Anhydrotetracycline and 4-Epianhydrotetracycline in Market Tetracyclines and Aged Tetracycline Products

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Abstract ☐ A large number of tetracycline samples were tested for the presence of anhydrotetracycline and 4-epianhydrotetracycline when they were fresh and after being stored under normal and adverse conditions. It was found that: (*a*) Newly manufactured tetracycline preparations contain only small amounts of anhydrotetracycline and 4-epianhydrotetracycline. (*b*) Storage under adverse conditions markedly increases the percentage of degradation products, but storage under normal conditions results in only a slow increase in anhydrotetracycline and 4-epianhydrotetracycline. In syrups, this can be correlated with loss in tetracycline potency. (*c*) Citric acid greatly increases the tendency of tetracycline to degrade. (*d*) Tetracycline hydrochloride is more stable than tetracycline phosphate. (*e*) Demethylchlortetracycline is the most stable of the tetracycline derivatives studied. (*f*) Rolitetracycline is extremely unstable.

Keyphrases ☐ Anhydrotetracycline determination—tetracycline products ☐ 4-Epianhydrotetracycline determination—tetracycline products ☐ Column chromatography—separation ☐ UV spectrophotometry—analysis ☐ Colorimetric analysis—spectrophotometer ☐ Turbidimetric analysis—biological potency

Degradation products of tetracycline (TC) and 4-epianhydrotetracycline (EATC), in particular, have been implicated in renal dysfunction (1–9). It was, therefore, of interest to determine the amount of degradation in TC products on the market. A large number of market TC products were examined for EATC content. Several lots were tested for stability under normal and adverse storage conditions to determine the amount of EATC that may reasonably be expected in newly manufactured TC products and to reveal what increase in EATC could be expected with time.

Several analytical methods for the determination of 4-epitetracycline (ETC), anhydrotetracycline (ATC), and EATC have been described (10–17). The most convenient are those of Dijkhuis (15), Kelly (16), and Selzer and Wright (17); these have been adopted for use in this study.

EXPERIMENTAL

Methods—*Column Chromatography*—The method of Kelly (16) is a column chromatographic separation of ATC and EATC from each other and from TC and other interfering substances. The column consists of acid-washed diatomaceous earth[1] moistened with 0.1 *M* ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.8; the compounds are eluted with chloroform and determined spectrophotometrically. For this study, the method was modified to ensure that the sample was at pH 7.8 when it was applied to the column. A 5-ml. sample of TC syrup containing 25 mg. of TC/ml. or a 2-ml. sample of pediatric drops containing 100 mg. of TC/ml. was diluted to 10 ml. with 0.1 *M* EDTA buffer, pH 7.8 (prepared by dissolving 0.1 mole of EDTA disodium salt in 800 ml. of water, adjusting the pH to 7.8 with ammonium hydroxide, and diluting to 1 l.). The sample was then brought to pH 7.8 with ammonium hydroxide–water (1:9) and diluted to 25 ml. with the EDTA buffer. One tablet containing 125 mg. of TC or capsule material containing 125 mg. of TC was ground in a mortar and pestle with 10 ml. of 0.1 *M* EDTA buffer, pH 7.8, brought to pH 7.8 with ammonium hydroxide–water (1:9), and diluted to 25 ml. with the EDTA buffer. Each 250-mg. capsule was blended with 25 ml of 0.1 *M* EDTA buffer, pH 7.8, brought to pH 7.8 with ammonium hydroxide–water (1:9), and diluted to 50 ml. with the EDTA buffer. Difficultly soluble samples were dissolved first in 10 ml. of 0.1 *N* HCl, brought to pH 7.8 with ammonium hydroxide–water (1:9), and then diluted to 50 ml. with 0.1 *M* EDTA buffer, pH 7.8.

A 1-ml. aliquot of the diluted sample was then mixed with 1 g. of dry diatomaceous earth, applied to the column, and covered with a 1-cm. layer of diatomaceous earth moistened with 0.1 *M* EDTA buffer, pH 7.8. The method was further modified in that no supporting circle of filter paper was used under the column, no layer of sand was used, and the 0.1 *M* EDTA buffer was not equilibrated with $CHCl_3$ before use.

