Stability of Various Penicillin Sources in Animal Feed Products

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The stability of the potassium and calcium salts of penicillin G, and of various sources of procaine penicillin G and benzathine penicillin G, was compared in laboratory tests at 37° C. and 88% RH and in autoclaving, as well as in animal feeds at 25°, 30°, and 30° C. and 70% RH and at 45° C. and 70% RH, both before and after commercial pelleting operations. Optimum stability in pelleted feeds was exhibited by intact procaine penicillin G crystals of the monoclinic hemimorphic system, having an average axial ratio of 2 to 1 and an average length of about 200 microns. Potency retention in 19 batches of meal feeds averaged 91% after 12 weeks' storage. The initial potency loss during 29 commercial pelleting runs averaged 8%. The effect of penicillin stability on animal growth responses, the limitations in assay methodology which influence stability data, and penicillin degradation mechanisms in feed products are discussed.

The earliest published papers on penicillin reported the inherent lability of the molecule (Abraham and Chain, 1942; Clutterbuck *et al.*, 1932; Fleming, 1929). Subsequent pharmaceutical studies showed that all the major penicillin salts were unstable in aqueous solutions. The optimum pH was 6.0 to 6.8 and any deviation from this narrow range greatly accelerated potency losses (Benedict *et al.*, 1945, 1946; Coulthard *et al.*, 1951; Scott *et al.*, 1954). While dry penicillin salts were stable indefinitely, their exposure to atmospheric humidity could result in 80% potency loss in a few months (Brindle and Keepe, 1947).

With the discovery of animal growth stimulation by antibiotics (Moore *et al.*, 1946), and the almost concurrent appearance of scientific reports on poor penicillin stability, research began in several laboratories to develop a penicillin premix which would be stable in manufactured feed products. The early prognosis for success was dubious because of the known moisture content of feeds ranging from 10 to 14%, and because most commercial feeds at that time were often stored in bags for several months before use. Even more discouraging was the fact that over 50% of commercial feed products were subjected to a pelleting process which involved preliminary steaming, plus exposure to high frictional heat during compression in the pellet mill die.

The early reports on penicillin stability in feeds presented a grim picture of high losses during commercial pelleting processes and from steam treatment in laboratory tests (Esposito and Williams, 1952a; Stokstad *et al.*, 1952; Williams *et al.*, 1953). These authors provided no data as to the physical or chemical properties of the penicillin sources used in their studies. This created the mistaken impression that all penicillin sources were equally unstable in feed products and processes.

Subsequent reports stated that a specially prepared type of procaine penicillin G was stable in feed products (Hollenbeck *et al.*, 1954; Wornick *et al.*, 1959). Neither of these reports described this improved product form, nor presented detailed information on potency losses in stored meal and pellets. These early reports could be interpreted as indicating that if a penicillin source were stable in the pelleting operation, it would also be stable in the pellets during subsequent storage before being fed.

After 18 years of penicillin usage in livestock and poultry feeds, there is still much confusion on the subject of penicillin stability. The increasing quality control activities by feed manufacturers, and inspection activities by state and federal regulatory agencies, have brought a renewed interest in potential penicillin stability problems. Penicillin decomposition in feed products during storage may reduce growth responses in animals, as well as prophylactic and therapeutic efficacy against various disease conditions. Inadequacies in feed assay methodology have contributed questionable stability data. This report, which summarizes 8 years of research, is designed to fill an obvious gap in the literature on this important and timely subject.

PROCEDURE

The data presented in this report were derived from a series of laboratory-scale tests, and from commercial feed pelleting operations. The preparation of feed blends in the laboratory and their storage under controlled temperature and/or humidity conditions are described in detail elsewhere (Wornick and Kuhn, 1962), as well as premix preparation and details of meal and pellet manufacture and sampling in local feed manufacturers' plants. Data on manufacturing equipment, process variables, and broiler feed formulations employed in each test have been presented (Wornick *et al.*, 1959). Special test changes are described below.

All penicillin assays shown were performed by the cylinder–agar plate–diffusion method using *Sarcina lutea* ATCC 9341. Feed samples were extracted with either formamide or 50% aqueous acetone solution. Each potency value shown in the accompanying tables represents an average of duplicate determinations performed on each of 2 to 4 successive days. The "test

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Table I. Stability of Various Penicillin Salts in Poultry Meal at 25° C.

