Tylosin–Urea Adduct Related to Tylosin Stability in Cattle Feed

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The interaction of tylosin and urea was studied, and a tylosin-urea adduct (TUA) was prepared from which nondegraded tylosin could be regenerated. The presence of tylosin-urea adduct in tylosin-containing cattle feeds to which urea had been added was indicated by chromatography. Microbiological and chemical studies indicated that the tylosin-urea reaction was reversible and that the apparent loss of antimicrobial activity in urea-rich feeds did not represent an actual loss of available tylosin.

Tylosin, a macrolide antibiotic marketed as TYLAN (Eli Lilly and Company, Elanco Division), is widely used as a feed additive for promoting growth in poultry and livestock. Its antimicrobial spectrum and physical-chemical properties have been summarized in a review (Morin and Gorman, 1967), and more recently its chemical structure (Figure 1) has been reported (Morin *et al.*, 1970).

Nutritional components added to experimental cattle feeds often evoke instability which necessitates a stabilization or elimination of the interacting substances. Tylosin exhibited an apparent inactivation in a number of experimental feeds which led to the preparation and study of the tylosin-urea adduct (TUA).

EXPERIMENTAL SECTION

Thin-layer chromatography was performed on precoated Brinkmann Silica Gel F254 plates using (A) 10% diethylamine in ethyl acetate and (B) 20% methanol in chloroform as the solvent systems. Nmr spectra were recorded with a Varian A-60 instrument using deuterochloroform solutions. Uv spectra were obtained using a Cary 14 recording spectrophotometer. Melting points are uncorrected.

Tylosin and Tylosin Tartrate. Commercial materials which met previously published specifications (Hamill *et al.*, 1961) were used.

Desmycosin. Using the procedure of Hamill and coworkers (1961), desmycosin was obtained by mild acid hydrolysis of tylosin.

TUA. A solution (pH 4.8) of 100 g of tylosin tartrate in 200 ml of saturated aqueous urea was allowed to stand at room temperature. The reaction was periodically checked for completion by tlc. In solvent systems A and B, the R_f's of TUA are, respectively, 0.1 and 0.3 those of tylosin. After 3 hr, the solution was diluted with 3 vol of water, adjusted to pH 8.5 with 10% aqueous NaOH, and saturated with NaCl. The precipitated TUA was removed by filtration and dissolved, without drying, in 1 l. of methylene chloride. The latter solution was washed thoroughly with water, dried over $MgSO_4$, and evaporated to give TUA as a pale yellow amorphous solid. The crude TUA was dissolved in 1 l. of acetone. The acetone solution was concentrated by boiling until crystallization had begun and was maintained at room temperature until the crystallization was complete. Filtration gave 68 g of crystalline TUA as an acetone solvate, mp 202–220°.

Electrometric titration of TUA in 66% dimethylformamide–water showed a neutralization equivalent weight of 1030, pK_a 7.2. The ultraviolet spectrum in methanol showed a maximum at 282 mμ (ϵ = 22,000). Repeated crystallization from boiling acetone afforded an analytical sample of TUA, as white needles, mp 220–222°.

Anal. Calcd for $C_{47}H_{83}N_5O_{18}$: C, 56.22; H, 8.39; N, 6.96; O, 29.53. Found: C, 56.08; H, 8.42; N, 7.05; O, 29.31.

Tylosin from TUA. A solution of 1 g of TUA in 100 ml of 10% ethanolic phosphate-citrate buffer (pH 5.0) was heated at 95° for 30 min. Examination of thin-layer chromatograms in both solvent systems A and B indicated that the hydrolysis of TUA was complete.

The solution was adjusted to pH 8.5, saturated with NaCl, and extracted thoroughly with methylene chloride. The methylene chloride solution was dried over $MgSO_4$ and evaporated at reduced pressure to give 0.8 g of amorphous solid, which was shown to be identical with tylosin by comparison of its nmr and uv spectra and its R_f values in a number of solvent systems to those of an authentic sample.