Spectrophotometric Screening Method—The method of Dijkhuis (15) was used to screen powder, tablet, and capsule samples for total anhydrotetracycline content (ATC + EATC). The sample preparation was modified to ensure the dissolution of samples containing TC base or TC phosphate. A 50-mg. sample of TC powder was weighed into a 50-ml. volumetric flask, and 10 ml. of 0.1 *N* HCl was added. The flask was shaken until the sample dissolved; the contents were then diluted to the mark with water and assayed. Capsule powder or finely ground tablet powder equivalent to 250 mg. of TC hydrochloride was placed in a 250-ml. volumetric flask; 50 ml. of 0.1 *N* HCl was added, and the flask was shaken on a mechanical shaker for 5 min. The sample was then diluted to volume with water and filtered through a fluted-filter paper. The first 20 ml. of filtrate was discarded and the remainder was collected for assay. The calculations were modified in that the formula recommended by Dijkhuis (15) for the calculation of the percent ATC in dosage forms was also used for TC powder.

Paper Chromatography—The method of Selzer and Wright (17) was used. It is an ascending paper chromatographic method for the separation of TC compounds by a resolving solvent of chloroform–nitromethane–pyridine (10:20:3) on 20.3 × 20.3-cm. (8 × 8-in.) Whatman No. 1 paper moistened with McIlvaine's buffer, pH 3.5; the spots are observed by their fluorescence under UV light. To vary the relative positions of the different TC and degradation products on the chromatogram and to facilitate their identification, the chromatographic paper was moistened with any of three buffers: McIlvaine's buffer, pH 3.5; 0.1 *M* EDTA buffer, pH 4.5; or 0.1 *M* EDTA buffer, pH 7.7.

Table I—Total Anhydrotetracyclines Present in Fresh Tetracycline Powder[a]

Sample Number	Manufacturer	ATC + EATC, %
1–4	A	0.17, 0.14, 0.31, 0.17
5–8	B	0.97, 0.86, 0.90, 0.92
9–12	C	0.47, 0.48, 0.57, 0.98
13–18	D	2.06, 1.44, 1.98, 1.59 1.69, 1.27
19–21	E	0.25, 0.71, 0.62
22–25	F	0.24, 0.25, 0.23, 0.21
26–29	G	0.39, 0.46, 0.36, 0.34
30–33	I	0.20, 0.17, 0.66, 0.06
34	J	0.11
35	K	0.25
36–38	L	0.41, 0.09, 0.81

[a] Determined by the method of Dijkhuis (15).

[1] Celite 545, Johns-Manville, New York, N.Y.

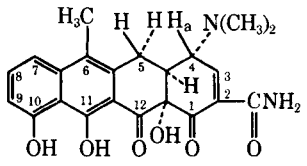
Table II—Total Anhydrotetracyclines Present in Fresh Tetracycline Phosphate Powder[a]

Sample Number	Manufacturer	ATC + EATC, %
1	H	1.44
2	H	1.58
3	H	1.05
4	H	1.07
5	H	1.05

[a] Determined by the method of Dijkhuis (15).

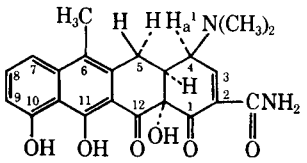
Potency Determination—The biological potencies of the samples of TC used in these studies were determined by the turbidimetric assay method (18) with *Staphylococcus aureus* (ATCC 6538P) as the test organism.

