〃	1.9(94.7 ~104.7)	1.4	138.1(95.5 ~104.7)	98.6	140.0	1.4(82.6 ~121.0)	3.8	35.5(91.2~ 110.1)	96.2	36.9
〃	4.3(85.5 ~117.0)	4.1	100.7(82.6 ~118.2)	95.9	105.0	2.2(86.4 ~115.6)	13.2	15.0(83.5~ 119.9)	87.2	17.2
〃	0.6†	1.5	39.5(94.4~ 106.0)	98.5	40.1	0.3†	13.6	1.9(91.1~ 109.7)	86.4	2.2
Average	3.2	2.1	89.8	97.9	91.9	1.1	5.7	18.2	94.3	19.3
S. U.	17.6	44.9	21.6	55.1	39.2	19.6	46.6	19.9	50.4	39.5

P. M. U.……Pregnant mare urine.
S. U.……Stallion urine.
† ……Dilution Method.
( )……f.l.e. % with degrees of freedom and $p=0.95$.
* ……Free estrone+estrone after hydrolysis were 100%.
**……Free estradiol+estradiol after hydrolysis were 100%.

Summary

Two-and-two dose assay method was applied for the quantitative analysis of estrone and estradiol by paper chromatographic analysis, measuring the area of spots, and fairly satisfactory results were obtained. In a fresh urine of a pregnant mare, an average 2% of free estrone and 6% of free estradiol, 98% of conjugate estrone, and 94% of conjugate estradiol were found by this method. About equal amounts of estrone (39 γ/cc.) and estradiol (40 γ/cc.) were determined in the stored stallion urine.

(Received January 31, 1955)

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28. **Morizo Ishidate and Takeichi Sakaguchi** : Metal Chelate Compounds of Tetracycline Derivatives. I. Aureomycin.

(Pharmaceutical Institute, Medical Faculty, University of Tokyo, and Pharmaceutical Faculty, University of Chiba**)*

A patent[1] of American Cyanamid Co. claims that Aureomycin (chlorotetracycline) can be precipitated with calcium or magnesium hydroxide at pH 8.5. Albert[2] reported that Aureomycin and Terramycin (oxytetracycline) combine strongly with ions of heavy metals, particularly with ferric ion. Weidenheimer *et al.*[3] prepared Aureomycin-metal complexes having therapeutic properties superior to those of the free base or its acid salts, and Kämmerer[4] reported that the presence of Co^{++} diminished the activity of Aureomycin.

During our investigation of color reactions between Aureomycin or tetracycline and metal ions, especially with Zr, Th, and UO_2, it became apparent that the reaction represented a specific instance of a very general reaction involving those compounds which contain hydroxyl and carbonyl groups in the *peri*-position or an enolized hydroxyl group of 1,3-diketone, and that the form of metal complexes is similar to that of 1-hydroxyanthraquinone and its derivatives.

The present paper deals with the application of this chelation to the determination

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* Hongo, Tokyo (石館守三).
** Inohanacho, Chiba (坂口武一).

[1] Brit. Pat. 690,381 (April 22, 1953); Japan. Patent Publication No. 6049/1954.
[2] A. Albert: Biochem. J. (1953) (in press).
[3] F. Weidenheimer, *et al.*: U. S. Pat. 2,640,842 (June 2, 1953).
[4] H. Kämmerer, A. Eberle: Klin. Wochshr., **30**, 1083 (1952).

of Aureomycin. The same reaction should analogously be applicable to that of Achromycin and Terramycin.

I. Chelate Compounds of Aureomycin with Common Metals:

Nearly all of the metal ions were tested to determine their behavior with Aureomycin under the optimum conditions. The absorption spectra indicated that such metal ions as Al, Fe, Co, Cu, Mg, and Ca formed chelates with Aureomycin in aqueous solution. It is possible that Hg and Bi also form chelates with Aureomycin.

The optimum pH, the maximum absorbance and its wave length for each ion are summarized in Table I.

TABLE I.

