Discussion. Collagen fibrils with tapered ends occurring in sufficient numbers to be found easily in electron microscope preparations were associated with rapidly growing sources of connective tissue. They were abundant in preparations from the human embryo. They were almost invariably present in the rapidly growing mesenchymal tumors examined and in samples of human endometrium. In preparations of adult human skin. Achilles tendon and dura tapered collagen fibrils were rare to absent and in the more slowly growing human mesenchymal neoplasms their presence was variable. This suggests that an abundance of tapered ends is one of the earmarks of newly formed collagen.

Randall, Fraser, Jackson, Martin, and North(4) observed tapered collagen fibrils in preparations made from the umbilical cord of the rat. From this observation they suggested that growth of the collagen fibril may take place by a process of pyramidal accretion. If one assumes that the true end of the native collagen fibril is tapered in contrast to the square or frayed fractured end, then in sites of apparent active collagen formation one is seeing more true fibril ends than in adult "resting" connective tissue. One explanation for this is that the fibrils from these sites are shorter, thus more true ends are seen. In the human being then, short collagen fibrils with tapered ends are relatively common where it would seem that collagen is being actively formed.

Summary. Preparations of adult human skin, Achilles tendon, dura and endometrium; human embryonic skin and Achilles tendon; and benign and malignant mesenchymal neoplasms were examined in the electron microscope for the relative frequency of occurrence of collagen fibrils with tapered ends. It was found that tapered fibrils occurred with greater frequency where collagen apparently was being actively formed.

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A Method for the Assay of Penicillin in Animal Feeds. (19978)

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The usage of antibiotics in animal nutrition is rapidly increasing. It is obviously desirable to have an assay method for the determination of antibiotics in feeds at the levels commonly employed. A companion paper describes a method for aureomycin(1). Standard turbidimetric and cup-plate methods can not be used for the determination of antibiotic activity in feeds due to the presence of non-antibiotic materials in the unsupplemented feeds which inhibit the assay organism or modify its response to the antibiotic. The presence of these non-specific materials gives erroneously high antibiotic values for supplemented feeds and an apparent value for feeds to which no antibiotic has been added. The successful assay of antibiotics in feeds has been made possible by the use of a simple technic. The feed extracts containing antibiotics are pipetted on thick paper pads which are placed on a solidified inoculated medium. This is similar to the technic originally described by Vincent and Vincent(2) but it has been considerably refined.

The method has gone through a lengthy process of development. An early form of the method was briefly discussed in abstract(3) and enabled the determination of 4 g of procaine penicillin per ton of feed. The change from thick double layers of medium to a