Desmycosin from TUA. A solution of 1 g of TUA in 100 ml of pH 3.2 phosphate-citrate buffer was heated at 95° for 1.5 hr. The reaction was processed as described above to give 0.7 g of amorphous desmycosin. Identification was made by comparison with an authentic sample as described above.

MICROBIOLOGICAL

Antimicrobial activity of tylosin and desmycosin in feeds was measured using the test organism *Sarcina lutea*, ATCC 9341, cultured in 4 ml of nutrient agar (pH 8.5) in a Petri plate (Kavanagh, 1963). The solution of tylosin or TUA was diluted to 0.20 μg/ml in 40:60 methanol–phosphate buffer (pH 8.0) and applied to the seeded bacteria in Penicylinder cups. The plates were incubated overnight at 30°, and the zones of inhibition were measured and averaged by standard procedures.

Bioautographs. The antimicrobial activity of tylosin and derivatives on thin-layer chromatograms was visualized by an overlay of *S. lutea* seeded agar according to the procedure of Kline and Golab (1965).

Preparation of Livestock Feed Extracts for Assay. Feed extracts containing tylosin were prepared by adding 100 ml of phosphate buffer (pH 8.0) previously heated at 80° to 20 g of feed. The extract was allowed to stand for 10 min and diluted with 64 ml of anhydrous methanol. The suspension was then blended on an Omni-mixer and filtered, and the filtrate was appropriately diluted for assay. Extracts containing inactivated tylosin were treated by various methods, described in Results, to regenerate the activity prior to assay.

RESULTS AND DISCUSSION

Tylosin-containing feeds of various composition on stability tests were assayed microbiologically for tylosin. The microbiological activity was noted to be influenced significantly by the presence of urea, added as a nonprotein nitrogen source. As seen in Figure 2, cattle feed which contained the basic ingredients 54% ground corn, 29% soybean meal, 4% fish meal, 4% animal fat, 2.5% alfalfa

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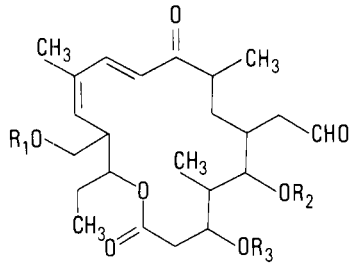

R_1 = Mycinose

R_2 = Mycaminose–mycarose R_3 = H

or R_2 = H R_3 = Mycaminose–mycarose

Figure 1. Structure of tylosin.

meal, 2% dicalcium phosphate, 1% dried whey, and 3.5% vitamin-mineral combination was maintained at 37° for 5 months without significant loss of potency. Addition of solid urea to a final level of 3% produced a 50% decrease in the tylosin assay during the next 5-month period.

THE REACTION OF TYLOSIN WITH UREA

Preliminary experiments were designed to study the fate of tylosin in the presence of extremely large excesses of urea in the absence of other feed components. When allowed to stand in open air at room temperature, dry mixtures of tylosin tartrate and urea in 1:15 molar ratios were found to decline slowly in antimicrobial activity; however, when tylosin tartrate was dissolved in saturated aqueous urea, it was rapidly converted to an antimicrobially inactive substance, TUA.

One of the first recognizable properties of TUA was that it is hydrolyzed back to active tylosin when subjected to a variety of pH and temperature conditions. Low pH's produced a more rapid release of tylosin from TUA; however, low pH's also favor cleavage of the sugar mycarose from tylosin to give the antibiotic desmycosin (Hamill *et al.*, 1961). Using a combination of thin-layer chromatographic isolation and microbiological assay techniques, it was determined that by rigidly controlling pH and temperature conditions, TUA could be hydrolyzed selectively to tylosin or desmycosin. Thus, by heating TUA in aqueous solution at 95°, it was hydrolyzed to tylosin in 30 min at pH 5.0 and to desmycosin in 1.5 hr at pH 3.2.

