Inactivation of Tetracycline with Cupric-Morpholine Complex

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Cupric-morpholine complex degrades and inactivates tetracycline in methanol or methanol water solutions. No other agent has been reported to inactivate tetracycline so rapidly and completely at the mild conditions employed. Kanamycin is not affected. This selectivity of inactivation is used as part of a biological assay for kanamycin in the presence of tetracycline.

BROCKMAN and Havinga (1) have reported on the metal amine oxidation and substitution of various phenols. For example, α- and β-naphthol were reacted with cupric-morpholine complex (Cu-M) to yield 4-morpholino-1,2-naphthoquinol or quinone. The similarity between this reaction and oxidation of phenolic substances by copper-tyrosinase was also considered.

By analogy, the phenolic nature of the D ring of tetracycline prompted us to react this antibiotic with Cu-M in anticipation of obtaining a biologically active morpholine derivative. Instead, a biologically inactive[1] markedly degraded product containing copper was isolated.

No other agent has been reported to inactivate tetracycline so rapidly and completely and at such mild conditions as Cu-M. In methanol-water or methanol solutions, tetracycline is almost instantaneously inactivated by 0.5 mole or more of Cu-M. Cupric ion (as cupric acetate) or copper tyrosinase did not inactivate tetracycline. Kaplan *et al.* and Sakaguchi *et al.* (2) have also been successful in isolating fully active copper tetracycline complexes upon reaction of tetracycline and Cu^{2+} in water or methanol at various pH's.

This unique and most rapid inactivation process has been utilized successfully as part of an assay procedure for the selective degradation of tetracycline in the presence of antibiotics. Kanamycin has been assayed in the presence of tetracycline by using a strain of *Staphylococcus aureus* which is resistant to tetracycline. Now, with the use of Cu-M, both tetracyline and kanamycin may be assayed in the presence of each other with a single organism, *St. aureus* 209P. This paper reports this assay technique and also the preparation and properties of the Cu-M tetracycline degradation product.

EXPERIMENTAL

In Situ Preparation of Cupric-Morpholine Complex.—A 0.1 *M* Cu-M solution may be prepared conveniently by dissolving 19.9 Gm. of cupric acetate monohydrate and 10 Gm. of morpholine in sufficient methanol to make 1 L. of solution.

Inactivation of Tetracycline with Cupric-Morpholine Complex for Assay Purposes.—This procedure as used here is primarily for assay of physical mixtures of tetracycline and kanamycin. A mixture containing an arbitrary equivalent of activity equal to 200 mg. of tetracycline HCl and 200 mg. of kanamycin base is dissolved in 50 to 100 ml. of 0.1 *N* HCl. This solution is then made up to 250 to 500 ml. with pH 4.5 phosphate buffer and assayed for tetracycline content by the method described by Grove and Randall (3). Under these conditions, kanamycin does not interfere with the tetracycline assay. For the kanamycin assay, the above described tetracycline-kanamycin mixture is added to 20–100 ml. of methanol. If necessary, triethylamine may be added to adjust the apparent pH to a range of 7.5 to 8.5. Five to eight milliliters of 0.1 *M* Cu-M solution is then added, and the mixture is stirred at ambient temperatures for 0.5 to 1 hour to inactivate the tetracycline. The mixture is then diluted to 250 to 500 ml. with pH 8.0 phosphate buffer and assayed for kanamycin content by the method described by Lamoy and Lannon (4).

Preparation of Cupric-Morpholine Degraded Tetracycline.—Thirty grams of tetracycline base trihydrate and 13 Gm. of cupric acetate monohydrate are added to 500 ml. of methanol with vigorous stirring. Morpholine is then added to maintain an apparent pH of 9.0 to 9.8 (electrodes of Beckman zeromatic pH meter are immersed directly in methanol solution). The resultant solution is held at ambient temperatures for 1 to 4 hours. The dark brown crystals which precipitate are removed by filtration, washed with 200 ml. of methanol, and air-dried at 40° for 3 hours to yield 30 Gm. of solids. These crystals are slurried with 300 ml. of methanol plus concentrated HCl to an apparent pH of 1.3 to 1.8 for 10 minutes. An insoluble residue is discarded, and the filtrate is added to 3 L. of water with moderate stirring. This mixture is stirred for 5 minutes, and the resultant crystals are removed by filtration and washed with 300 ml. of methanol. The damp cake is reslurried with 300 ml. of methanol plus added concentrated HCl at an apparent pH of 1.3 to 1.8 for 10 minutes. An insoluble residue is discarded, and the filtrate is added to 300 ml. of a 50% v/v mixture of normal and tertiary butyl alcohols. This solution is concentrated under reduced pressure at 20–25° to about 100–125 ml. The resultant crystalline precipitate is removed by filtration, washed with 50 ml. of normal butyl alcohol and 100 ml. of ethyl ether, and dried under high vacuum—50° for 24 hours. A yield of 14 Gm. of dark brown crystals is obtained. These crystals (and the original crystalline precipitate), when examined by circular paper strip chromatography (5), showed only a single, brown nonfluorescing zone at the solvent front.