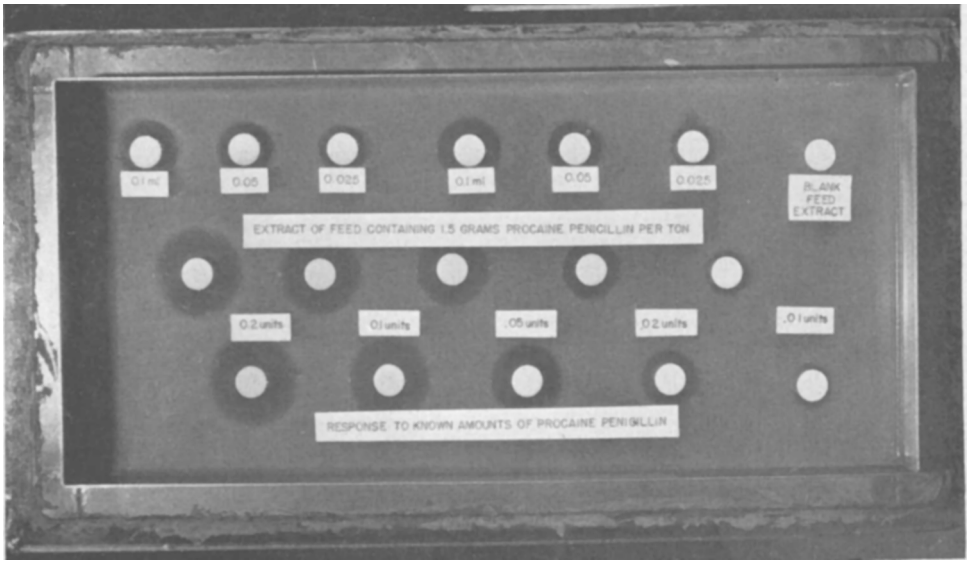

FIG. 1. A typical pad-plate assay after 16 hr incubation at 37°C. The top row of pads contain aliquots of an extract of a feed. The lower 2 rows contain aliquots of the standard extract.

single thin layer increased the sensitivity so that 2 g of procaine penicillin per ton could be accurately determined. The observation by Bigger(4) that sulfonamides increase the sensitivity of staphylococci to penicillin led to the inclusion of 0.15% succinyl sulfathiazole (Sulfasuxidine) in the assay medium which further diminished the minimum amount which can be assayed. The method in its present form is sufficiently sensitive to determine 0.5 g of procaine penicillin per ton while amounts as low as 0.1 g per ton can be detected. The method has been applied to the study of the stability of antibiotics in feed and these results will be reported(5,6).

Methods. Equipment. Flat plates, 14″ x 6″ consisting of stainless steel edges cemented to plate glass bottoms (Fig. 1); a platform with adjusting screws in each corner to provide a level area; filter paper discs No. 740-E, ½″ in diameter (available from Carl Schleicher and Schuell, N. Y.); pointed vernier calipers; and sintered glass funnels of medium porosity (available from A. H. Thomas, Philadelphia. Catalog No. 5593-C.) *Assay organism.* The assay organism is *Staphylococcus aureus* 209-P. It is maintained on agar slants of the same composition as the assay medium (Table II) except that the Sulfasuxidine is omitted and the agar is raised to 1.5%. To prepare the inoculum for the assay the organism is transferred to inoculum broth (Table II); after 18 to 24 hours incubation at 37°C one ml of the broth culture is used to inoculate 100 ml of assay medium at a temperature of 45°C to 48°C. *Extraction of Samples.* Ten grams of the feed to be assayed are placed in a sintered glass funnel. Twenty-five ml of 99% methanol (1% water) are added to the samples and stirred. The suspension is allowed to filter by gravity into a suction flask for 10 minutes and vacuum is applied. This process is repeated and the combined extracts are diluted with 95% methanol to 50 ml. The extract is further diluted with 95% methanol to give an estimated potency of 0.5 to 1.0 unit of penicillin activity per ml. One-tenth ml of this dilution is pipetted on a filter-paper pad resting on a clean glass plate. This extract is then diluted 1 to 2, 1 to 4, and 1 to 8 with 95% methanol and 0.1 ml of each of the dilutions is pipetted immediately on a filter-paper pad. The solvent is allowed to evaporate and the pads are placed on the assay plate with the aid of a pair of forceps.

Standard. Extracts of feeds which do not contain an antibiotic do not give zones of inhibition; *i.e.*, there is no blank value for an unsupplemented feed. However, if penicillin is added to a methanol feed extract, larger

TABLE I. Comparison of Zones of Inhibition Obtained Using Constant Amount of Penicillin in 3 Variations of the Pad-Plate Method.

Units of penicillin per pad	Double layer, mm	Zone diameter, single layer, mm	Single layer sulfasuxidine, mm
.1	22.2	27.2	33.9
.05	19	23.4	29.7

TABLE II.

