_4O_{12}$: C, 49.46; H, 6.14; N, 10.03; potency, 859 μg./mg. Found: C, 49.69; H, 6.42; N, 9.97; potency, 840 μg./mg.

Five grams of tetracycline-urea trihydrate was dissolved with warming in 100 ml. of methanol, and the solution was filtered while warm. Dilution with 100 ml. of water caused a slow precipitation of crystals. A first crop of 1.00 g. was obtained after the mixture had stood at room temperature for three hours. The chilled filtrate (4°) deposited a further 2.88 g. of crystals. Both fractions, assaying 1009 μg./mg. and 870 μg./mg., respectively, represent hydrated tetracycline base.

Conversion of tetracycline-urea hydrates to tetracycline hydrochloride was accomplished by slurrying 9.0 g. of tetracycline-urea in 45 ml. of butanol/Cellosolve (2:1) and adding concentrated hydrochloric acid to reduce the *p*H to 1.7. The mixture was stirred for 60 hr. at room temperature and then filtered. The crystals were washed with 2-propanol and dried in a vacuum oven at 40°, yielding 7.49 g. of tetracycline hydrochloride, assaying 982 μg./mg. (90.2% yield).

B. *From tetracycline neutral.* Five grams of tetracycline neutral assaying 1030 μg./mg. (compared to tetracycline hydrochloride) was slurried in 25 ml. of saturated aqueous urea solution and then shaken on a rotary shaking machine for two hours. The crystals were filtered and washed with water, and dried *in vacuo* over phosphorus pentoxide. A yield of 5.72 g. (95%) of tetracycline-urea trihydrate was obtained, assaying 820 μg./mg.

Ultraviolet spectra of the tetracycline-urea compounds in 0.1*N* sulfuric acid exhibited maxima at 218 mμ, 268 mμ, and 355 mμ, with minima at 233 mμ and 300 mμ. The ratio of the extinctions at 268 mμ and 355 mμ is 1.28; at 255 mμ and 268 mμ, 0.84 (no epimerization[12]). Tetracycline hydrochloride and base have maxima at the same wave lengths with ratios at 268 mμ and 355 mμ of 1.28 and at 255 mμ and 268 mμ of 0.87.[12] In 0.1*N* sodium hydroxide tetracycline-urea compound has the same ultraviolet spectra as tetracycline hydrochloride, with maxima at 248 mμ, 268 mμ, 290 mμ (shoulder), and 380 mμ, and minima at 228 mμ, 255 mμ, and 323 mμ.

Infrared spectra of tetracycline urea trihydrate in potassium bromide disks indicate bands at 2.93 μ, 3.35 μ, 5.77 μ, 6.00 μ, 6.20 μ, 6.55 μ, 6.87 μ, etc.

Moisture determinations on the tetracycline-urea trihydrate preparations cannot be made by hot air drying and loss of weight determination as the tetracycline-urea compounds char easily on heating above 100°. Karl Fischer determinations have generally given higher results than the calculated. Urea determinations by a urease digestion have given slightly higher results than the calculated, mainly due to varying blank determinations.

Isolation of tetracycline as tetracycline-urea monohydrate. Two liters of a *S. aureofaciens* fermentation broth containing tetracycline as the main antibiotic was acidified with 25% sulfuric acid to *p*H 1.8 and filtered with 200 g. of Hyflo filter-aid. The solids were reslurried with 1.5 l. of warm water (45°) at *p*H 1.85, and refiltered. The combined acid filtrates were treated with 88.2 g. of ammonium oxalate, stirred for 30 min. at room temperature, and stored overnight at 4°. The precipitated calcium oxalate was filtered and the cake was washed with 50 ml. of water. To 2.97 l. of aqueous filtrate was added 300 ml. of methyl isobutyl ketone and 15 ml. of 50% cetyltrimethylammonium chloride (Arquad 16)[13] in 2-propanol.

The *p*H was adjusted to *p*H 8.8 with 23 ml. of 18*N* sodium hydroxide solution, and the mixture stirred for 20 min., at which time the phases were separated. The aqueous phase was re-extracted with 100 ml. of methylisobutyl ketone with 5 ml. of Arquad 16 at *p*H 8.8. The combined organic extracts were washed twice with 40 ml. of water.

To 200 ml. of the methyl isobutyl ketone extract containing 40,000 μg./ml. of antibiotic activity was added 32 ml. of a saturated aqueous urea solution and 32 ml. of water.

(13) Arquad 16 is predominantly cetyltrimethylammonium chloride; obtained from Armour Chemical Division, Armour and Co., Chicago, Ill.

Hydrochloric acid was added to bring the *p*H to 5.5. The mixture was shaken on a rotary shaker for 16 hr., at which time the *p*H was adjusted to 3.0 with HCl, and the mixture was agitated for an additional 48 hr. The product accumulated as a precipitate in the aqueous phase, and was filtered without phase separation. The tetracycline-urea compound was dried *in vacuo* at 50°, weight 7.80 g. (90% from organic extract), assay 938 μg./mg. as tetracycline hydrochloride.