Standards—*ATC–EATC Mixture*—A standard mixture of 50:50 ATC–EATC was prepared as follows: a mixture of 2 g. of TC HCl, 1 ml. of glacial acetic acid, and 20 ml. of water was incubated overnight at 37°. One milliliter of concentrated HCl was added, the mixture was heated on a steam bath for 30 min., and the liquids were removed by lyophilization. The dry powder was dissolved in methanol, and an aliquot was chromatographed on paper as described previously, using each of the three buffers mentioned to moisten the paper. In each case, two spots of equal intensity, corresponding to ATC and EATC, were detected. Analysis of the powder by the column chromatographic method indicated that it contained 48.9% ATC and 50.9% EATC. Comparison of the 100-Mc.p.s. NMR spectrum of a CF_2COOH solution with data in the literature (19) confirmed the identity of the mixture. The bands in a 100-Mc.p.s. spectrum were better resolved, and those bands due to H_a and H_a^1 (Structures I and II) could be integrated. Comparison



anhydrotetracycline

I



4-epianhydrotetracycline

II

of the peak areas indicated that the powder contained 45% ATC and 55% EATC.

Additional quantities of mixed ATC and EATC were prepared by heating TC HCl for 5 min. in 2 *N* HCl (15). The compositions of these materials were determined by the method of Kelly (16) and by comparison of their absorptivities at 356 and 430 mμ.

Anhydrotetracycline HCl Hydrate—Anhydrotetracycline HCl hydrate (Lot No. 63 F 2052, Bristol Laboratories, Syracuse, N.Y.) was used. Paper chromatography in all three buffer systems indicated that the sample contained only a trace of EATC; none could be detected by column chromatography. Integration of the bands corresponding to protons H_a and H_a^1 (Structures I and II) in the 100-Mc.p.s. NMR spectrum of a DMSO-d_6 solution of the sample indicated that the amount of ATC was on the order of 23 times as much as the amount of EATC.

4-Epianhydrotetracycline Sulfate—4-Epianhydrotetracycline sulfate (Lot No. 57 F 536, Bristol Laboratories) was used. Paper chromatography of this compound in all three systems indicated that only a trace of ATC was present; none could be detected by column chromatography. Integration of the bands corresponding to protons H_a and H_a^1 (Structures I and II) in the 100-Mc.p.s. NMR spectrum of a DMSO-d_6 solution of the sample indicated that the amount of EATC was on the order of 10 times as much as the amount of ATC. However, the presence of traces of one or more other compounds was indicated.

Samples—All of the TC samples used in this study had been received as part of the antibiotic certification program.

Table III—Total Anhydrotetracycline and 4-Epianhydrotetracycline Present in Fresh Tetracycline Hydrochloride Tablets[a]

Sample Number	Manufacturer	ATC + EATC, %
1–15	F	0.50, 0.46, 0.53, 0.52, 0.54, 0.46, 0.47, 0.63, 0.59, 0.59, 0.57, 0.58, 0.63, 0.49, 0.54
16–19	B	1.81, 1.62, 1.45, 1.59
20–22	G	0.14, 0.11, 0.69

[a] Determined by the method of Dijkhuis (15).

Table IV—Total Anhydrotetracycline and 4-Epianhydrotetracycline Present in Tetracycline Capsules[a]

Sample Number	Type of Tetracycline	Manufacturer	ATC + EATC, %
1–3	HCl	B	1.38, 1.28, 0.83
4–5	HCl	G	0.29, 0.27
6–15	PO_4	H	1.73, 1.66, 1.62, 1.50 1.76, 1.44, 2.89, 3.05 1.13, 1.14
16–18	PO_4	M	1.58, 0.60, 1.32
19	HCl	M	0.57
20	Base	N	0.65
21–24	HCl	N	0.57, 0.50, 0.46, 0.44
25–28	HCl	O	0.23, 0.32, 0.12, 0.55
29–31	HCl	P	0.21, 0.29, 0.24
32–33	HCl	Q	0.85, 0.68
34	HCl	R	0.33
35–38	HCl	S	0.63, 0.71, 0.71, 0.34
39	HCl	T	0.68
40–49	HCl	U	1.14, 1.09, 1.29, 1.35 1.60, 1.45, 1.41, 1.24 1.31, 1.31

[a] Determined by the method of Dijkhuis (15).