Assay medium		Inoculum broth	
Peptone	.6	Peptone	.5
Trypticase	.4	Yeast extr.	.15
Yeast extr.	.3	Beef extr.	.15
Beef extr.	.15	NaCl	.35
Glucose	.1	Glucose	.1
Sulfasuxidine	.15	K_2HPO_4	.37
Agar	1	KH_2PO_4	.13
H_2O	100	H_2O	100

zones are obtained than with the same amount of penicillin added to 95% methanol. This is illustrated in Fig. 2 which will be discussed later. This phenomenon necessitates the use of a standard prepared in either of the following two ways. 1) Fifty mg of procaine penicillin is ground with a few grams of a feed which is of the same general type as the feed being assayed. This thoroughly mixed preparation is mixed with enough of the same feed to give 1 kilo. This is the *Concentrated Standard Feed.* It may be refrigerated and used over a period of 2 months. On the day of assay 10 g of the concentrated standard feed is added to 90 g of a sample of the feed which is of the same composition as the sample to be assayed but which does not contain added antibiotic. This is the *Standard Feed* and if refrigerated, it may be used for 2 months for repeat assays or for assays of the same feed containing different amounts of penicillin. This is extracted in exactly the same manner as the samples. The extract will contain 1 unit of penicillin per ml. One-tenth ml is pipetted on duplicate pads resting on a glass surface. The extract is then diluted 1 to 2, 1 to 4, 1 to 10, and 1 to 20 with 95% methanol and duplicate pads are prepared from each dilution. The solvent is allowed to evaporate before the pads are placed on the assay plate. 2) Ten g of antibiotic-free feed of the same composition as the samples is extracted in the same manner as that of a sample. One ml of 95% methanol containing 0.02 mg of procaine penicillin is added to 19 ml of the feed extract giving a solution containing 1 unit per ml. This is then diluted as described under(1). The above two standards represent a feed containing 4.5 g of procaine penicillin per ton and are satisfactory for the assay of feeds containing from 1.0 to 20 g per ton. If a considerably

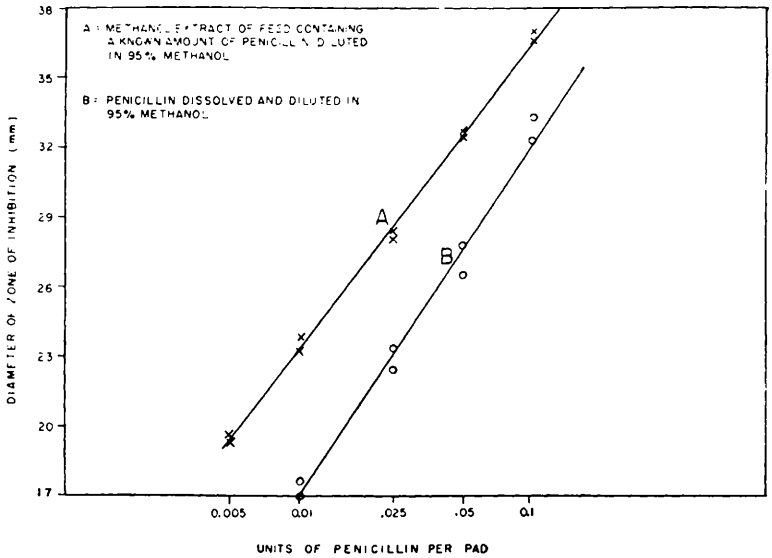

FIG. 2. Comparison of response lines obtained from penicillin contained in a feed extract and in 95% methanol.

TABLE III. Assays of Feeds Containing Known Amounts of Penicillin Salts.

Penicillin salt	Amt added, g/ton	Amt found by assay, g/ton	% recovery
Procaine	23	23	100
	23	21	91
	23	23	100
	10	10	100
	4.6	5	108
	4.6	5	108
	.9	.8	89
NN′ dibenzyl ethylene diamine	4.6	4	87
	4.6	4	87
	23	23	100
Di-p-chlorophenylbiguanide	23	22	96
Guanyl urea	23	24	104
		Avg recovery	98%