Aureomycin, $10^{-4} M$ Metal Ion, $5 \times 10^{-3} M$

Ion	pH	max. (mμ)	Absorbance at max.	Aureomycin alone at same pH		Possibility of complex formation
				max. (mμ)	Absorbance at max.	
Hg^{++}	1.2	360	0.58	370	1.08	+
Sn^{++++}	1.2	370	1.08	370	1.08	−
Bi^{+++}	1.2	370	0.88	370	1.08	+
Zn^{++}	5.2	370	1.05	370	1.03	−
As^{+++}	3.0	370	1.08	370	1.07	−
Mn^{++}	3.0	366	0.90	370	1.07	±
Co^{++}	5.2	390	1.35	370	1.03	+
Al^{+++}	3.4	390	1.20	370	1.07	+
Cu^{++}	4.6	390	1.63	370	1.08	+
Ca^{++}	5.2	371	1.12	370	1.03	+
Mg^{++}	5.2	385	1.60	370	1.03	+

Some metal ions react with Aureomycin in nonaqueous solvent to yield a colored complex. When an absolute ethanolic solution of anhydrous aluminum chloride is added to a butanolic solution of this antibiotic, an orange color is produced. The aluminum-Aureomycin color in the aqueous solution is yellow. However, either in butanolic or in aqueous solution Anhydroaureomycin gives an orange red coloration.

Aureomycin adsorbed on or diluted with alumina gave a yellow powder. When antimonyl chloride in anhydrous chloroform solution was added to this yellow powder, followed by heating, a red precipitation formed. This sensitive reaction is useful in detecting Aureomycin in capsuls which has been diluted with aluminum phosphate.

II. Spectrophotometric Evidence of Aureomycin-Metal Chelates:

An aqueous solution containing metals such as Zr(IV), Th(IV), UO_2(II), or Al(III) gives yellow colored chelate with Aureomycin. The yellow chelate develops its full color intensity at a pH between neutral and slightly acidic.

The present experiments indicated that the *peri*-standing hydroxyl and ketone  $(-\underset{\overset{\|}{O}}{C}-CH=\underset{\overset{|}{OH}}{C}-)$ in Aureomycin was able to chelate with these metal ions.

In Tables II and III are illustrated the absorption spectra of Aureomycin and 1-hydroxyanthraquinone chelates.

TABLE II. Absorption Spectra of Aureomycin Chelates

Metal	pH	max. (mμ)	Molar Extinction
Zr	4.6	395	11300
Th	4.0	405	18000
UO_2	4.6	415	9000
Al	3.4	390	12000
Mg	5.2	380	16000
Co	5.2	390	13500
Cu	4.6	380	16300

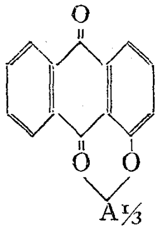
Table III. 1-Hydroxyanthraquinone Chelates

Metal	max. (mμ)		Molar Extinction	
Co	410	480	6080	3190
Cu	400	500	4830	940
Mg	400	500	2160	1440
UO_2	410	510	13900	2020
Ni	400	500	8100	6900

As illustrated for Aureomycin and 1-hydroxyanthraquinone, the choice of metal has no significant effect on the wave lengths of the absorption maxima whereas it greatly affects the intensity of absorptions.

Consequently, it is evident that the color produced by Aureomycin is due more likely to the presence of a chelate ring and is not to the characteristics of the particular metal in the ring. This hypothesis was proposed by Geyer and Smith⁵⁾ in 1-hydroxyanthraquinone chelates, because wave lengths of the maximum absorbance were practically independent of the metals employed.

The 1-hydroxyanthraquinone or alizarin chelates with aluminum and zirconium have already been investigated by several workers, especially by Feigl and his co-workers.⁶⁾ According to these workers, the complex is most likely a chelate type of the following structure :



Similarly, the same chelate structure may be demonstrated for the Aureomycin-metal complexes in a solution and they are shown in the following section.

Anhydroaureomycin, produced by boiling with 3 *N* hydrochloric acid and precipitating from the acid solution, also gives characteristic colors with ions, i.e. red coloration with uranyl in neutral and orange red or orange coloration with thorium, zirconium, and aluminum.

The results of the present experiments might possibly account for the formation of chelates between the functional group in Aureomycin and a metal ion.

Another type of Aureomycin complex is based on the precipitation reaction of $(BiI_4)^-$ and a tertiary amine in slightly acidic medium containing potassium iodide, but this point has been excluded from the present paper.

III. Complexes of Aureomycin with Rare Earths Metals :

1. Chelate of Zr(IV) with Aureomycin—When Aureomycin is added to a solution of zirconium buffered with sodium acetate and acetic acid to pH 4.6, an intense yellow color is obtained. The absorption curves (Fig. 1) show that the Aureomycin-zirconium chelate has its maximum absorption at 395 mμ.