Compound	Activity as Sodium Penicillin G, Grams/ Kg. Initial	1 week	2 weeks	3 weeks	3-Week Retention, %
1. Potassium penicillin G, crystalline	1.58	0.27	0.17	0.12	7.6
2. Calcium penicillin G, amorphous	1.40	0.43	0.23	0.15	10.7
3. Procaine penicillin G, crystalline	1.49	1.34	1.29	1.33	89.2
4. Beryllium penicillin G, powder	0.98	0.60	0.40	0.28	28.5

Table II. Properties of Major Penicillin Salts

Compound	Potency, Units/Mg.	Melting Point, °C. (Dec.)	$[\alpha]_D^{25}$ H_2O	H_2O Solubility, Mg./Cc., 25° C.
1. Penicillin G, sodium	1667	215	+301°	> 1000
2. Penicillin G, potassium	1595	214–17	+285–310°	> 1000
3. Penicillin G, procaine	1009	106–10	+173° [a]	6.8
4. Benzathine penicillin G	1211	123–4	+206° [b]	0.15

[a] In 50% aqueous acetone.
[b] In formamide.

variation" encountered was approximately ±10%, which includes errors due to nonuniformity of the heterogeneous feed products, plus the inherent low precision of the microbiological assay.

Since penicillin G was known to be the most stable type, it was used exclusively during this project. The term "penicillin" in this paper refers only to benzylpenicillin or penicillin G. The term "benzathine penicillin" refers to *N,N'*-dibenzylethylenediamine dipenicillin G.

RESULTS

Penicillin Premix Development. The earliest attempts to produce a penicillin-containing premix for feed use involved vacuum drying of fermentation broths, or of solvent extracts or eluates, either as is or on various cereal carriers. Assays on the resulting products showed rapid potency losses—e.g., 75% in one day. Since freeze-drying, as utilized in pharmaceutical processing, was prohibitive in cost for agricultural applications, this approach was quickly dropped. Attempts were then made to prepare adsorbates of penicillin on such materials as carbon, fuller's earth, etc., followed by drying and blending with diluents into premixes. However, large potency losses occurred in a few hours, so the use of solutions as starting penicillin sources was abandoned.

The next early approach was to incorporate available pharmaceutical types of a pure dry penicillin salt into a cereal or mineral diluent. The sodium, potassium, and calcium salts were fairly stable in low moisture premix formulations, but lost potency rapidly in complete feed products. After the discovery of procaine penicillin (Salivar *et al.*, 1948; Sullivan *et al.*, 1948), emphasis shifted to the evaluation of water-insoluble penicillin salts. Early testing showed the procaine salt to be a more promising compound for further research because of its superior feed stability (Table I). Shortly thereafter, *N,N'*-dibenzylethylenediamine dipenicillin was developed (Elias *et al.*, 1951). This compound exhibited aqueous solubility about 1/45 that of procaine penicillin (Szabo *et al.*, 1951). It was therefore included in the early stability studies in feed products. The pertinent physical properties of these early penicillin salts are summarized in Table II.

Benzathine Penicillin Evaluation. Stability studies during 1951–2 on this new compound showed 4-week potency retention of 96% in poultry meal, which contained about 10% moisture, and was stored at 30° C. and 60% RH (Chas. Pfizer & Co., 1952). Six production premix batches diluted in poultry feed were compared to the pure derivative, and to commercial premixes containing procaine penicillin, in commercial feed pelleting tests. The results (Table III) indicate good stability, which contradicted several published reports and encouraged further research.

Since the steam employed in feed pelleting operations was then believed to be responsible for potency losses, a series of rapid laboratory tests was performed in an autoclave. Small samples of undiluted penicillin salts were exposed in Petri dishes to a steam pressure of 5 p.s.i.g. for periods up to 60 minutes. Results from

Table III. Stability of Benzathine *vs.* Procaine Penicillin during Feed Pelleting

Penicillin Source	Lot No.	Potency Retained in Fresh Pellets,[a] %
1. Benzathine, pure crystalline	31452	91
2. Benzathine, production batch	078-1	94
3. Benzathine premix P-2[b]	V9-0722	88
4. Benzathine premix P-2	V9-0832	75
5. Benzathine premix P-2	V9-0842	79
6. Benzathine premix P-2	V9-0852	84
7. Benzathine premix P-2	V9-0862	99
8. Benzathine premix P-2	V9-0872	75
9. Procaine, pure crystalline	0632-Z-SP	97
10. Supplier 1, procaine premix	V-862	89
11. Supplier 1, procaine premix	5PF15683I	68

[a] Feed potency = 1000 units per gram.
[b] Benzathine penicillin premix potency = 2 grams per pound.

Table IV. Stability of Benzathine vs. Procaine Penicillin after Autoclaving at 5 P.S.I.G.

