Table II. Recovery of Procaine Penicillin from Commercially Available Premixes

Replication	Found, P.P.M.	
	Premix A	Premix B
1	11,415	15,540
2	11,081	15,340
3	11,430	15,885
4	11,016	14,400
5	10,545	14,498
6	10,920	15,430
7	10,856	14,598
8	10,502	15,440
9	11,119	14,995
10	10,897	14,845
Av.	10,878	15,187
Mean dev.	235	520
Std. dev.	310	652
Av. % recovery	98.8	110.3
Coeff. of variation	2.8	4.3

Premix A tag guarantee: 5 grams/pound or 11,013 p.p.m. of procaine penicillin and 15 grams/pound of streptomycin.

Premix B tag guarantee: 6.25 grams/pound or 13,765 p.p.m. of procaine penicillin and 18.75 grams/pound of streptomycin.

25-ml. volumetric flask. One milliliter of the mixed color reagent is added and the solution made up to volume with distilled water. After a 10-minute color development period, determine the intensity of the color. Centrifuge prior to determining the color intensity, if necessary. The difference between the absorbance value for the sample and that of the blank can be attributed to penicillin.

The 10-minute color development period in the modification was chosen because the reduction of the arsenomolybdate reagent by penicillin is minimal, while the reduction of the arsenomolybdate by the oxidation of penicillamine to the disulfide is maximal (Figure 1). Any penicilloic acid extracted from the premix will be cleaved to produce penicillamine and penaldic acid. Any penicillamine extracted as well as produced will be oxidized rapidly to the disulfide prior to significant reduction by penicillin alone.

In the premixes studied, the absorbance values in the modification ranged from 0.015 to 0.022 with the exception of premix II, which had a high value of 0.059. This abnormally high value is probably attributable to the high concentration of penicillin, 37,479 p.p.m.

In comparing the procedure and the modification, extremely small differences were found for the penicillin levels in the laboratory prepared premixes or the commercial premixes. Because of the minute differences, the determination of breakdown products was ignored in the determinations.

The analyst has considerable latitude in choosing sample size and volume of extraction solvent, but an aliquot of greater volume than 2 ml. is not recommended.

The advantages of this method are: simplicity, accuracy, a multiplicity of samples can be analyzed, and procaine penicillin can be determined accurately without the use of microbiological procedures.

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FEED ADDITIVES

Stability of Several Oleandomycin Derivatives in Livestock and Poultry Feed Products

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Since its discovery in 1954, oleandomycin has proved to be a highly effective chemotherapeutic agent. It also elicits substantial growth responses in livestock and poultry. However, the common pharmaceutical derivatives were unstable in feed products, which made their agricultural application unfeasible. Extensive laboratory screening and plant-scale evaluation resulted in the development of a new oleandomycin resin adsorbate for agricultural use. This product exhibits feed stability which equals or surpasses that of many other commercial antibiotics, while retaining full biological activity.

OLEANDOMYCIN (Matromycin, Chas. Pfizer & Co.), first announced by Sobin, English, and Celmer in 1954 (*18*), has received wide acceptance in clinical therapy. When this antibiotic was evaluated as a growth promoter for chickens and turkeys, excellent responses were obtained at levels as low as 2 grams per ton of feed (*2, 4, 13, 16, 17, 19–21*). Substantial growth increases over control birds were observed during 5 years of repeated tests under conditions where penicillin fed to parallel groups often elicited only slight responses. Oleandomycin also exerted strong growth-promoting action in swine, cattle, and sheep (*8, 9, 14, 15*). When it appeared that oleandomycin might find application in commercial feeds as a growth promoter, the product formulation laboratory at the Pfizer Agricultural Research Center attempted to develop a suitable feed supplement containing this antibiotic.

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Materials and Methods

Stability studies comprise laboratory-scale tests and commercial pelleting operations.