The method of continuous variations introduced by Job⁷⁾ was applied to this chelate. From Figs. 2 and 3, the ratio between Aureomycin and zirconium is found to be 1 to 2. On heating the chelate (395 mμ) with 3 *N* hydrochloric acid in a boiling water bath there was produced an orange red solution which had the absorption maximum at about 490 mμ. The orange red chelate is also obtained by heating Aureomycin with zirconium and 3 *N* hydrochloric acid at the same time. This compound shows a maximum at

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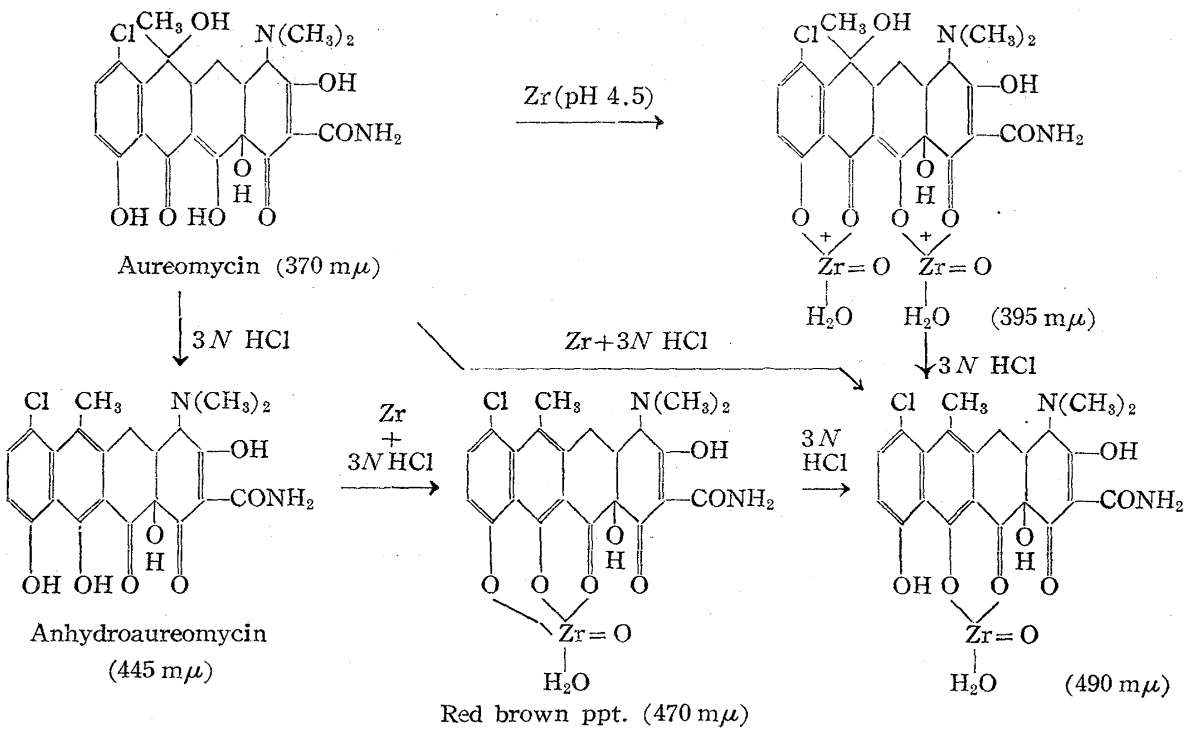
5) P. Geyer, McP. Smith : J. Am. Chem. Soc., **64**, 1649(1942).
6) F. Feigl, R. Stern : Z. anal. Chem., **60**, 9(1921).
7) P. Job : Ann. chim., **9**, 113(1928).



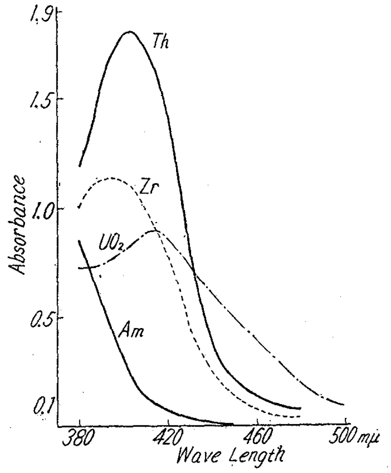


Fig. 1.

Aureomycin + Th(IV), Zr(IV), or UO_2^{++}

-·-·-·-; UO_2^{++} chelate

------------; Zr(IV) chelate

━━━━━; Th(IV) chelate

at pH 4.6 adjusted with a buffer

AM·HCl 50 γ/cc.

————; AM·HCl 50 γ/cc.