Exposure Time, Min.	Benzathine Penicillin, %		Procaine Penicillin, Micronized Crystals, %	
	Pure crystals, lot 31452, 1180 units/mg.	Production run, lot 265-1, 1020 units/mg.	Lot LBQ-520129, 1000 units/mg.	Lot LBQ-520128, 990 units/mg.
1. 0	100	100	100	100
2. 5	100	104	100	101
3. 10	99	99	101	102
4. 30	99	76	0	0
5. 60	85	0	0	0

Table V. Stability of Benzathine *vs.* Procaine Penicillin at 37° C. and 88% RH

Penicillin Source	Potency Retention,% [a]						Final Moisture, %
	0 days	6 days	10 days	13 days	17 days	24 days	
1. Benzathine, pure crystalline lot 31452	1270 units/mg.	94	92	96	82	96	6.9
2. Procaine, pure crystalline lot JBQ-512521	1090 units/mg.	97	94	95	94	—	3.6
3. Benzathine P-2 premix lot V9-0722F	2.32 g./lb.	77	68	69	59	—	6.2
4. Procaine premix supplier 1, V-862	3.48 g./lb.	84	80	77	66	—	20.4

[a] Initial assays and interim potencies expressed on dry basis.

a typical run (Table IV) suggest that benzathine penicillin may have somewhat greater temperature resistance than procaine penicillin. This would be expected, since it has a higher melting point. Commercial feeds are exposed to steam for less than a minute in a pellet mill conditioning chamber. It would therefore seem unlikely that either procaine or benzathine penicillin potency is significantly affected by steam in so short a time. Early stability reports, based on 30-minute steaming, undoubtedly produced far greater losses than would actually occur in normal feed pelleting operations.

In another series of laboratory tests, the influence of humidity was studied. Small samples of pure penicillin salts, and of premixes, were exposed in desiccators at 37° C. and 88% RH (over saturated K_2CrO_4 solution). Table V shows that humidity exerted a negligible effect on the pure penicillins during this test, whereas the activity in the two premixes fell gradually, undoubtedly as the result of moisture pickup by the premixes due to the hygroscopic properties of the diluents used. Such high moisture increases would obviously not occur in bags of premixes during normal warehouse storage.

While these studies were being conducted, procaine penicillin premixes became available from a number of suppliers (Ott, 1956). Commercial pelleting tests on seven such premixes diluted in poultry feed showed potency losses from 12 to 60% during pelleting (Chas. Pfizer & Co., 1952). An unexplainable variation in stability existed between different suppliers' premixes. Test results through 1953 showed benzathine penicillin to exhibit stability characteristics equal or superior to those of procaine penicillin. As a result, benzathine penicillin premixes were well accepted and widely used in the feed industry. At that time, these premixes were marketed under the trade name of diamine penicillin and described thus in the literature. Additional commercial pelleting tests on 16 lots of benzathine penicillin premixes diluted in poultry feed showed an average retention of 88% in fresh pellets (Chas. Pfizer & Co., 1953).

During these early years several other penicillin compounds were evaluated in feeds by various researchers. These included the paludrine and the di-*p*-chlorophenylbiguanide derivatives (Esposito and Williams, 1952a) and 1-erythroephenamine penicillin G (Buckwalter, 1956). None of these products was ever commercially accepted for use in the feed industry.

Procaine Penicillin. Because of the increasing use of procaine penicillin in human and veterinary medicine, process economics began to favor it over the benzathine salt for use in feeds. Effort was therefore directed toward development of a type of procaine penicillin which would show improved potency retention in commercial feed products. Our earlier results (Tables I, III, and V) had suggested that pure crystalline procaine penicillin showed promising stability in poultry feeds, commercial pelleting, and high humidity. A series of commercial feed pelleting tests was conducted to evaluate a wide variety of pharmaceutical grades of procaine penicillin.

TEST 4.7-7. One early test (Table VI, batches 1 to 6) quickly revealed that the initial pelleting loss was not a reliable criterion of procaine penicillin stability in pelleted feed products after storage. Retention was far better in meal than in pellets. Micronized procaine penicillin, U.S.P., in batch 1, was unstable in both media, however. Crystals finer than 25 microns were therefore excluded from use in feed premixes. Pre-