Laboratory-Scale Tests. Five-pound portions of a master basal ration were supplemented with each test antibiotic product and thoroughly mixed in a Patterson-Kelley twin-shell blender. About 0.5 pound was withdrawn for initial assays, and the remaining portion transferred to amber glass screw cap bottles for elevated temperature storage. Bottles were kept sealed during storage

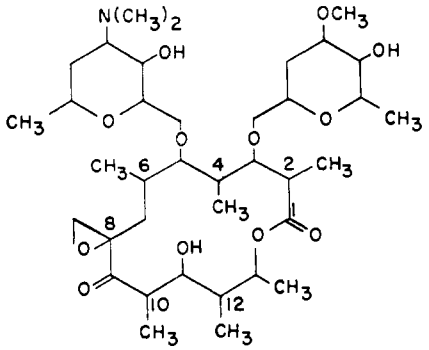

Figure 1. Oleandomycin base

and allowed to cool to room temperature prior to interim sampling. Moisture determinations were performed (5 hours, 100° C., 20 mm. of Hg) on each initial and interim sample, and assay results were corrected accordingly as required.

If tests were also to be conducted under controlled humidity, portions of each blend were transferred to 6 × 15 inch porous cotton bags. The bag material, the same as that used for commercial cotton feed bags, permitted free moisture passage during storage. Storage conditions were 70% R.H. at 30° C. Under these conditions, in rapidly circulating air, the test feed blends of Basal Ration 27 exhibited an equilibrium moisture content of about 13.25%.

Earlier studies in our laboratories indicated that commercially available laboratory incubators were inadequate to handle the dozens of test feed bags in any one test. Space requirements were substantial since each bag required unobstructed air flow on all sides to ensure uniformity in moisture from bag to bag. Several Robbins Egg Incubators, Model 26 I, were modified for this work. Egg trays were replaced by special trays having bottoms of coarse wire mesh. Alternate tray spaces were kept vacant to promote free air passage. Bags were laid on their sides, an inch between adjacent bags. Temperature was controlled thermostatically. Humidity was recorded on a humidigraph, and adjustments were made manually as required.

In more elaborate tests under three or more storage conditions, batch size was increased to 20 pounds, and mixing was performed in a 20-quart capacity Hobart mixer. All basal feeds were premilled to pass a U. S. No. 20 Sieve to avoid microingredient segregation.

Commercial Pelleting Operations. Ten-pound premixes, containing sufficient quantities of vitamins and of each test antibiotic product to fortify 1000 pounds of poultry feed, were prepared on the day the feed was to be pelleted in a commercial feed mill. After thorough mixing of the mash, representative ten-pound samples were drawn from the blender just prior to pelleting. Processing data such as temperatures, moistures, per cent fines, etc., were recorded for each batch. Data on equipment used and on operating variables are described elsewhere (*22*).

Ten-pound portions of the cooled $^3/_{16}$-inch pellets were sampled "across the batch" by a grab cup as they streamed down a chute from the sifter. All samples of mash and pellets were stored in amber glass screw cap bottles. Stability studies were conducted on pelleted feed which had made only a single pass through the pellet mill die. Fines were not recycled to eliminate this variable. Both mash and pellet samples were reblended to ensure uniformity before storage and assay. Pellets, about 1 pound, were freshly ground and reblended for assay initially and at each interim period.

The estimated variation in each specific stability test is shown below the corresponding table. Values for per cent retention of antibiotic potency in each table are averages of at least four microbiological assays performed on separate samples on successive days. The value for "test variation" therefore includes errors due to nonuniformity of the heterogeneous feed products and to inherent low precision of the microbiological assay.

Table I. Feed Supplement Diluent Formulas

Ingredient	All-Cereal Formula, %	Part-Cereal Formula, %	All-Mineral Formula, %
Soybean meal, 44%	30	5	..
Soybean mill feed	70	55	..
Granular limestone	..	40	48.5
Powdered limestone	..	..	48.5
Tricalcium phosphate	..	..	3.0
Moisture, %	11.0	7.0	0.1

Results

Although a number of derivatives of oleandomycin have been described in the literature (*5–7, 10, 12*), only four chemical forms were selected for initial tests. These were the phosphate, a chloroform solvate, the triacetyl ester, and the base of oleandomycin (Figure 1). In a preliminary screening, each of these four forms was blended into three feed supplement formulations consisting of all-cereal, all-mineral, and part-cereal part-mineral diluents. The antibiotic sources were added at a level of 1 to 4 grams of activity per pound. Formulas of the diluents are shown in Table I and stability results in Table II. Potency losses were greatest in the all-cereal diluent and least in the all-mineral diluent. This might be a function of the moisture content, since this was highest in the all-cereal formula.