Fig. 2.
AM-Zr Molar Ratio (pH=4.0)

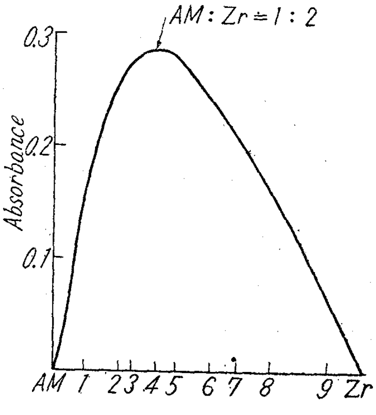
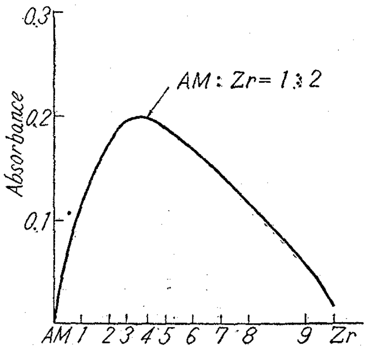


Fig. 3.
AM-Zr Molar Ratio (pH=5.0)



490 mμ and 1:1 molar ratio. The results are shown in Figs. 4 and 5.

It was possible to remove zirconium when disodium ethylenediaminetetraacetate (EDTA) was added to the above chelate solution having the maximum at 490 mμ. Anhydroaureomycin was thus spectrophotometrically obtained (cf. Fig. 6).

Anhydroaureomycin-zirconium chelate has, however, a peak at 470 mμ in 3 N hydrochloric acid. It is gradually precipitated from a concentrated solution and it shows

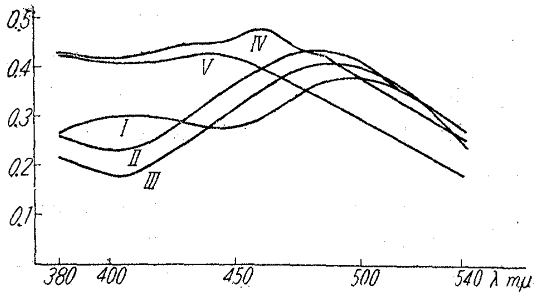


Fig. 4.
Variation of HCl Concentration
AM-Zr-HCl

I. 1 N HCl
II. 2 N HCl
III. 3 N HCl AM·HCl 50 γ/cc
IV. 4 N HCl 1% $Zr(NO_3)_4$ 1 cc./25 cc.
V. 5 N HCl

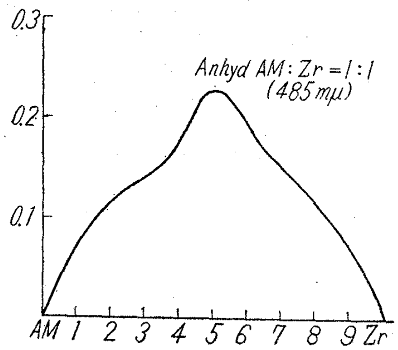


Fig. 5.
AM-Zr Chelate
(after heating with 3 N HCl)

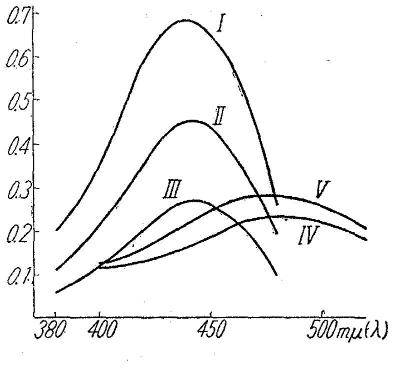
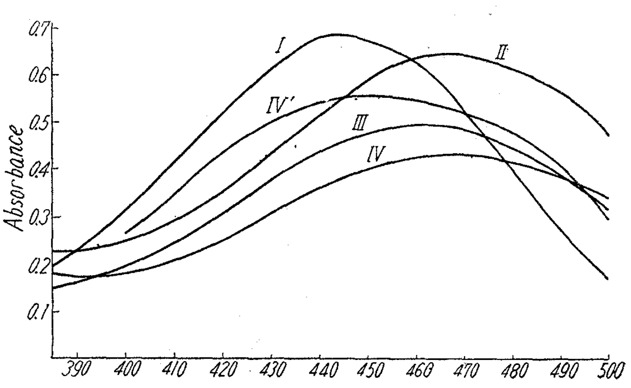


Fig. 6.