Anal.—Calcd. for $(C_{17}H_{16}NO_8)_2Cu$: C, 51.7; H, 4.07; Cu, 8.08; N, 3.6. Found (anhydrous solid): C, 51.8, 51.6; H, 4.06, 4.02; Cu, 8.04, 8.07; N, 3.36, 3.66.

Chlorine was not detected in the crystals. Melting point (capillary); progressive degradation begins at about 170° (not melted completely at 190°). Absorption maxima (0.1 *N* HCl in methanol) at

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[1] No activity by turbidimetric assay using *St. aureus* 209P as test organism.

267 mμ, ϵ = 44136; $[\alpha]_D^{25}$, −2° (C = 0.1 in 0.1 *N* HCl in methanol). Infrared analysis (KBr pellet) showed two new bands, 5.64 and 5.8 μ, related to carbonyl moieties not present in intact tetracycline.

SUMMARY

Cupric-morpholine complex rapidly inactivates tetracycline in methanol or methanol water solutions. Kanamycin is not affected. This selectivity of inactivation has been used as part of an assay for kanamycin in the presence of tetracycline.

Analytical data of the isolated crystalline reaction product indicate extensive degradation of the tetracycline molecule has occurred. An apparent empirical change from $C_{22}H_{24}N_2O_8$ to $C_{17}H_{16}NO_8$ was noted.

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Cast Electrode Mount for Self-Stimulation Electrodes

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In a previous communication (1), the construction and implantation of self-stimulation electrodes was described. Recently, it was discovered that when stereotaxic equipment other than the Lab-Tronics apparatus was used, the mount would not conform to the skull and loss of both the electrode and the animal resulted. Moreover, the amount of handwork required in producing the Lucite mounts was excessive. These disadvantages have been overcome by casting epoxy resin mounts as described below.

The dimensions in inches of the brass pattern used in preparing the mold for casting the epoxy resin mount are given in Fig. 1. The angles shown are critical since any deviations will result in a mount which will not conform to the skull. The completed brass pattern is shown in Fig. 2A. The mold shown in Fig. 2B is made from a RTV-11 silicone rubber compound (General Electric Co.) mixed according to package directions and poured around the brass pattern in an aluminum form $^7/_8$ in. in diameter and $^5/_8$ in. in depth. This mold sets in 24 hours; several should be prepared. The epoxy mounts are made from Wilhold clear epoxy glue by filling the mold and allowing 4 hours for hardening. All bubbles should be removed by stirring with a fine wire. The mount is removed from the mold by flexing the latter. All excess resin should be removed by sanding with fine emery paper. The completed mount is attached to the rat's skull as previously described (1), and the electrode is inserted and cemented firmly in place.

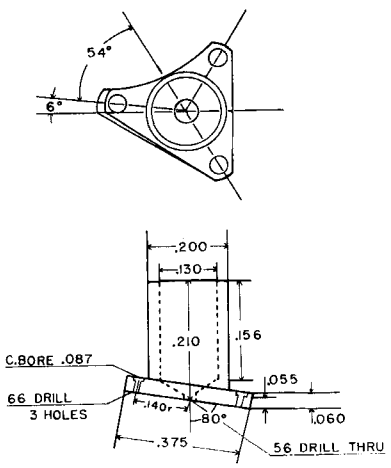

Fig. 1.—Brass pattern of epoxy resin mount with dimensions in inches.

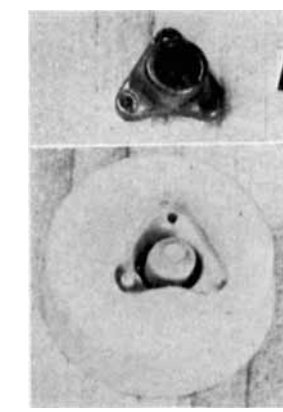

Fig. 2.—Key: A, completed brass pattern; B, completed silicone rubber mold for preparing epoxy resin electrode mounts.

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