RESULTS AND DISCUSSION

Because pharmaceutical dosage forms of TC contain a number of other ingredients, the column chromatographic procedure and the screening method were tested for interference from such materials. Phenyltoloxamine, aspirin, phenacetin, caffeine, salicylamide, and chlorothen citrate did not interfere with either method. Amphotericin B, neomycin, nystatin, oleandomycin, and triacetyloleandomycin had no detectable effect on the results of the column chromatographic procedure. Although novobiocin caused the EATC band to follow the ATC band quite closely in the column chromatographic procedure, it did not affect the results. However,

Table V—Anhydrotetracycline and 4-Epianhydrotetracycline Found in Fresh Tetracycline Syrups[a]

Sample Number	Manufacturer	ATC, %	EATC, %
1	B	0.27	0.28
2	B	1.81	1.12
3	F	0.06	0.13
4	H	0.13	0.13
5	T	0.13	0.30
6	T	0.09	0.26
7	V	0.39	0.43
8	V	0.72	0.70
9	V	0.11	0.16
10	V	0.11	0.12
11	W	2.93	4.18
12	O	0.19	0.47

[a] Determined by the method of Kelly (16).

Table VI—Comparison of the Loss of Potency of Tetracycline Syrup after Storage[a] with the Amount of Anhydrotetracycline and 4-Epianhydrotetracyclines

Sample Number	Potency Lost, mg./dose	ATC Found, mg./dose	EATC Found, mg./dose
1	0	0.16	0.25
2	0	0.26	0.52
3	0	0.38	0.30
4	0	3.7	4.6
5	2	0.49	0.62
6	5	0.53	0.32
7	5	0.98	1.29
8	9	3.5	3.9
9	10	0.35	0.50
10	11	0.49	0.62
11	12	1.2	0.72
12	13	0.32	0.32
13	13	0.38	0.59
14	16	4.8	4.1
15	17	0.44	0.82
16[b]	17	1.4	1.5
17	23	1.2	0.99
18	25	2.0	2.6
19	28	2.0	2.1
20	61	3.6	4.1
21	32	5.3	5.1

[a] Storage ranged from 12 to 20 months at 25°. [b] An experimental formula.

amphotericin B and nystatin, both of which absorb light at 430 mμ, caused unduly high results in the screening method when they were present in concentrations comparable to those found in commercial products. For this reason, TC products containing amphotericin B or nystatin were tested by the column chromatographic method.

A survey was conducted to find out how much ATC and EATC were present in fresh TC powder. As shown in Table I, the ATC + EATC content of 38 samples of fresh TC HCl bulk material from 11 manufacturers ranged from 0.11 to 2.06%. However, all of the six results over 1% were obtained with material from one manufacturer. Table II shows the total ATC + EATC found in newly manufactured TC PO_4 analyzed by the method of Dijkhuis (15). All of the five samples tested contained between 1 and 2% ATC + EATC.

Another survey was made of the ATC and EATC present in newly manufactured samples of various TC dosage forms. Table III shows that of 22 TC HCl tablets tested, only five contained more than 1% total ATC + EATC. These five samples were produced by one manufacturer and contained less than 2% ATC + EATC. As shown in Table IV, all but two of the 49 TC capsules tested contained less than 2% total ATC + EATC. Table V shows that only two of the 12 fresh TC syrups tested contained more than 1% of either ATC or EATC.

It can thus be seen that the amounts of ATC and EATC found in fresh TC products were quite low. On the basis of these results, limits of 2% EATC in TC powders and 3% EATC in finished TC products have been proposed for inclusion in the *Code of Federal Regulations* (20).

Several studies were conducted to determine the stability of TC products under various conditions. In one study, TC syrups were examined for ATC and EATC after they had been stored for different periods of time. The solid matter in the syrups was either in suspension or easily resuspendable. As shown in Table VI, there is some correlation between loss of biological potency and increase in ATC and EATC content.