Table VI. Stability of Various Procaine Penicillins in Broiler Meal and Pellets

Batch No.	Procaine Penicillin Source	Procaine Penicillin, Grams/Ton at 25° to 30° C. Initial meal	Initial pellets	4-week pellets	8-week meal	8-week pellets	8-Week Pellet Retention, %
1.	31102-98, micronized, U.S.P.	403	364	22	17	10	3
2.	54002-RZ, crystalline	440	353	79	157	64	15
3.	Premix X-0607-D, as is	423	394	84	164	48	11
4.	Premix X-0607-D, stearated	452	394	65	175	66	15
5.	Supplier 1, 50404 premix	457	407	149	272	111	24
6.	SPZ-ML, X-0607-G	370	362	33	76	17	5
7.	Supplier 1, M-137-4	507	475	368	455	290	57
8.	Supplier 2, P1-200	450	423	100	167	36	8
9.	190-8, crystalline	502	473	276	426	177	35
10.	190-9, crystalline	480	457	158	366	72	15
11.	54328-Z, crystalline	485	444	145	249	68	14

treatment of the penicillin in batch 4 with calcium stearate, a common pharmaceutical moisture repellent, was completely ineffective in reducing potency losses. The types of pure crystalline procaine penicillins used in batches 1 and 2 were no more stable than a semi-refined grade used in batch 6. A commercial procaine penicillin premix from another major supplier in batch 5 also showed poor stability during storage in feed.

TEST 4.7–10. Concurrent studies in the plant and laboratory had provided experimental batches of procaine penicillin with modified crystallographic characteristics. These were compared to premixes from two major suppliers in another commercial pelleting test (Table VIII, batches 6 to 10). The initial pelleting losses again showed no correlation with stability in pellets during storage, so these initial data were disregarded in all further tests. The crystals from lot 54030-RZ in batch 8 showed promising stability. The stability of premixes from the two different suppliers in batches 6 and 9 varied widely. Potency retention in meal was again far superior to that observed in pelleted feed.

TEST. 4.7–11. A similar commercial feed pelleting study (Table VI, batches 7 to 11) compared three new experimental lots of crystalline procaine penicillin to commercial premixes from two major suppliers. The premix in batch 7 from supplier 1 showed better stability than the other products tested. However, retention was poorer than with another lot of premix from the same supplier, evaluated in the previous test (Table VIII, batch 6). Potency retention of premixes from the two different suppliers in batches 7 and 8 again varied widely. Stability in meal-type feed was again far superior to that in pellets.

TEST 4.7–13. Two laboratory batches of crystalline procaine penicillin, with and without an edible wax coating, were compared for stability in another commercial feed pelleting test (Table VII). The 1-82-A crystals in batch 3 showed the most promising stability, but were not significantly benefited by coating. Crystalline laboratory batches which had received a variety of other surface-coating treatments were also stability-tested concurrently. These treatments included waxes, chelating agents, silicone oils, vegetable oils with and without gelling agents, antioxidants, hydrophobic powders, and pharmaceutical shellac. None of these treatments afforded significant protection to crystalline procaine penicillin in pelleted feed products during storage. The crystal form of lot 1-82-A, without surface treatment, was selected for use in future production for feed premixes and is referred to hereafter as "feed grade." The stability data in this test again showed that the crystal form was critical for optimum stability in pelleted feeds. However, in meal-type feeds, many crystal types gave excellent stability.

TEST 4.7–14. A production lot of the improved "feed grade" procaine penicillin, 55021-RZ, was compared to premixes from two other suppliers in a follow-up commercial feed pelleting test (Table VIII, batches 1 to 5). The feed grade in batch 3 was superior, while the samples from the two other suppliers in batches 1, 2, and 5 varied widely in stability.

Table VII. Effect of Crystal Coating on Procaine Penicillin Stability in Broiler Meal and Pellets

Batch No.	Procaine Penicillin Source	Procaine Penicillin, Grams/Ton, 30° C. Initial meal	Initial pellets	4-week pellets	8-week pellets	12-week meal	12-week pellets	12-Week Pellet Retention, %
1.	55015-RZ, crystals	436	393	312	279	410	245	56
2.	55015-RZ, coated	438	394	330	332	403	326	74
3.	1-82-A, crystals	491	445	378	368	448	373	76
4.	1-82-A, coated	490	469	367	360	467	334	68
5.	Supplier 1, M-137-4	471	385	304	273	410	287	61
6.	Premix, PS-1595-A	401	396	144	82	—	—	—

Table VIII. Stability of Various Procaine Penicillins in Broiler Meal and Pellets