higher potency feed is to be assayed, adjustments in the potency of the standard should be made so that the amount of feed extract pipetted on the paper pads is within 5-fold of that present on the standard pads. The standard should be prepared from the same type of penicillin salt as is present in the feed or one which approximates it in units per milligram. Procaine penicillin has been used as a standard in assays of penicillin salts which ranged from 895 to 1158 units per milligram with no apparent difference in the response lines when drawn on a unit basis. However, when assaying for a salt which had a potency of 1300 units per mg a definite difference in the slope of the response lines was observed, which may be due to a difference in diffusibility produced by differences in molecular weight. *Assay procedure.* One hundred ml of assay medium (Table II) is prepared and sterilized in a 250 ml Erlenmeyer flask. The medium is cooled to 45°C to 48°C and inoculated with 1 ml of an 18 to 24 hour broth culture of *S. aureus* 209-P. The medium is then poured into a flat bottom plate which is resting on a level surface. After the medium has solidified, the dry filter-paper pads containing the sample and standard dilutions are placed on the agar surface. The assay plate is covered with either a glass plate or a special stainless steel cover made for that purpose and then incubated at 37°C for 15 to 18 hours. After incubation the zones of inhibition are measured with pointed calipers. The standard response line is drawn on semi-log graph paper and the sample values are calculated from it. Line A in Fig. 2 is an example of a typical standard response line. Fig. 1 illustrates a typical assay plate after incubation.

Results and discussion. In order to test the effectiveness of the extraction procedure used in the pad-plate method a number of determinations were made on feeds containing known amounts of various pure penicillin salts. The standard in these experiments was prepared by adding pure penicillin salt to a methanolic feed extract. The results of these tests are given in Table III and show good recovery of known amounts of added penicillin salts.

Repeated tests on refrigerated samples of known content consistently gave an average value within a few percent of the amount of penicillin added. Although earlier reports have shown that penicillin is unstable in the presence of alcohol(7), methanol has been found to be fully adequate as a solvent to extract penicillin from feeds and superior to either water, pyridine, or ether. This apparent anomaly is explained by the data shown in Table IV which shows that two pure penicillin salts added to 1) 95% methanol, 2) an anhydrous methanol extract of undried feed, and 3) an anhydrous methanol extract of *dried* feed were essentially stable. The pure salts added to anhydrous methanol were rapidly destroyed. Water added to the methanol is obviously a stabilizing factor. Since the penicillin salts were stable in an anhydrous methanol extract of feed which had been dried at 100°C for 2 hours, it appears that materials

TABLE IV. Stability of Penicillin in Methanol.

Solvent	Penicillin salt*	% of penicillin remaining After 1 hr	After 4 hr
Anhydrous methanol	Procaine	0	0
	DBED†	0	0
Methanol extr. of undried feed	Procaine	88	93
	DBED	94	92
Methanol extr. of feed dried 2 hr at 100°	Procaine	98	93
	DBED	93	102
Methanol 95% + water 5%	Procaine	102	79
	DBED	92	60

* 5 mg of pure procaine or DBED penicillin was dissolved in 25 ml of solvent and allowed to stand at room temperature.

† Dibenzyl ethylene diamine dipenicillin.

are contained in the feed extracts other than water which protect the penicillin from rapid destruction by anhydrous methanol. The protecting effect of materials in the feed extracts is apparent even after a 25-fold dilution of the extracts with anhydrous methanol. Procaine penicillin retained full activity in such diluted solutions held at room temperature for 4 hours. In a separate experiment procaine penicillin was found to be stable in 99% methanol for 1 hour at room temperature. Formamide also has been found to be satisfactory for extracting procaine and diamine penicillin from feed. This solvent was substituted for methanol in the method and identical assays were obtained on a number of penicillin-containing feeds which had been stored 4 weeks in stability studies.

Fig. 1 shows the assay plate. For demonstration the pads were widely spaced. Routinely 30 pads are put on such a plate permitting the assay of 5 samples; *i.e.*, 4 pads for each sample plus a standard curve in duplicate. The pad showing no zone of inhibition contains 0.1 ml of an extract of blank or unsupplemented feed demonstrating the complete absence of non-antibiotic interference in the pad-plate method from such feeds. Plates larger than the 14" x 6" plate shown can be conveniently used since more samples can be assayed with each standard curve.