Further tests were made with these four forms of the antibiotic in a commercial broiler mash with results as shown in Table III.

The compositions of the basal rations employed in the various stability tests are shown in Table IV.

High antibiotic levels were necessitated here because an assay sensitive enough to measure accurately 2 grams of oleandomycin per ton of feed was still being developed. This and other early feed tests were therefore conducted using levels of 200 to 500 grams per ton.

Effect of pelleting on stability was tested with two experimental lots of oleandomycin chloroform solvate blended into a second broiler mash and pelleted on standard commercial equipment. Results are shown in Table V.

Data in Table V indicate that the steam, heat, and pressure of feed pelleting contribute substantially to potency losses. The principal difference between the two lots in Table V was in crystallography. Upon microscopic examination, Lot 203 showed a structure of microcrystals of about 5-micron size, cemented together into agglomerated

Table II. Stability of Oleandomycin Derivatives in Various Diluents

Oleandomycin Form	Type of Diluent	Antibiotic Potency Retention at 45° C.,[a] % 30 days	60 days
Base	All cereal	26	5
	Cereal-mineral	42	13
	All mineral	..	..
Chloroform solvate	All cereal	34	24
	Cereal-mineral	45	30
	All mineral	100	104
Phosphate	All cereal	23	8
	Cereal-mineral	39	23
	All mineral	..	..
Triacetyl ester	All cereal	58	39
	Cereal-mineral	70	71
	All mineral	92	92

[a] Estimated test variation, ±10%.

Table III. Stability of Oleandomycin Derivatives in Broiler Mash (Basal Ration 25A)[a]

Oleandomycin Form	Lot No.	Initial Potency, G./Ton	Potency after 30 Days at 45° C., G./Ton	Potency Retention,[b] %
Base	101	486	224	46
Base	102	460	277	60
Chloroform solvate	201	495	275	56
Chloroform solvate	202	526	264	50
Triacetyl ester	301	495	96	19
Phosphate	401	432	281	65

[a] See Table IV for mash composition.
[b] Estimated test variation, ±15%.

Table IV. Composition of Experimental Feeds

Ingredients	Broiler Ration Basal 25A, %	Broiler Ration Basal GM-1, %	Swine Concentrate Basal 27, %
Ground yellow corn	55.45	55.5	...
Soybean oil meal, 44%	26.94	20.0	73.7
Alfalfa meal, 17%	2.00	3.75	9.5
Ground oats	...	5.0	...
Fish meal, 60%	4.00	2.5	...
Meat scrap, 60%	...	3.75	...
Meat and bone scrap, 50%	...	...	6.0
Corn gluten meal	2.50	...	...
Wheat mixed feed	...	2.5	...
Stabilized animal fat	2.50	3.0	...
Brewers yeast	1.50	...	...
Dried corn distillers solubles	1.00	...	...
Delactosed whey, 50%	1.00	...	...
Dicalcium phosphate	1.00	...	5.0
Defluorinated rock phosphate	...	0.75	...
Iodized salt	0.40	0.35	...
Trace mineralized salt	...	...	2.5
Limestone flour	...	1.50	...
Delamix trace mineral mix	0.10	0.15	...
3-Nitro powder, 10%	...	0.0375	...
Nicarbazin, 25% premix	...	0.25	...
Vitamin-antibiotic premix	1.61	0.97	3.3
Av. moisture content, %	11.9	13.2	8.0

Table V. Stability of Oleandomycin Chloroform Solvate in Broiler Mash and Pellets (Basal GM-1)[a]

Lot No.	Initial Potency, G./Ton		Retention in Pellets, %	Potency after 30 Days at 30° C.[b]			
				Mash		Pellets	
	Mash	Pellets		G./ton	Retention, %	G./ton	Retention, %
203	502	468	93	254	51	116	23
204	541	450	83	107	20	145	27

[a] Table IV for mash composition.
[b] Estimated test variation, ±15%.