Batch No.	Procaine Penicillin Source	Procaine Penicillin, Grams/Ton at 25° C.						12-Week Pellet Retention, %
		Initial meal	Initial pellets	4-week pellets	8-week pellets	12-week meal	12-week pellets	
1.	Supplier 1, 50964	535	447	338	218	493	199	37
2.	Supplier 1, K-0909-K	501	457	384	305	486	267	53
3.	55021-RZ, crystalline	505	474	392	338	496	345	68
4.	Premix, PS-2665-A	611	552	407	362	573	343	56
5.	Supplier 3, 7871-65-0100	469	449	300	186	379	162	35
6.	Supplier 1, M-189-4	492	480	386	360	467	328	67
7.	54018-RZ, crystalline	493	482	305	270	422	228	46
8.	54030-RZ, crystalline	498	482	385	351	457	333	67
9.	Supplier 2, 33508	455	452	111	61	177	31	7
10.	211-Z, crystalline	470	438	220	134	322	107	23
11.	Supplier 1, 52066	412	365	272	259	401	246	60
12.	P-50 premix, 66646[a]	442	430	356	333	439	335	67

[a] Contained 50% procaine penicillin.

Table IX. Stability of Procaine Penicillin in Poultry Meal at Various Temperatures and 70% RH

		Procaine Penicillin, Grams/Ton at 30° C. and 70% RH				
		Initial	1 month	2 months	3 months	6 months
1.	Premix, PS-2665-A	500	462	456	451	494

		Procaine Penicillin, Grams/Ton at 45° C. and 70% RH		
		Initial	7 days	Retention, %
1.	Supplier 1, 51114	515	475	92
2.	55021-RZ, crystalline	519	469	90
3.	54328-Z, crystalline	462	295	64
4.	190-8, crystalline	509	417	82
5.	Supplier 2, P1-200	511	221	43
6.	55028-AZ, crystalline	528	475	90

TEST 4.7–21. Commercial pelleting tests in feed mill equipment are laborious, and 1000 to 2000 pounds of feed must be processed per batch to obtain the few pounds required for laboratory storage and assays. Tests therefore were conducted to determine the possibility of obtaining comparable data by storing laboratory blends of meal-type feeds under elevated temperature and humidity conditions. Feed blends containing a variety of procaine penicillins were stored in cloth bags in a forced-air humidity cabinet. The same procaine penicillin premix was used as in batch 4 of Table VIII for comparison. Potency values in meal after 3 and 6 months' storage under 30° C. and 70% RH (Table IX) were similar to those observed in Table VIII, batches 1 to 5, after 12-week storage of meal in closed jars at 25° C. The test conditions were obviously too mild to produce the more rapid losses encountered in stored pellets.

In a follow-up test, the temperature was raised to 45° C., while maintaining 70% RH, to make conditions more severe. Two lots of feed grade procaine penicillin in batches 2 and 6 showed good retention after 7 days, as did supplier 1 premix in batch 1 (Table IX). The penicillins in batches 4 and 5 showed losses after 7 days under these severe conditions, of the same magnitude as those observed after 8 weeks in the same broiler meal at 25° C. in batches 8 and 9 (Table VI). However, even the more severe storage conditions did not produce the losses encountered in stored pellets. The testing had to be terminated at 7 days, since the absorbed moisture caused heavy mold growth in all feed blends. Consequently, our attempts to decompose feed grade procaine penicillin rapidly in meal-type feeds using elevated temperatures and humidity were unsuccessful. Consequently, we did not develop an accelerated laboratory-scale test which would simulate the potency losses in commercial pelleting operations and subsequent storage.

TEST 4.7–27. A final commercial feed pelleting test in this series showed the improved feed grade procaine penicillin in batch 12 (Table VIII) to be comparable in stability to a sample from supplier 1. Stability data on several years' production of premixes containing the improved feed grade procaine penicillin were recently published (Wornick, 1967a). The rate of potency loss at 25° C. ranged from 0.3 to 0.6% per month over an 18-month storage period. It is probable that these apparent losses, based on computed averages, are not significant in view of the variation inherent in the microbiological assay.

Crystallography. An important consideration during this project was the determination of the optimum form and dimensions of the procaine penicillin crystals. A very fine particle size facilitates uniform mixing into commercial feed products. At approved nutritional use levels of 4 to 10 grams per ton, this requires many

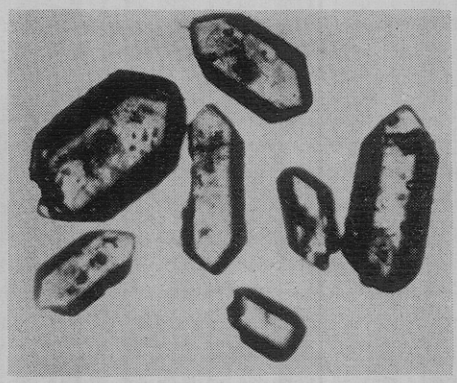

Figure 1. Procaine penicillin crystals, feed grade, magnification approximately 200 ×

active penicillin particles per gram of premix. Unfortunately, a fine particle size generally results in a dusty premix with poor flow properties. Of even greater concern is the unsatisfactory stability of the fine crystals, as shown by micronized procaine penicillin in batch 1 (Table VI). These fine particles can also acquire static electric charges, which cause them to separate out of feed mixtures during processing in feed manufacturing equipment.

