	jours							
Injection intraveineuse de methyle-(hexabitale	15	〃	2500	5.8	14	1380	460	3.0
		〃	3000	6.5	18	1190	440	2.7
	31	〃	1450	5.9	11	670	760	0.9
		〃	1600	5.5	11	440	260	1.7
		〃	2500	6.6	13	610	450	1.4
Injection intraveineuse de l'acide aspartique	1	〃	2300	7.2	20	1360	430	3.1
		〃	2360	6.5	20	1370	400	3.4
	10	〃	2100	10.0	20	1080	260	4.2
		〃	2100	8.7	20	1060	330	3.2

qui avaient subi ; celle d'acide aspartique.

Tableau I donne les résultats de cette expérience, montrant une baisse de la proportion de ces deux acides aminé à ⅓ environ.

Cette baisse de la proportion de l'acide glutamique et de l'acide aspartique libres dans le cerveau suppose, à notre avis, qu'un changement soit survenu, sous l'action du médicament, au métabolisme des deux acides aminé dans le cerveau où l'acide glutamique exerce une action importante ; soit que la faculté de synthèse de l'acide glutamique s'affaiblisse, soit que son utilisation s'intensifie, si la proportion entre l'acide glutamique —facteur du système respiratoire ainsi que de la composition de protéine—et l'acide aspartique, son pendant, va s'abaissant, c'est qu'un trouble jeté par le medicament dans l'exercice normal du métabolisme nécessite un recours à un autre système métabolique.

L'injection intraveineuse d'acide aspartique n'entraine de variation ni á la quantité de l'acide aspartique libre dans le cerveau ni à sa proportion avec l'acide glutamique. Ce phénomène n'est pas sans intérêt, si l'on le rapporte aux résultats d'une experience faite par. Waelsch et d'autres,[22] qui affirment qu'aucune variation sensible de quantité n'a été provoquée, pour l'acide glutamique dans le cerveau, par l'injection intraveineuse de cette drogue.

Je tiens à exprimer mes remerciements sincères à M. le docteur Den-ichi Mizuno, directeur de la Section chimique de l'Institut d'Hygiène, Tokio, et à M. Kiyosi Suzuki, licencié en agriculture, pour leurs précieux conseils et leur direction bienveillante.

Experimentale

Le lapin a été soumis à l'expérience après 10~15 jours d'élevage qui suivaient ses achats.

Après l'extraction rapide de tout son sang, on a dégagé ses cerveaux (cerveaux et cervelets) pour les mettre au mélangeoir pendant une minute en les refroidissant à la glace. On les a réchauffés en les remuant dans un bain d'eau tiéde et y a ajouté de l'alcool jusqu'à 70%. Le précipité en ayant été enlevé, on a distillé sous pression réduite cette solution pour en obtenir une quantité déterminée de liquide èpais, dont ou a analyse l'acide glutamique et l'acide aspartique à l'essai microbien à *Leuconostoc mesenteroides* P-60.

(On a donc la quantité respective des substances correspondant à l'acide glutamique et à l'acide aspartique en proportion avec ce microbe plutôt que la quantité même de ces deux acides aminés.)

1) Le tableau suivant donnera la quantité de l'acide glutamique et de l'acide aspartique et leur proportion dans 1 g. de cerveau d'un lapin normal, traité ainsi (malgré la variation individuelle de la quantité absolue de ces deux acides aminés, leur proportion reste assez fixée).

2) Lapin soumis à l'injection intraveineuse de méthyle-hexabitale soluble : Même traitement après 15 et 31 jours d'injection de 50 mg. par jour de méthyle-hexabitale soluble dans les veines auriculaires. Au bout de 31 jours, diminution de l'acide glutamique et baisse de sa proportion.

3) Lapin soumis à l'injection d'acide aspartique : Même traitement effectué 1 heure après un coup ou dix coups par jour d'injection de 0.5 g. pas de changement dans la quantité de l'acide glutamique ni de l'acide aspartique, ni dans leur proportion.

Resumé

Nous avons observé pendant longtemps les variations du méthyle-hexabitale soluble avec radical méthyle à l'azote, dont on a injecté dans les veines de lapins. Nous avons obtenu de l'urine du lapin comme produits métaboliques, uréthane, Fp. 62°, acide oxalique,

22) Waelsch : J. Biol. Chem., **184**, 37(1950).

acide benzoïque, urée, acide hydroxybenzoïque-*m*, Fp. 137~144°, acide hippurique, oxalate d'ammonium, et autre substances huileuses.