Table VI. Stability of Experimental Oleandomycin Products in a Feed Supplement and a Broiler Mash

Oleandomycin, Experimental Form	Lot No.	Potency Retention,[a] %			
		All-Mineral 25% Supplement[b]		Broiler Mash 25A	
		3 Weeks, 50° C.	6 Weeks, 25° C.	3 Weeks, 50° C.	6 Weeks, 25° C.
Lauryl sulfate	501	106	104	40	81
Lauryl sulfate	502	102	98	31	79
Methylene disalicylate	601	92	100	10	80
Methylene disalicylate	602	87	103	11	84
Tannate	701	82	96	46	82
Tannate	702	77	92	50	91
Trichloroethane solvate	801	104	98	7	51
Pamoate	901	98	87	78	105
Clay adsorbate	1001	20	..	57	..
Phosphate in wax	1101	92	95	68	86
Phosphate in gelatin	1201	73	95	40	88
Resin adsorbate	1301	90	87	97	111
Chloroform solvate (control)	205	104	97	7	51

[a] Estimated test variation, ±10% in supplement, ±15% in feed.
[b] Supplement contained 250 grams of oleandomycin activity per kilogram.

balls. Under low-power magnification (25X), these masses looked like snowballs. Lot 204 was crystallized more slowly to produce larger, intact crystals of approximately 100-micron size. While stability in mash in this test might suggest superiority of the snowball type of crystal, the combined effects of steaming, compression, and high temperature during pelleting rendered both lots equally unstable in pellets. Since it is estimated that over 70% of all poultry feeds are pelleted or crumbled (*22*), the chloroform solvate was unsuitable for feed use. The poor stability of the base, phosphate, and triacetyl ester forms of oleandomycin in mash feeds indicated that these forms also would be unsuitable for addition to feeds on a commercial basis.

However, the low potency retentions observed in feed blends suggested that the antibiotic was being bound by some feed component resulting in incomplete extraction. A variety of solvents and solvent mixtures was evaluated in an attempt to increase oleandomycin recovery from aged feed blends. None of these procedures raised the assay values significantly.

Further work with oleandomycin crystals coated with moisture-resistant films, such as fatty acid compounds, vegetable oils, mineral oils, etc., showed no improvement in stability. Since an oxidative inactivation might be involved, several phenolic-type and other commercial antioxidant products were applied to the oleandomycin crystals as intimate dry mixtures or as oil solutions. These treatments showed no advantages over controls.

In addition, the effect of decreasing exposed surface area of the crystals was investigated. Feed blends were prepared containing one type of chloroform solvate crystals varying in length from 75 to 300 microns. No significant differences in stability were attributable to crystal surface area.

An anomaly seemed to exist with reference to the stability of the triacetyl ester of oleandomycin. In complete feeds, bio-inactivation took place rapidly, but this ester was stable in mineral diluent formulations. It was likewise stable in pharmaceutical capsules and tablets. This difference could be attributed to the low moisture of the mineral and dry pharmaceutical vehicles as contrasted with that of complete feeds. However, a factor other than moisture must be involved in deterioration in feeds inasmuch as no significant potency loss was observed from an aqueous preparation of triacetyloleandomycin after 3 weeks of storage at 37° C. (*5*). Feed pH is apparently not a factor as optimum stability of this ester is in the pH range of 5.0 to 7.0, which is the normal range for complete feeds.

Table VI presents stability results

Table VII. Stability of Experimental Oleandomycin Resin Adsorbates in Broiler Mash (Basal Ration 25A)

Resin Adsorbate Lot	Potency Retention,[a] % 3 Weeks, 50° C.	12 Weeks, 25° C.
1302	92	91
1303	95	87
1304	77	86
1305	82	92
1306	77	97
1307	90	90

[a] Estimated test variation, ±15%.

with nine other experimental oleandomycin products after storage in a broiler mash and in an all-mineral diluent feed supplement. Initial potency in this supplement was 250 grams of oleandomycin activity per kilogram.

While potency retention by these experimental forms of oleandomycin was generally good in all-mineral, low-moisture carriers, only a few showed satisfactory antibiotic potency retention in a practical poultry ration. The excellent stability exhibited by the adsorbate on ion exchange resin prompted further investigation of this form. Several experimental resin adsorbates were prepared. Results from a series of batches of these experimental resin adsorbates in a broiler mash are shown in Table VII.