I. Anhydroaureomycin
II. Anhydroaureomycin produced from V by removing Zr
III. Anhydroaureomycin produced from IV by removing Zr
IV. AM-Zr-3 N HCl
V. AM-3 N HCl, after heating

Fig. 7.
AnhydroAM-Zr Chelate

I. Anhyd. AM
II. Zr(IV) Chelate pH 4.0 } after
III. Zr(IV) Chelate pH 1.6 } 48 hrs.
IV. Zr(IV) Chelate 3 N HCl 5 cc./25 cc. }
IV'. IV measured after 15 mins.



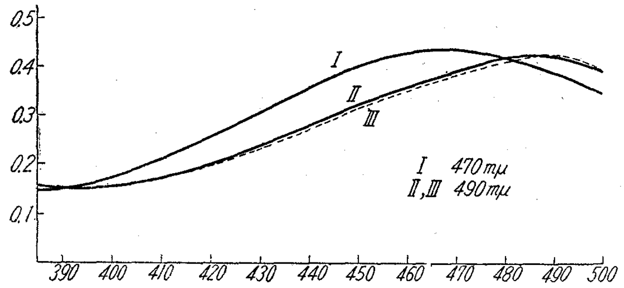


Fig. 8.
Transformation of AM-Zr into Anhyd. AM-Zr Chelate

various absorbances in different acidities (cf. Fig. 7). Consequently, it was different from the orange red chelate of 490 mμ (cf. Fig. 8). The results obtained by these experiments strongly indicated that the above formulae seem to be very satisfactory.

2. Chelate of Th(IV) with Aureomycin—Aureomycin forms a yellow-colored chelate with thorium at pH 4.0, which shows a maximum at 405 mμ (cf. Fig. 1).

The fact shown by Fig. 10 obviously indicate that the chelate compound is formed by an atom of thorium to a molecule of Aureomycin. The orange yellow chelate obtained by heating the Aureomycin-thorium complex with dilute hydrochloric acid has maximum absorption at 450 mμ (Fig. 11).

When 2 *N* hydrochloric acid is added to a solution containing Anhydroaureomycin and thorium, the resulting solution gives the same absorption maximum at about 450 mμ, as illustrated in Figs. 12 and 13, which shows 490 mμ at pH 4.4.

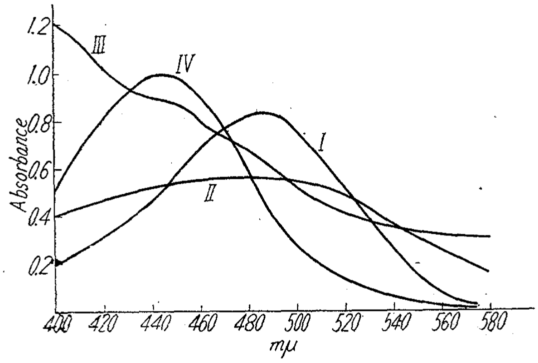


Fig. 9.
Anhydroaureomycin-Metal Chelate

I.	AnhydroAM-Al^{+++}	pH 4.0
II.	AnhydroAM-UO$_2^{++}$	pH 6.5
III.	AnhydroAM-Fe^{+++}	pH 2.0
IV.	AnhydroAM	pH 4.2

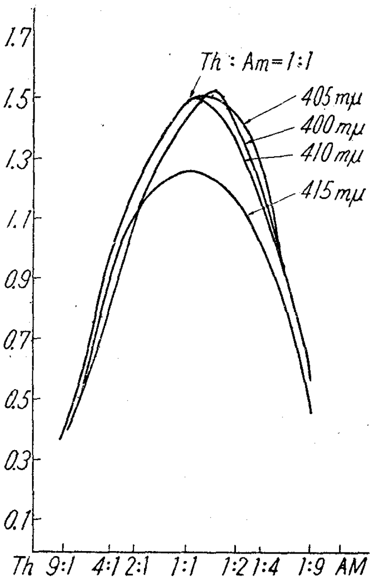
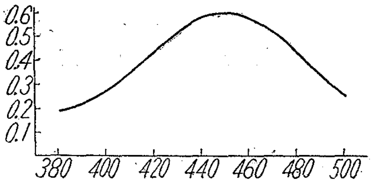


Fig. 10.
AM-Th Molar Ratio

Fig. 11.
AM-Th-2 *N* HCl Method
Aureomycin-HCl 50 γ/cc.