The physical and chemical properties of TUA suggest that it is a bisurea derivative, arising from the reaction of two urea molecules with the tylosin aldehyde group, as shown in Figure 3. The molecular formula, $C_{47}H_{83}N_5O_{18}$, based on that reported for tylosin (Hamill *et al.*, 1961), is consistent with the proposed structure. An additional

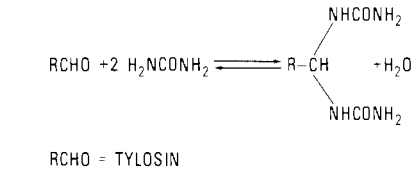

Figure 3. Reaction of tylosin with urea.

band at 1610 cm^{-1} in the infrared spectrum can be attributed to an amide carbonyl. The fact that the nmr spectrum of TUA does not exhibit a signal in the δ 9.7 region, where the aldehyde-proton signal characteristically occurs, indicates that urea reacts with tylosin at the aldehyde position. In addition, other macrolide antibiotics, such as spiramycin and magnamycin, which contain aldehyde groups were found to react with urea under similar conditions to yield analogous urea adducts. The bisurea structure, which is well documented (Zigeuner *et al.*, 1961) for simpler aldehydes, also serves to explain the ease with which TUA is hydrolyzed to tylosin.

Since the antibiotics used in this study were usually isolated in amorphous form and were difficult to crystallize, it should be mentioned that comparison with authentic samples by melting point was useless. Ultraviolet and infrared spectra were not particularly useful either; however, thin-layer chromatographic and nmr measurements were definitive in identifying substances isolated in this study.

Identification of TUA in Feeds Containing Inactive Tylosin. An understanding of the reversible nature of the tylosin–urea reaction allowed a reexamination of tylosin-containing feeds for the presence of TUA. Using synthetic TUA, as assay procedure was devised whereby 100% of the theoretical activity as tylosin and desmycosin could be regenerated from TUA; the data on which this assay was based are shown in Figure 4. This procedure involved autoclaving (120°, 15 psi) solutions of TUA at pH 5.5 for 20 min, followed by plate assay with *S. lutea*. This organism is useful to measure antibiotic concentration by plate assay, since the average zone size is virtually the same for tylosin and desmycosin between 0.05–3.0 μg/ml.

As illustrated by representative data in Table I, various feed samples were extracted and subjected to those conditions applicable to the regeneration of activity from synthetic TUA. In all of the samples tested nearly complete regeneration of antimicrobial activity was demonstrated, while chromatography and bioautography of the samples prior to and after the regeneration procedure yielded components with R_f values similar to TUA, tylosin, and desmycosin. TUA, tentatively identified by thin-layer chromatography, was lifted from the plate and eluted from the silica gel for further comparisons.

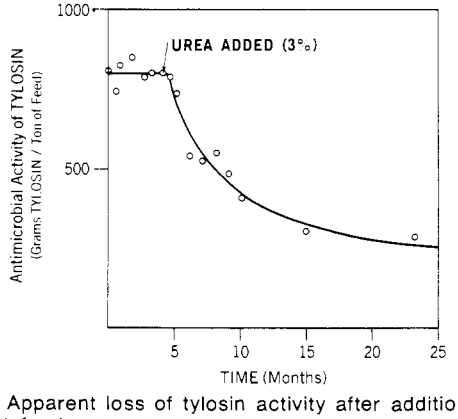

Figure 2. Apparent loss of tylosin activity after addition of urea to livestock feed.

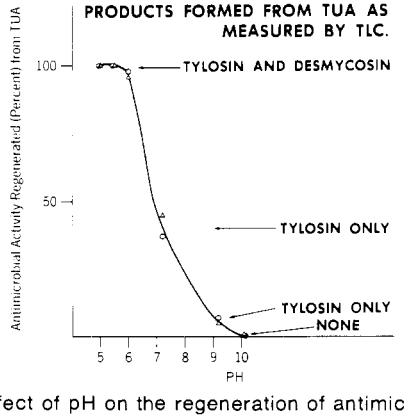

Figure 4. Effect of pH on the regeneration of antimicrobial activity from TUA.