In another study, the ability of the TC antibiotics to resist unfavorable storage conditions of elevated temperature and high humidity was investigated. The TC samples were stored at 37° in desiccators containing water (100% relative humidity) or a saturated sodium bromide solution (66% relative humidity) in the desiccant chamber. The samples were tested for potency by the microbiological turbidimetric method (18) and for degradation products by the paper chromatographic method on paper moistened with McIlvaine's buffer, pH 3.5, and by the column chromatographic method.

Several conclusions may be drawn from the data presented in Tables VII and VIII. Demethylchlortetracycline (DMCTC) is the most stable of the TC derivatives when stored under 100% relative humidity at 37°; it showed no loss of potency after storage for 1 month. Chlortetracycline (CTC) is somewhat less stable than DMCTC; after 1 month at 37° and 100% relative humidity, the potency of CTC powder had diminished by 17% and that of capsule material by 14%. Neither CTC nor DMCTC showed evidence of anhydrolike degradation products by the paper chromatographic method. However, TC was less stable; TC products stored 1 month at 37° and 100% relative humidity gave visible evidence of degradation by becoming partially liquid and turning dark brown. Potency losses of from 17 to 79% occurred, and the column chromatographic procedure revealed ATC levels ranging from 2.2 to 7.1% and EATC levels ranging from 3.3 to 14.1%. Paper chromatography indicated that approximately one-half of the TC was converted to the inactive epimer form, ETC. Rolitetracycline (RTC) was even more unstable. Storage at 37° and 100% relative humidity resulted in almost complete destruction of this antibiotic; the only recognizable fragment was a small amount of the epimeric form of RTC. Paper chromatography of degraded oxytetracycline indicated the possible presence of an anhydrolike product.

TC HCl powder and dosage forms were quite stable when stored 2 months at 66% relative humidity and 37°, as shown in Table IX. Only a small fraction of the TC was converted to the anhydro forms. However, TC HCl with added citric acid was almost completely inactivated and formed large amounts of ATC and EATC. Citric acid thus has an adverse effect on TC stability. TC PO_4 is somewhat

Table VII—Results of the Storage of Tetracycline Derivatives for 30 Days at 37° and 100% Relative Humidity

Product	Description after Storage	Potency, mcg./mg. Before Storage	Potency, mcg./mg. After Storage	Paper Chromatography[a]
Pooled demethylchlortetracycline hydrochloride powder	Brown powder	1000	1030	Strong fluorescent spot (DMCTC) at R_f 0.63
Demethylchlortetracycline capsule powder	Light-brown powder	490	480	Two minor spots at R_f 0.19 (Epi-DMCTC) and R_f 0.37
Oxytetracycline hydrochloride powder	Dark-brown	1000	490	Strong fluorescent spot (OTC) at R_f 0.20
Oxytetracycline base powder	Dark-brown liquid with few solid particles		185	Strong blue-green fluorescent spot at origin; R_f 0.39 and 0.96
Rolitetracycline powder	Very dark-brown tar	1000	15	No TC detected. Weak fluorescent spots at R_f 0.38, 0.20, and 0.96. Strong spot at origin.
Chlortetracycline hydrochloride powder	Yellow powder	490	420	Strong fluorescent spot (CTC) at R_f 0.74
Chlortetracycline capsule powder				Weak TC spot at R_f 0.43. No degradation indicated

[a] On paper moistened with McIlvaine's buffer, pH 3.5.

Table VIII—Results of the Storage of Tetracycline Powder and Capsule Materials for 30 Days at 37° and 100% Relative Humidity[a]

Product	pH before Storage	Description after Storage	Potency, mcg./mg. Before Storage	Potency, mcg./mg. After Storage	ATC, %	EATC, %
TC PO_4 powder		Very dark-brown viscous liquid	800	215	2.2	3.3
TC HCl powder	2.38	Dark-brown powder	550	350	2.2	3.7
TC HCl with glucosamine (capsule powder)	2.30	Partially liquid, yellow-brown powder	520	430	7.1	6.5
TC PO_4 capsule powder (Company H)	2.58	Partially liquid, very dark-brown powder	620	130	2.3	5.2
TC PO_4 capsule powder (Company M)	2.46	Very dark-brown liquid	570	160	5.7	14.1

[a] Chromatography was done on paper moistened with McIlvaine's buffer, pH 3.5; strong fluorescent spots for TC at R_f 0.46 and Epi-TC at R_f 0.14; spots for ATC at R_f 0.98 and EATC at R_f 0.60.