Anal. Calcd. for $C_{23}H_{30}N_4O_{10}$: C, 52.80; H, 5.75; N, 10.72. Found: C, 52.85; H, 5.68; N, 10.70.

Determination of solubilities. An unweighed portion of the tetracycline derivative was suspended in the solvent and placed on a rotary shaker for two hours at room temperature, the undissolved material filtered, and the filtrate assayed spectrophotometrically.

Determination of distribution coefficients. Samples were dissolved in *p*H 2.5/0.25*M* sodium dihydrogen phosphate buffer (previously equilibrated against the organic phase) at a final concentration of 50 μg./ml. and equilibrated with a mixture of 80% chloroform-20% butanol (10 ml. of each phase). Each phase was assayed spectrophotometrically after thirty inversions. The distribution coefficient for tetracycline urea compound was 0.14 (organic phase/aqueous phase), for tetracycline base, 0.13.

Fractionation of mixtures of tetracycline and 7-chlorotetracycline. Mixtures containing 7-chlorotetracycline neutral and tetracycline neutral were prepared, and 5.0 g. of the crystal mixture was slurried in 100 ml. of saturated aqueous urea solution. After one hour of shaking the undissolved material was filtered and analyzed.

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Preparation of α-Hydroxyguanamines from Cyanohydrins

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Lactonitrile, a typical cyanohydrin, was not stable in an attempted base-catalyzed condensation with dicyandiamide to an α-hydroxyguanamine. The acid-catalyzed condensation of cyanohydrins with vinyl ethers gave α-cyanoacetals, I, which were stable in this condensation. Acid-catalyzed hydrolysis of the resulting diaminotriazines, II, gave the desired α-hydroxyguanamines, III (2,4-diamino-*s*-triazine-6-alkanols). A homologous formal, 2,4-diamino-6-ethoxymethoxymethyl-*s*-triazine, was either recovered quantitatively or converted to resins under comparable conditions.

The base-catalyzed reaction of dicyandiamide with nitriles[1] is usually one of the best methods of synthesizing guanamines (2,4-diamino-6-alkyl-*s*-triazines). However, when a cyanohydrin, such as lactonitrile, was used in the method, no hydroxyguanamine was obtained. It was probable that reversion of the cyanohydrin to the aldehyde and HCN occurred with subsequent base-catalyzed polymerization reactions.

The desired α-hydroxyguanamines[2] (III) were therefore prepared by a sequence of reactions (Fig. 1) involving in the first step the stabilization of the cyanohydrins to basic conditions, in the form of their addition products to various vinyl ethers. The convenience and high yields of this reaction sequence provide a practical and general synthesis for this series of compounds. It is illustrated in the accompanying table and in the experimental section for cyanohydrins from three aldehydes (formaldehyde, acetaldehyde, and benzaldehyde) and two ketones (acetone and cyclohexanone). The only previous synthesis (for lactoguanamine)[3] was from a corresponding α-hydroxy-

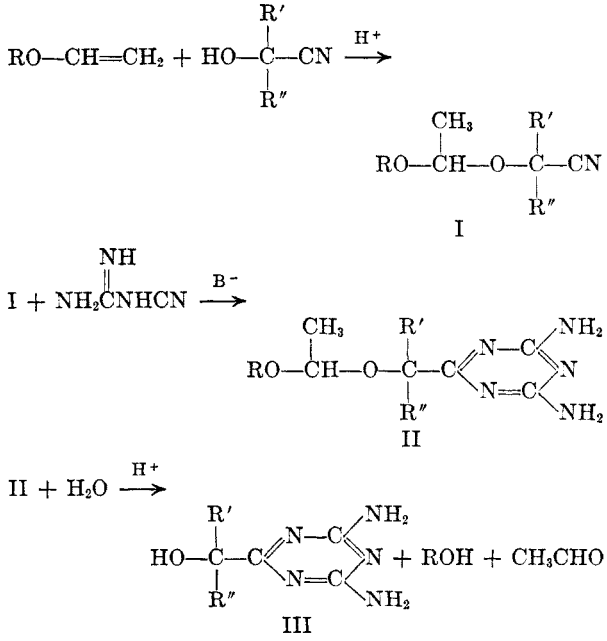

Figure 1

(1) (a) W. Zerweck and W. Brunner, U. S. Patent 2,302,163; *Chem. Abstr.*, **37**, 2016 (1943); (b) J. K. Simons, U. S. Patent 2,532,519; *Chem. Abstr.*, **45**, 3429 (1951).

(2) The non-systematic term "guanamine" will be used in the discussion section. Compounds III are named after the corresponding hydroxyacids, thus: glycologuanamine (R, = R′ = H), α-hydroxyisobutyroguanamine (R′ = R″ = CH_3), etc.

(3) J. T. Thurston, U. S. Patent 2,394,526 (1946); *Chem. Abstr.*, **40**, 5776 (1946).