Resin adsorbates were also evaluated in poultry and livestock growth tests. While this paper does not include feeding data, it can be stated that the resin adsorbates give growth increases equivalent to the four less stable forms tested earlier. In feeding trials, experimental diets were mixed biweekly and were not pelleted. Hence, stability was not a test variable.

In the pharmaceutical industry, stabilization by adsorption on ion exchange resins has been accomplished with many types of drugs. These include amphetamine, fungicides, antihistamines, antibiotics, tranquilizers, prednisolone, hydrocortisone, aspirin, and vitamin B_{12} (*3*). So far as is known, the present report is the first which describes the commercial application of this technique for the stabilization of a feed additive. The finished oleandomycin resin adsorbate is milled to a range of 80 to 200 mesh to minimize segregation and to ensure optimum distribution in complete feeds (Figure 2).

The problem of a more sensitive assay procedure for oleandomycin in feeds had been under investigation concurrently with the stability studies. At this point in the testing program, a workable procedure was devised which permitted determination of oleandomycin at the low levels used in feeds (1 to 10 grams per ton) (*11*). This procedure follows normal microbiological plate diffusion assay techniques. Briefly, a 20-gram feed sample is extracted with 2% sodium bicarbonate–phosphate buffer solution (pH 7.8–8.0), filtered, and diluted to 0.1 μg. per cc. Aliquots of feed extract are pipetted into cylinders placed on agar plates previously inoculated with *Sarcina lutea* (ATCC 9341). Zones of inhibition are read after 16 to 18 hours incubation at 37° C.

Results are calculated from a standard curve prepared by adding oleandomycin assay standard to inactivated feed extract. This extract is prepared by autoclaving 100 grams of the same feed at 20 p.s.i. for 90 minutes. The autoclaved feed is then extracted and diluted like the original sample. Feeds containing oxytetracycline (Terramycin, Chas. Pfizer & Co.), are treated similarly using *Staph. epidermidis* A.T.C.C. 12228 (formerly designated *Staph. albus* P.C.I. 1200 A.) as a test organism for oleandomycin.

Table VIII summarizes results from two commercial pelleting tests in which seven lots of oleandomycin resin adsorbate were evaluated for stability at levels suitable for use in feeds. For comparison, results are given on stability of five other antibiotics currently used in animal feeds. All antibiotics were tested in Basal Ration GM-1. Antibiotic activity levels employed were: oleandomycin, 200 grams per ton; oxytetracycline, 175 grams per ton; Aureomycin, 200 grams per ton; Bacitracin, 160 grams per ton; erythromycin, 200 grams per ton; and procaine penicillin, 200 grams per ton. Assays were

Table VIII. Stability of Oleandomycin Resin Adsorbate in Broiler Feed (Basal Ration GM-1)

Product	Lot No.	Potency Retention,[a] % 30 Days, 30° C. Mash	Pellets	60 Days, 30° C. Mash	Pellets
Oleandomycin resin adsorbate	1308	92	83	90	78
	1309	99	93	98	83
	1310	76	71	70	63
	1311	78	76	71	66
	1312	74	68	70	60
	1313	79	90	73	79
	1314	93	77	90	70
Oleandomycin chloroform solvate	204	31	27	15	10
Oxytetracycline	PF-3578-A	94	91	73	76
Chlortetracycline	21220-2	99	76	84	67
Bacitracin, zinc salt	7-B-4	94	74	74	55
Erythromycin thiocyanate	697-1577-22	86	64	65	20
Procaine penicillin	55028	96	63	91	56

[a] Estimated test variation at low use levels is ±20%.

Table IX. Stability of Oxytetracycline-Oleandomycin 8 + 2 Mixture in a Swine Concentrate (Basal Ration 27)[a]

Antibiotic	Lot No.	Potency Retention after 90 Days Storage,[b] % 25° C.	37° C.	70% R.H. at 30° C.
Oxytetracycline	1401	98	83	81
Oleandomycin resin	1315	79	62	64
Oleandomycin resin +	Same lots	77	74	70
Oxytetracycline		86	76	90
Oxytetracycline	1402	97	76	63
Oleandomycin resin	1310	80	77	55
Oleandomycin resin +	Same lots	90	77	61
Oxytetracycline		89	86	65
Oxytetracycline	1403	88	78	66
Oleandomycin resin	1316	84	83	67
Oleandomycin resin +	Same lots	90	65	67
Oxytetracycline		95	84	65
Av. oleandomycin resin	All 3 lots	83	73	64
Av. oxytetracycline	All 3 lots	92	81	72

[a] Table IV for ration composition.
[b] Estimated test variation, ±15%.