Although large crystals are more stable, they may also separate physically from dry feed mixes because of vibration during processing, and they do not provide a sufficient number of active particles per gram of final feed to ensure optimum distribution. Bloom and Livesey (1953) showed that additives employed at levels of 10 to 50 grams per ton should possess particle sizes of 100 to 170 microns.

Our experience indicates the optimum procaine penicillin crystal to be of the monoclinic hemimorphic system. The axial ratio (length to thickness) should average about 2 to 1. The optimum crystal dimension is approximately 200 microns long. Typical crystals of feed grade procaine penicillin are shown in Figure 1. Comparable crystals may be produced by processes such as those described by Deans and Scarrow (1955). The optimum dimension refers to intact single crystals only. The achievement of an "average particle size" of 200 microns by milling larger coarse crystals, or crystal agglomerates, cannot be recommended. Any such milling operation can generate an excessive amount of fine crystal fragments, in the 1- to 25-micron range. These fines exhibit poor stability in both meal-type and pelleted feeds, as illustrated above. However, carefully controlled crystallization processes will always produce batches in which the size of intact individual crystals will vary normally around the desired mean value.

The average number of procaine penicillin crystals of various sizes per gram of feed is shown in Table X. These calculations are based on an average axial ratio of 2 to 1, a penicillin level of 4.0 grams per ton, and an absolute crystal density of 1.255 as reported by Rose (1955). Since baby chicks consume about 10 grams of feed per day during the first week of life, the 200-micron average size is adequate. Medicated feed products containing 50 to 200 grams of penicillin activity per ton will contain proportionally higher numbers of crystals per gram of feed. The use of an even larger average crystal size in medicated feeds would therefore be feasible.

The corresponding U. S. sieve numbers are also shown in Table X for reference. Sieving analyses using automatic screen shakers often produce misleading results on procaine penicillin crystals, and the data must be interpreted with caution. Experience in our laboratories has shown that the short "stubby" crystal habit permits the crystals to fall endwise through screen openings. The fraction passing a U. S. No. 40 sieve and retained on a No. 50 sieve should theoretically measure from 297 to 420 microns, with an average of about 360 microns. Actual microscopic observations of this screen fraction have shown crystal lengths of over 500 to 600 microns. We therefore prefer to employ direct microscopic examination using a micrometer eyepiece or a microprojector.

DISCUSSION

Stability *vs.* Growth Responses. Early reports in the literature suggested that animal growth may be a useful criterion for measuring antibiotic potency in feed products. Blakely *et al.* (1952) found comparable growth in turkey poults whether the procaine penicillin was added to feed before or after the pelleting process, and suggested that this indicated satisfactory stability. Identical findings were reported in poults by McGinnis and Stern (1953) for both benzathine and procaine penicillin added as pure antibiotics and as premixes. However, Stokstad *et al.* (1952) found that when the penicillin in feed was steamed in the laboratory to inactivate it to microbiological assay, it did not produce a growth response in chicks. Williams *et al.* (1953) reported a possible direct correlation between microbiological assay and chick growth as the penicillin decreased in potency in feed.

Conversely, Elam *et al.* (1951) found that penicillin inactivated by autoclaving elicited chick growth by parenteral administration, but not orally. Fell and Stephenson (1953) found that penicillamine produced a growth response in chicks when administered parenterally, but not when given in the feed. Penicillamine is a degradation product of the penicillins under certain

Table X. Procaine Penicillin Distribution in Feed *vs.* Crystal Size

(4.0 grams/ton of feed; axial ratio 2:1)

Crystal Length, Microns	Corresponding U. S. Sieve No.	No. of Crystals per Gram of Feed
600	30	0.06
300	50	0.5
200	70	1.8
150	100	4.1
100	140	14
50	325	112
10	—	14,000

conditions, and shows no antibiotic activity. Taylor and Gordon (1955) reported growth promotion in swine when inactivated penicillin was given either parenterally or through the feed. Sauberlich (1956) reported no response to oral L- or D-penicillamine in thiamine-deficient rats, but observed a good response from these rats to procaine penicillin.