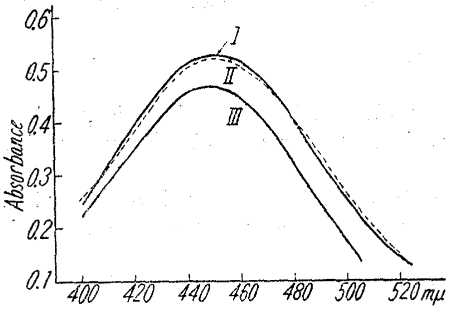


Fig. 12.
AnhydroAm-Th

I. AnhydroAM-Th-2 *N* HCl (heated)
II. AnhydroAM-Th-2 *N* HCl
III. AM-Th-2 *N* HCl (heated)

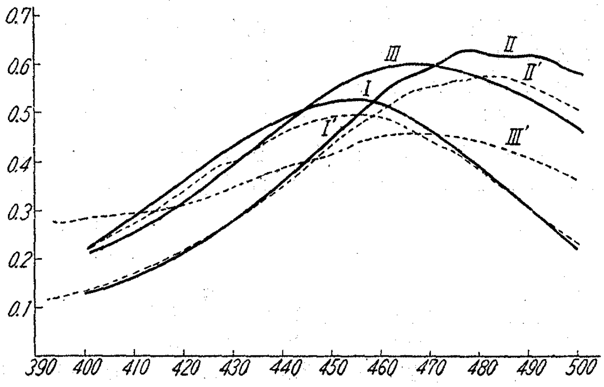


Fig. 13.
Anhydroaureomycin-Th Chelate

I. AnhydroAM 50 γ/cc. + 0.5% $Th(NO_3)_4$ 0.5 cc./25 cc. + 2 *N* HCl 5 cc./25 cc.
II. AnhydroAM 50 γ/cc. + 0.5% $Th(NO_3)_4$ 0.5 cc./25 cc. + pH adjusted to 2.5 with dil. HCl
III. AnhydroAM 50 γ/cc. + 0.5% $Th(NO_3)_4$ 0.5 cc./25 cc. + pH adjusted to 5.2 with dil. NaOH

Fig. 14.
AM-UO_2 Molar Ratio

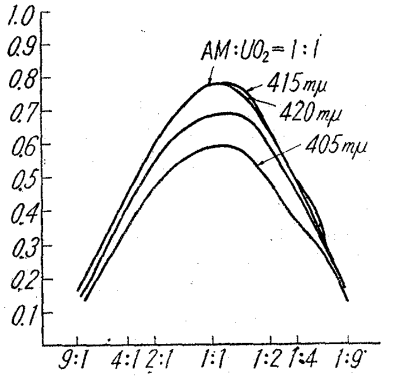
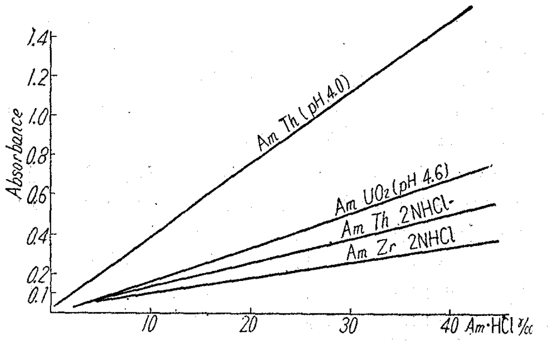


Fig. 15.
Calibration Curves of Metal Chelates



3. Complex of uranium(VI) with Aureomycin—Aureomycin forms a slightly yellow colored complex with uranyl ion at pH 4~8, whose absorption maximum is at 415 mμ (Fig. 1).

The molar ratio between Aureomycin and uranium is 1 to 1 (Fig. 14). The chelate compound of Anhydroaureomycin with uranyl ion has a red brown color (480 mμ at pH 6.5) and the similarly colored precipitate is formed after long standing. The precipitate is soluble in butanol.

IV. A Comparison of Slopes of Calibration Curves:

Plotting the absorbance against concentrations of Aureomycin gives a straight line for the chelate containing Zr, Th, and UO_2. A comparison of slopes of these straight lines proved that the thorium chelate at pH 4.0 had the highest absorbance and that of zirconium obtained by heating with 3 *N* hydrochloric acid had the lowest at the same concentration. Therefore, the colorimetric determination of Aureomycin by thorium is the most sensitive, as shown in Fig. 15.

The authors are indebted to Mr. K. Taguchi for carrying out the photometric measurements.