Table IX—Results of Storage of Tetracycline Powder and Capsule Materials at 37° and 66% Relative Humidity

Product	Days Stored	Description after Storage	Potency, mcg./mg. Before Storage	Potency, mcg./mg. After Storage	Loss in Potency, %	ATC, %	EATC, %	Paper Chromatography[a]
TC HCl	70	Yellow-brown powder	1000 (est.)	900	10	0.02	0.2	No ATC or EATC detected
TC HCl capsule powder	70	Yellow-brown powder	520	600	0	0.15	None	No ATC or EATC detected
TC HCl with citric acid capsule powder	70	Orange-brown cake	480	35	93	18.5	56.7	Large amount of ATC and EATC. No TC detected
TC PO_4 powder	62	Yellow-brown powder	800	480	40	2.0	3.2	Spots for ATC and EATC visible; large TC and ETC spots
TC PO_4 capsule powder (Company H)	62	Yellow-brown powder	680	450	34	1.3	1.8	Spots for ATC and EATC visible; large TC and ETC spots
TC PO_4 capsule powder (Company M)	62	Yellow-brown powder	600	450	25	1.7	2.3	Spots for ATC and EATC visible; large TC and ETC spots

[a] On paper moistened with McIlvaine's buffer, pH 3.5.

less stable than TC HCl under conditions of 37° and 66% relative humidity; TC PO_4 products stored under these conditions had potency losses ranging from 25 to 40% and small amounts of ATC and EATC were present.

The effects of other active ingredients on the stability of TC PO_4 were also investigated. Samples from two lots of TC PO_4 capsules made by the same manufacturer were stored under adverse and control conditions for 3 years. One lot (A) contained TC PO_4 and no other active ingredients; the other lot (B) contained TC PO_4, phenyltoloxamine citrate, aspirin, phenacetin, and caffeine. Control capsules were stored at room temperature in closed bottles, and the test capsules were stored under adverse conditions in open beakers in a desiccator at 32°. The desiccant chamber was filled with a saturated solution of $Na_2Cr_2O_7$ to ensure a constant relative humidity of 63%. At intervals the capsules were tested for ATC and EATC content. As shown in Table X, the test capsules of both lots at all times contained greater amounts of ATC and EATC than the control capsules; and at all times, Lot B contained greater amounts of ATC and EATC than did Lot A. The final potency of Lot A after being stored under adverse conditions was 24.4% of the labeled potency; after being stored under control conditions, it was 80% of labeled potency. The final potency of Lot B after being stored under adverse conditions was 13.4% of labeled potency; under control conditions, it was 85%. The combination of phenyltoloxamine citrate, aspirin, phenacetin, and caffeine apparently had some additional deteriorating effect on the stability of TC PO_4 capsules stored under adverse conditions.

Table X—Percent of Labeled Potency Present as Anhydrotetracycline and Epianhydrotetracycline in Tetracycline Phosphate Capsule Powder Stored under Normal Conditions and at 32° and 63% Relative Humidity

Months Stored	Normal Conditions ATC, %	Normal Conditions EATC, %	32° and 63% R.H. ATC, %	32° and 63% R.H. EATC, %
		Lot A[a]		
0	0.52	0.27		
4	0.8	0.3	2.0	2.6
9	1.4	0.88	3.3	3.4
32	3.9	2.9	8.6	10.6
		Lot B[b]		
0	1.5	0.44		
4	2.3	0.6	2.7	2.2
9	2.6	1.2	5.7	3.7
	2.8	1.1	3.5	4.0
32	5.1	3.7	13.0	22.1

[a] No other active ingredients present. [b] Contained phenyltoloxamine citrate, aspirin, phenacetin, and caffeine.