Table X. Stability of Oxytetracycline-Oleandomycin 8 + 2 Mixture in Pelleted Swine Concentrate (Basal Ration 27)

Antibiotic	Lot No.	Potency Retention at 25° C.,[a] % 30 Days Storage Meal	Pellets	90 Days Storage Meal	Pellets
Oleandomycin resin +	1315	93	75	74	72
Oxytetracycline	1401	83	77	77	77

[a] Estimated test variation, ±15%.

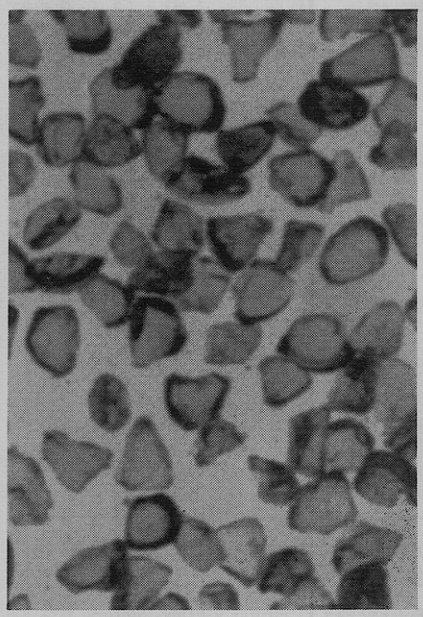

Figure 2. Oleandomycin resin adsorbate after milling—magnification 20×

performed microbiologically using A.O.A.C. methods (*1*) for oxytetracycline, chlortetracycline, penicillin, and bacitracin. Erythromycin assays were comparable to those for oleandomycin, except for substitution of erythromycin in standard curve preparation.

Recent research (*14*) has shown that a mixture of oleandomycin and oxytetracycline (TAOmyxin, Chas. Pfizer & Co.) is highly effective as a growth stimulant for swine. Two stability experiments were conducted with antibiotic mixtures of this type in a swine protein concentrate. Table IX summarizes stability data obtained with three lots of oleandomycin resin adsorbate in a swine concentrate under three conditions of storage. Oleandomycin was added at 10 grams per ton and oxytetracycline at 40 grams per ton. The stability of the antibiotic mixture was compared to that of each antibiotic alone. The swine concentrate was in meal form.

These results indicate that oleandomycin resin adsorbate stability is approximately equivalent to that of oxytetracycline, and that mixtures of the two antibiotics have the same stability characteristics as their components.

Table X shows stability results of an 8 + 2 mixture of oxytetracycline and oleandomycin resin adsorbate in the same swine concentrate after commercial pelleting and extended storage. The 8 + 2 mixture contained 8 grams of oxytetracycline, and 2 grams of oleandomycin activity as the resin adsorbate per pound, in an all-cereal diluent. Levels were the same as in the previous experiment.

Although the degree of stability at 30 days was somewhat greater in meal, there was no significant difference between meal and pellets at 90 days.

Conclusions

Thirteen oleandomycin derivatives and/or product forms were evaluated for stability in poultry and livestock feeds and in feed supplement formulations. While potency retention was generally good in all-mineral, low-moisture carriers, only a few oleandomycin sources showed satisfactory antibiotic potency retention in practical poultry rations. Attempts to improve stability by using coatings, stabilizers, and crystallographic modifications were unsuccessful. The best stability was exhibited by oleandomycin absorbed on ion exchange resin. Consistently high stability was found with a number of lots of oleandomycin resin adsorbate incorporated into poultry feed at use levels. Comparative data showed stability of the oleandomycin resin adsorbate to be equal to or better than that of many other antibiotics commonly used in animal feeds. No adverse stability interaction occurred when oxytetracycline-oleandomycin mixtures were incorporated into a swine concentrate at use levels of 10 grams of oleandomycin and 40 grams of oxytetracycline per ton.

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