Our results show that initial losses of procaine or benzathine penicillin activity during feed pelleting are generally minor, and that significant decomposition is seldom detectable until after several weeks' storage. In meal-type feeds, the penicillin activity has often been relatively stable for 3 to 6 months. Information on the exact age and microbiological assay of the meal and pellets at time of feeding, and on the quality of penicillin used, is lacking in these early reports. However, it is very doubtful that growth responses in poultry are sufficiently sensitive to detect a gradual 10 to 30% potency loss over a 4- to 12-week feeding period. This is the magnitude of loss which our data would predict for feed grade procaine penicillin in stored pellets during this period.

Antibiotic growth responses are greatest in young animals, and tend to decrease as the animal matures. The interesting possibility therefore exists that growth response in poultry over a 4- to 12-week feeding period would be comparable whether the same original batch of pellets was fed continuously for 4 to 12 weeks, or freshly pelleted feed was supplied daily. The early data of both Blakely and McGinnis suggest this, since it is reasonable to assume a combined penicillin potency loss of 10 to 30% due to initial pelleting plus subsequent storage of the feed used in their studies. Neither researcher could detect a significant effect on growth response when birds fed the pellets were compared to birds fed the same feed in the more stable meal form. If poultry and other species cannot differentiate, growth-wise, between penicillin levels which vary from 10 to 30%, this fact should help to minimize some of the concern which exists today in the feed industry and in regulatory circles concerning the acceptable magnitude for penicillin assay tolerances in feeds.

The data on penicillamine are interesting, although somewhat contradictory. Unfortunately, there are no definitive data which establish penicillamine as the major degradation product in the complex systems represented by the innumerable manufactured feed formulations in use today, nor is it known whether penicillamine is stable in pelleted feeds during storage.

Stability *vs.* Assay Methodology. Esposito and Williams (1952b) proposed a method for penicillin assay which employs methanolic extraction of feed. Their penicillin stability data, and those of Stokstad *et al.* (1952) and of Williams *et al.* (1953), were developed using this method. Penicillin was first reported to be unstable in methanol by Abraham and Chain (1942). Subsequent work by Kersey and Leghorn (1953) and Price and Boucher (1954) documented this basic incompatibility in greater detail as it relates to feed assays. When one considers the low penicillin levels employed for growth promotion, and the wide diversity in the composition of commercial feed formulations, it would seem prudent to eliminate the possibility of questionable penicillin stability data by avoiding methanol extraction. Some laboratories may still occasionally use this solvent, but its use is not recognized in the official AOAC method for procaine penicillin (Wright, 1968).

Another common procedure over the years has been to extract feed samples containing procaine penicillin with a pH 6.0 phosphate buffer. The former official AOAC method listed the buffer as an approved extractant. Simpson and Lees (1956) reported poor recovery using aqueous buffers, and this was recently confirmed by Mayernik and Fiori (1965). Buffer extraction has been used in many laboratories and may still be today, which raises serious questions about the accuracy of some penicillin assay values. We have experienced low recoveries using buffers on feed samples containing the improved large crystal sizes of feed grade procaine penicillin. This may be explained by the slower solubility rate of the large intact crystals in pH 6.0 buffer, compared to acetone. The data in this report were developed using either 50% aqueous acetone, or formamide, as the feed sample extractant.

There are numerous reports of inactivation of penicillin by metallic salts. Of particular relevance are the papers of Niebergall *et al.* (1966) on cupric salts, of Eisner and Porzecanski (1946) on zinc salts, and of Unterman and Schwarz (1960). Some commercial formulas for custom premixes and feed concentrates specify high levels of these and other soluble mineral salts. We have encountered fresh samples of these types containing minerals which showed low penicillin recovery by plate assays. Soluble mineral salts in such sample extracts can sometimes cause potency losses during the assay. Commercial and regulatory laboratories seldom know the composition of samples which they receive. This, therefore, is a possible deficiency in the present official AOAC method. We have employed chloroform extraction of such samples in our laboratories, followed by transfer to the pH 6.0 buffer, to reduce the solution of the metallic salts while dissolving the penicillin. Feed samples of unknown composition should always be checked for quantitative penicillin recovery.

Many workers in the field of microbiological penicillin assays have stressed the need for blank feed extracts to use in preparing standard curves. Blank feeds are seldom available, which casts further doubt on some penicillin feed stability reports. Interfering substances may cause potency loss in sample extracts, may affect the assay organism, and/or may reduce the diffusion rate of penicillin on the agar plates. As now employed, the AOAC method makes no provision for correcting such problems. The collaborative study by Mayernik (1967) gave no indication that such correction was needed, but gave no details on composition of the three feeds employed, which prevents comparison with known feed formulas. Our recommended approach has always been to add known increments of procaine penicillin to feed samples of unknown composition, or to samples which show unexpectedly low potencies.