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NMR Analysis of Synthetic Corticosteroids of the 1,4-Dien-3-one Type

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Abstract □ A procedure for the analysis of synthetic corticosteroids of the 1,4-dien-3-one type is described. The method is based upon NMR spectroscopy. Spectra are determined in dimethyl sulfoxide containing an internal reference substance, fumaric acid. Both bulk drugs and formulations can be assayed using this method, and comparison is made with results obtained from official assays on the steroids and their formulations. The average deviation obtained in the NMR method was 0.6%. A procedure for water-soluble 1,4-dien-3-ones is also described. This method uses triethylamine hydrochloride as an internal standard.

Keyphrases □ Corticosteroids, 1,4-dien-3-one type—analysis □ NMR spectroscopy—analysis □ Fumaric acid—internal standard

Synthetic corticosteroids of the 1,4-dien-3-one type are at present assayed (1, 2) by colorimetric methods based on the reduction of certain tetrazolium derivatives by the α-ketol side chain at C-17. Such methods do not distinguish between 1,4-dien-3-ones and related corticosteroids of the 4-en-3-one type, which may be present as impurities and which frequently possess different corticoid activity to that desired in the 1,4-dien-3-one. NMR spectroscopy affords a method of distinguishing easily between the two groups of steroids. 1,4-Dien-3-ones possess three vinylic protons, of which the chemical shift usually differs from that of the single vinylic proton of 4-en-3-ones. It is, therefore, theoretically possible to detect the two groups of compounds in the presence of each other and, hence, to develop a much more specific assay procedure.

Present methods used in the determination of prednisolone sodium phosphate (PSP) (1, 2) are also relatively nonspecific. The BP method (1) for the bulk drug relies upon dissolution in water and measurement of the extinction of the solution at 247 mμ; for

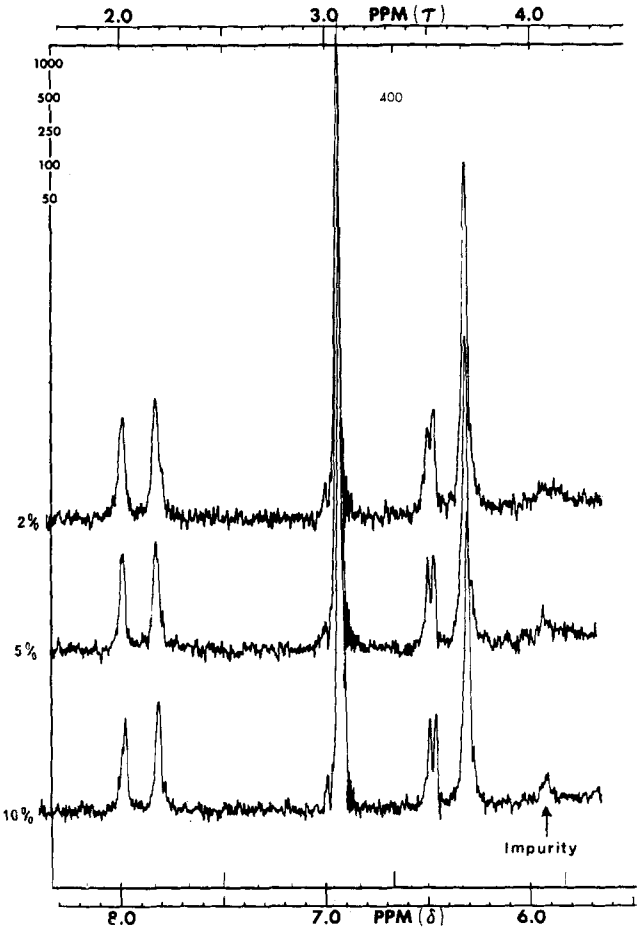

Figure 1—*Partial NMR spectrum of steroidal 1,4-dien-3-one in dimethyl sulfoxide containing fumaric acid and 2, 5, or 10% of added steroidal 4-en-3-one.*