Experimental

The absorption curves of colored chelates of Aureomycin (AM) with zirconium(IV), thorium(IV), and uranium(VI) are shown in Fig. 1. The concentration of Aureomycin hydrochloride used in this experiment is 50 γ/cc. ·The pH is adjusted to 4.6.

Molar Ratio of Zirconium (IV) and aureomycin—The method of continuous variations proposed by Job and extended by Vosburgh and Cooper[8] was tried. These results are summarized in Tables IV and V.

TABLE IV.

No. of Tubes		1	2	3	4	5	6	7	8	9
$10^{-3}M$ AM·HCl	(cc.)	0.4	0.8	1.0	1.33	1.6	2.0	2.4	2.8	3.6
$10^{-3}M$ $Zr(NO_3)_4$	(cc.)	3.6	3.2	3.0	2.67	2.4	2.0	1.6	1.2	0.4
1 M Acetate buffer	(cc.)	2	2	2	2	2	2	2	2	2
Distilled water	(cc.)	14	14	14	14	14	14	14	14	14

i) At pH 4.0 : 1 M acetate buffer (I) was prepared by mixing 164 cc. of 1 M AcOH with 36 cc. of 1 M AcONa solution. The concentrations of the sample and the contrast are identical in each cell.

TABLE V.
(cf. Fig. 2)

No. of Tubes		1	2	3	4	5	6	7	8	9
AM-Zr.	$A_{400m\mu}$	0.256	0.421	0.474	0.540	0.565	0.61	0.64	0.67	0.70
Contrast	$A_{400m\mu}$	0.080	0.160	0.190	0.250	0.290	0.365	0.435	0.505	0.64
	$A_{400m\mu}$	0.176	0.260	0.285	0.290	0.275	0.245	0.205	0.165	0.060

Thus from Table V and Fig. 2, it is seen that the peak occurs at the tube No. 4 containing 1.33 cc. of $10^{-3}M$ AM·HCl. Therefore, $n=\frac{1.33}{4-1.33}=\frac{1}{2}$ and the ratio of Aureomycin to zirconium ion is 1 to 2.

ii) At pH 5.0 : 1 M acetate buffer (II) was prepared by mixing 59 cc. of 1 M AcOH with 141 cc. of 1 M AcOEt solution.

TABLE VI.
(cf. Fig. 3)

No. of Tubes		1	2	3	4	5	6	7	8	9
AM-Zr	$A_{400m\mu}$	0.180	0.300	0.345	0.390	0.405	0.430	0.440	0.460	0.470
Contrast	$A_{400m\mu}$	0.065	0.125	0.250	0.190	0.220	0.265	0.310	0.345	0.410
	$A_{400m\mu}$	0.115	0.175	0.195	0.200	0.185	0.165	0.130	0.115	0.060

The molar ratio of Aureomycin to zirconium is also 1 to 2.

Aureomycin-Zirconium Chelate Produced by Heating with dil. HCl— The curves in Fig. 4 show the results for a fixed concentration of Aureomycin and Zr ion obtained by the addition of the increasing concentration of HCl, followed by heating on a water bath for 10 mins. Use of 2 N and 3 N HCl might be desirable. The concentration of HCl higher than 3 N results in the decomposition of this chelate. AM·HCl : 50 γ/cc., 1 cc. of 1% $Zr(NO_3)_4$ is added to 25 cc. of AM·HCl solution.

Molar Ratio of Aureomycin-Zirconium Chelate produced by Heating with 3 N HCl— The series of experiments are shown in Table VII and the results are shown in Table VIII and Fig. 5.

TABLE VII.

No. of Tubes		1	2	3	4	5	6	7	8	9
10^{-3}AM·HCl	(cc.)	0.3	0.6	0.9	1.2	1.5	1.8	2.1	2.4	2.2
$10^{-3}Zr(NO_3)_4$	(cc.)	2.7	2.4	2.1	1.8	1.5	1.2	0.9	0.6	0.3
3 N HCl	(cc.)	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2

The mixture was heated 10 mins. on a boiling water bath and then cooled to the room temperature. To the solution in each tube was then added 0.5 cc. $Ba(OH)_2$ to prevent any precipitation and the reading of absorbances was measured.

TABLE VIII.

No. of Tubes		1	2	3	4	5	6	7	8	9
Chelate	$A_{485m\mu}$	0.106	0.200	0.263	0.338	0.420	0.437	0.450	0.440	0.440
Contrast	$A_{485m\mu}$	0.035	0.08	0.123	0.155	0.190	0.240	0.295	0.323	0.370
	$A_{485m\mu}$	0.071	0.120	0.140	0.183	0.230	0.190	0.155	0.117	0.070

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8) W. C. Vosburgh, G. R. Cooper : J. Am. Chem. Soc., **63**, 437 (1941).