As mentioned earlier unknown materials in feeds extracted by methanol markedly affect the size of zones of inhibition obtained with various amounts of penicillin, although the feed extracts do not have antibiotic activity in the absence of penicillin. This effect is shown in Fig. 2. To construct line A, 10 g of a poultry feed which contained 4.5 g of penicillin per ton was extracted with methanol, diluted and pipetted as described for samples. Line B was obtained by suitable dilution of a solution of crystalline procaine penicillin in 95% methanol. The mechanism of the effect of the feed extractives on zone size is not known. It appears likely that the extractives either affect the extent of diffusion of the penicillin or enhance its antibiotic action against *S. aureus*.

Crystalline procaine penicillin dissolved in 95% methanol is used for the assay of penicillin feed supplements and a standard response line such as line B (Fig. 2) is constructed. Penicillin is extracted from the feed supplement by suspending 1 g of supplement in 100 ml 95% methanol and allowing contact for 30 minutes at room temperature with occasional mixing. Further dilutions are made with 95% methanol until a 1 unit per ml solution is obtained.

In the aureomycin method the necessity for using a feed for the Standard Feed of exactly the same composition as the samples has been clearly shown(1). With penicillin, however, preliminary results indicate that a single Standard Feed may suffice for the assay of penicillin in a variety of feeds of the same general type; but further investigation is needed.

Some of the fundamental advantages and limitations of the pad-plate method are as follows: Strictly aseptic conditions are unnecessary. Only the assay medium and the inoculum broth are sterilized by autoclaving.

Contamination is rare in plates which have been thoroughly washed. It is not necessary to remove solvents from extracts of samples because they are allowed to evaporate from the pads before the pads are placed on the assay plates. The method is free from interference due to non-antibiotic materials since these non-specific inhibitory materials in the feed extracts do not diffuse into the agar medium but apparently remain on the pads.

Using the single layer technic, it is essential that the plate be resting on a level surface when the agar is poured. Differences in agar depth in different areas of the plate can cause a 2-fold difference in assay values calculated from duplicate pads. In the 2-layer technic, the level surface is not as important since the agar is approximately 6 times as deep and the variations in depth will be small in relation to the total thickness of the agar.

Summary. A rapid simplified technic utilizing inexpensive equipment has been developed for the determination of penicillin in feeds and feed supplements. The method is useful to determine the quantities of penicillin added to feeds for nutritional purposes since it can be used to assay accurately as little as 0.5 g of procaine penicillin per ton of feed. Feeds which are not supplemented with antibiotics do not give blank values. A sample of antibiotic-free feed of the same or similar composition as the samples to be assayed must be available. The test organism is *S. aureus* 209-P which is suspended in thin layers of agar medium upon which paper pads are placed containing methanol extracts of the feeds. The use of the paper pads to contain feed extracts and the use of Sulfasuxidine in the assay medium are the features of the method which permits the method to be used for the determination of the small amounts of penicillin added to feeds.

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Inability of Flavonoids to Modify Roentgen Ray Irradiation Mortality in Guinea Pigs.*† (19979)

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General disagreement exists regarding the usefulness of the flavonoid compounds in the prevention and treatment of Roentgen ray irradiation injury. Rekers and Field(1,2) reported that rutin modified such damage in dogs but not in rats. However, Griffith *et al.* (3) found rutin beneficial in rats. On the other hand, Kohn, Robinette, and Capp(4) reported that rutin had no effect on Roentgen ray irradiation lethality in rats, and Cronkite *et al.*(5) obtained similar results in mice. Sokoloff *et al.*(6) reported that a citrus flavonoid mixture decreased such lethality in a British brown strain of rats, but Cronkite *et al.*(7) were unable to obtain any benefit in mice. Clark *et al.*(8) reported that "calcium

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