These problems are generally, but not always, more common in feeds containing low penicillin levels. In the studies reported in this paper, we employed high penicillin levels, and obtained blank feed samples to avoid these difficulties.

The subject of interpretation of microingredient assays on feed products has been reviewed recently (Wornick, 1967b). Another report (Wornick, 1967a) reviews the literature on substances which are incompatible with penicillin or may interfere in the plate assay and thus affect stability values. Many factors besides the assay method itself can influence the results obtained on a feed sample: the quality of the premix used, the uniformity of the feed product, details of the feed pelleting operation, and sampling methods. Inattention to any of these factors can result in penicillin assay data of little practical value.

Penicillin Degradation Mechanism in Feeds. Penicillin salts are stable indefinitely when protected from moisture. Crystalline sodium and potassium penicillin are stable for 4 to 6 years (Buckwalter and Holleran, 1954). The U. S. P. standard of sodium penicillin G showed no loss after 11 years' storage (Kirshbaum *et al.*, 1965). However, a moisture content above 1.5% promotes decomposition (Hirsh and Putnam, 1958). If dry penicillin products are exposed to atmospheric humidity, an 80% potency loss can occur in just a few months (Brindle and Keepe, 1947).

In contrast, aqueous suspensions of benzathine penicillin show only 3% loss after 1 year at 37° C. (Elias *et al.*, 1951). Similarly, aqueous suspensions of procaine penicillin show only 1 to 5% loss after 2 years at 22° to 28° C. (Buckwalter *et al.*, 1953). The decomposition of penicillin in aqueous suspensions is associated with that portion which is in solution (Swintosky *et al.*, 1956a,b). Saturated solutions of procaine penicillin lose all activity within 2 weeks at 24° C. (Levin, 1953).

Crystals of procaine penicillin in animal feed undoubtedly acquire a surface film of moisture from the atmosphere and/or from the intergranular humidity between feed ingredient particles. The saturated film on such crystals is known to be a dynamic system, with penicillin transferring continuously from the solid to the film at a rate proportional to the rate of degradation (Scott *et al.*, 1954) and to ambient temperature. Once in solution in the film, penicillin degradation proceeds as an irreversible first-order reaction (Benedict *et al.*, 1945).

It follows that in premixes and in meal feeds of normal moisture content, from 10 to 13%, held at moderate temperatures, and containing intact procaine penicillin crystals of the optimum crystallographic characteristics described above, the reaction rate will be low. This is confirmed by our data on meal feeds, which average 91% retention after 12 weeks' storage. Microscopic examination of the few procaine penicillin sources which show poor stability in meal feeds has invariably revealed particles with greatly increased surface area. Either the crystals have been micronized or microatomized down to a pharmaceutical usage range of 1 to 25 microns, or they consist of elongated, slender needle crystals and fragments, or they have been milled and contain a high percentage of fine crystalline fragments.

In the feed pelleting process, the meal receives 2 to 6% added moisture from the steam and leaves the conditioning chamber of the pellet mill at about 60° to 75° C. The hot meal then acquires additional temperature due to the high compression and friction during the few seconds it passes through the die. Exit temperatures for the hot pellets may range from 70° to 85° C. or higher (Wornick *et al.*, 1959). While pure procaine penicillin crystals melt at 106° to 110° C. (Rose, 1955), one report indicates that decomposition commences as low as 70° to 80° C. (Hirsh and Putnam, 1958). The smaller crystals and fragments apparently soften, melt, or may even decompose, during feed pelleting. This forms a very thin penicillin film on adjacent feed ingredient particles during compression in the pellet mill die. This film further increases the exposed penicillin surface area and permits more rapid dissolution and degradation in the overlying moisture film during subsequent pellet storage. The larger optimum-sized crystals of feed grade procaine penicillin are apparently more resistant to the transient temperatures and compression in the pellet mill die. These factors, plus their decreased surface area for moisture-film formation, undoubtedly combine to explain their superior stability. Table X indicates the reason for strict product specifications if exposed crystal surface area, and potency losses in stored pellets, are to be held to a minimum.

CONCLUSIONS

A series of laboratory and commercial pelleting tests has shown benzathine penicillin and procaine penicillin to exhibit equivalent stability in feed products. A low initial pelleting loss is not a reliable criterion of subsequent potency retention in the pellets during storage. Some penicillin sources were apparently stable during pelleting, but decomposed rapidly during storage. Optimum stability in stored pellets was exhibited by a procaine penicillin source having carefully defined crystallographic characteristics and a minimum of fine particles.

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