The molar ratio of Aureomycin to zirconium is 1 to 1.

Anhydroaureomycin—Anhydroaureomycin was prepared by the following procedure: An excess of Aureomycin Hydrochloride was dissolved in a small volume of 0.01 N HCl and any insoluble material was removed by filtration. To the saturated solution of Aureomycin Hydrochloride thus obtained was added conc. HCl to the concenration of 10% and heated on a water bath at 80～90°. On cooling the resulting solution, a brown yellow solid precipitatd after 10 mins. The precipitate was filtered, washed with 5 N HCl, and dried in a vacuum desiccator.

When Anhydroaureomycin is added to an aqueous solution of Zr, Th, Al, or UO_2 ion, an orange or orange red color is produced. The absorption curves are shown in Figs. 7, 9, and 12.

Anhydroaureomycin-Zirconium Chelate—Fig. 7 illustrates the effect of pH and acidity on the wave lengths and absorbance of the Anhydroaureomycin-Zr complex. The curves I～IV were measured after 48 hrs. IV′ is the same curve as IV except that it was measured after 15 mins.' standing.

Molar Ratio of Th(IV) and Aureomycin—The results are summarized in Tables IX and X, and in Fig. 10.

TABLE IX.

No. of Tubes		1	2	3	4	5	6	7	8	9
$10^{-3}M$ AM•HCl	(cc.)	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25
$10^{-3}M$ $Th(NO_3)_4$	(cc.)	2.25	2.00	1.75	1.50	1.25	1.00	0.75	0.50	0.25
1/5 M Acetate buffer (I)		1	1	1	1	1	1	1	1	1
Distilled water		10	10	10	10	10	10	10	10	10

They were measured after 20 mins.' standing.

TABLE X.

No. of Tubes		1	2	3	4	5	6	7	8	9
AM–Th	$A_{395m\mu}$	0.250	0.580	0.860	1.00	1.30	1.40	1.20	0.86	0.46
〃	$A_{400m\mu}$	0.321	0.675	1.02	1.25	1.43	1.51	1.30	0.97	0.52
〃	$A_{405m\mu}$	0.348	0.760	1.10	1.30	1.50	1.50	1.38	1.00	0.54
〃	$A_{410m\mu}$	0.350	0.780	1.15	1.30	1.50	1.45	1.28	0.98	0.52
〃	$A_{415m\mu}$	0.320	0.720	1.075	1.20	1.25	1.20	1.12	0.86	0.46

From Table X, the molar ratio of Aureomycin to Th is 1 to 1.

Anhydroaureomycin-Thorium Chelate (cf. Figs. 11～13)—Anhydroaureomycin forms a colored complex in an acid or neutral solution of Th. Fig. 13 illustrates the absorption curves at various pH values. The curves I, II, and III were the results in the time interval of 15 mins., and I′, II′, and III′, 48 hrs. after the preparation.

TABLE XI.

No. of Tubes		1	2	3	4	5	6	7	8	9
$10^{-3}M$ AM•HCl	(cc.)	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25
$10^{-3}M$ $UO_2(Ac)_2$	(cc.)	2.25	2.00	1.75	1.50	1.25	1.00	0.75	0.50	0.25
0.2 M Acetate Buffer	(cc.)	1	1	1	1	1	1	1	1	1
Distilled Water		10	10	10	10	10	10	10	10	10

The molar ratio of Aureomycin to UO_2 ion is 1 to 1.

Calibration Curves of Each Chelate—The results are shown in Fig. 15.

Summary

Aureomycin forms chelate compounds with various metals, especially stable compounds with zirconium(IV), thorium(V), uranium(VI), aluminum, and magnesium. Cobalt and copper are also capable of forming the chelate compound with the antibiotic. A comparison of Aureomycin-metal chelates with 1-hydroxyanthraquinone chelates was made spectrophotometrically. The results of this experiment proved that the presence of the constituent functional groups, a *peri*-standing phenolic hydroxyl and carbonyl, in Aureomycin was important. The molar ratio of these complexes containing Zr, Th, or UO_2 was determined by the continuous variation method of Job, and structural formulae of these complexes are suggested. It is believed that some of these chelates may offer a sensitive method for the chemical determination of Aureomycin and the allied compounds.

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