The substance isolated from feed extracts and authentic TUA had identical R_f values in a number of solvent systems, and both were hydrolyzed under autoclave conditions to yield similar ratios of tylosin and desmycosin as a function of pH. In addition, controlled regeneration of both substances at constant pH and different temperatures yielded equivalent rate constants.

SUMMARY

The evidence presented in this report indicates that tylosin reacts with urea to form an antimicrobially inactive compound, which has been identified as TUA. The formation of TUA does not represent an actual loss of available tylosin.

Elucidation of this reversible tylosin–urea reaction affords the opportunity to investigate the application of TUA as a stabilized form of tylosin in urea-containing mixtures. Investigation is continuing to determine if TUA would be a useful therapeutic agent for ruminant production and management. The results of on-station and feedlot experience with TUA in cattle, as well as *in vitro* and *in vivo* hydrolysis and rumen function data, will be reported elsewhere.

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Effects of Different Classes of Pesticides on Pentobarbital Anesthesia and Toxicity in Japanese Quail

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Two hours after a single oral dose of DDT, methoxychlor, or malathion, Japanese quail responded with prolonged pentobarbital sleeping times. Prolonged sleeping times occurred 48 hr after DDT treatment. In contrast to DDT, sleeping times returned to control levels 24 hr after methoxychlor or malathion treatment. Abate reduced sleeping times, while Sevin had no effect. *Ad libitum* feeding of DDT or methoxychlor prolonged pentobarbital sleeping times, but malathion, Abate, or Sevin had little effect. Although very few birds died from the toxicity of the pesticide alone, birds died in many pesticide groups when pentobarbital was administered. Mortality was greatest during anesthesia when pentobarbibital had been administered 2 hr after a single oral dose of the pesticide. *Ad libitum* feeding of pesticides did not increase mortality during pentobarbital anesthesia.

In almost all species the liver is the major site for the metabolism of foreign chemicals (Bush, 1963). A standard dose of pentobarbital will produce a standard sleeping time, the duration of which is primarily dependent upon the detoxification of the barbiturate in the liver. An increase in liver microsomal enzyme activity will be reflected in greater metabolism of the barbiturate and a shorter sleeping time. An inhibition of liver microsomal enzyme activity would result in a longer barbiturate sleeping time.

We recently reported that *o,p'*-DDT and *p,p'*-DDT, as well as their metabolites, initially increased pentobarbital sleeping time in Japanese quail. After 2 weeks on the pesticide diet, sleeping times approached the control values, indicating that DDT effects on pentobarbital sleeping times were less, although pesticide lipid residues were accumulating (Bitman *et al.*, 1971).

In the present study we have compared compounds of several different pesticide classes (organochlorine, organophosphate, and carbamate) in their effects upon pentobarbital sleeping times in Japanese quail.

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EXPERIMENTAL PROCEDURE

Mature male and female Japanese quail, 40–80 days old, were housed individually on a schedule of 14 hr of light and 10 hr of dark. The quail were fed the pesticide in olive oil with a feeding needle or were fed *ad libitum* diets containing the pesticide. Two organochlorines (*p,p'*-DDT and methoxychlor), two organophosphates (malathion and Abate), and a carbamate (Sevin) were used. The pesticides were: *p,p'*-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethaned], 99+% pure, Aldrich Chemical Co.; technical DDT, Olin-Matheson Co. analyzed to be 70% *p,p'*-DDT; methoxychlor [1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane]; technical methoxychlor, Grade II, 90% Sigma; Sevin [1-naphthyl *N*-methylcarbamate], Union Carbide; Technical Sevin, 50% wettable powder, Union Carbide; malathion [*O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) dithiophosphate], 99.7%, American Cyanamid Co.; technical malathion, 92%, American Cyanamid Co.; Abate [*O,O,O',O'*-tetramethyl *O,O'*-(thiodi-*p*-phenylene) phosphorothioate], 99%, American Cyanamid Co.; technical Abate, 86.2%, American Cyanamid Co.

At appropriate times after administration of the pesticide, sodium pentobarbital was injected intramuscularly at a dosage rate of 50 mg/kg of male body weight or 60 mg/kg corrected female body weight. Body weights of the