Nous avons fait l'analyse de l'acide glutamique et de l'acide aspartique libres dans le cerveau des lapins, à l'essai Microbien à *Leuconostoc mesenteroides* P-60. Tableau I donnera les resultats de cette expérience montrant une baisse de la proportion de ces deux acides aminé à ⅓ environ.

(Reçu à 27 auguste, 1954)

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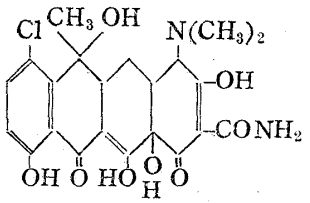
30. Takeichi Sakaguchi and Kiyomi Taguchi: Metal Chelate Compounds with Tetracycline Derivatives. II.* Colorimetric Determination of Aureomycin with Thorium(IV).

(*Pharmaceutical Institute, University of Chiba***)

Aureomycin (chlorotetracycline) is the most widely used antibiotic and the following structure has been reported by Waller, Hutchings, *et al.* in the Lederle Laboratories and also by Woodward, Stephans, *et al.*[1]



The quantitative determination of the antibiotic has been an important part of pharmaceutical and clinical analyses. A generally used, official method for the determination of Aureomycin is attributed to the fact that an intense yellow color is produced when it is heated with 0.2 *N* hydrochloric acid.[2] Other methods are through ultraviolet absorption spectra by Hiscox,[3] fluorometric analysis,[4] titration in nonaqueous solvent,[5] and polarographic determination.[6]

In this paper, a new sensitive method for the determination of Aureomycin is proposed.

I. Procedure by Th(IV)

Investigations indicated that the enolized hydroxyl group of 1,3-diketone, $-\overset{\overset{O}{\|}}{C}-CH=\overset{\overset{OH}{|}}{C}-$ in Aureomycin underwent chelation with metallic ions such as Th(IV), UO_2, Al(III), and Zr(IV).

In acidic medium, Aureomycin solution has peaks at 230, 262.5, and 367.5 mμ. When an aqueous solution of Th(IV) salts is added to an aqueous solution of Aureomycin Hydrochloride, an intense yellow color formed. This complex was studied spectrophotometrically and found to have maximum absorption at 405 mμ at pH 4.0 (Fig. 1).

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* Part I: This Bulletin, **3**, 147(1955).

** Inohana-cho, Chiba (坂口武一, 田口清水).

1) C. W. Waller, *et al.*: J. Am. Chem. Soc., **74**, 4978(1952); R. B. Woodward, *et al.*: *Ibid.*, **74**, 4976(1952).
2) J. Levine, *et al.*: J. Am. Pharm. Assoc., **38**, 473(1949).
3) Dorothy J. Hiscox: *Ibid.*, Sci. Ed., **40**, 237(1951).
4) M. Serenhe: C. A., **46**, 5112(1952).
5) Charles W. Pifer, *et al.*: J. Am. Pharm. Assoc., **42**, 509(1953).
6) Krestynova-Telupilova, *et al.*: Chem. Listy, **47**, 637(1953).

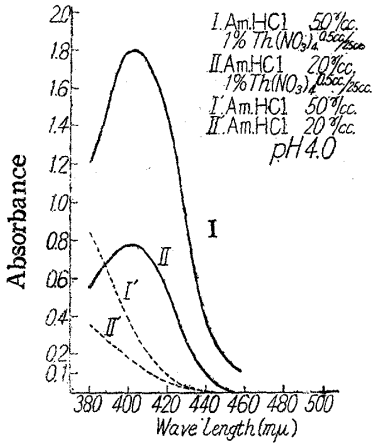


Fig. 1. Aureomycin–$Th(NO_3)_4$

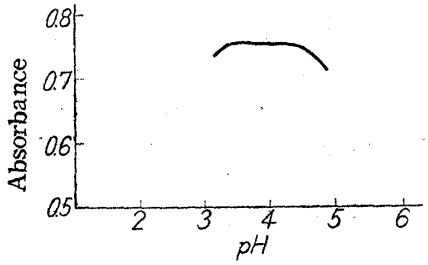


Fig. 2. Aureomycin–Th Chelate pH Variation

Absorption curves of aqueous solutions of these complexes at 405 mμ in various pH values were studied and from the curve represented in Fig. 2, it can be seen that the density reading remains constant between pH 3.5 and 4.5, its absorbance decreasing above and below this pH range. It was found that the pH could be maintained between 4.0 and 4.4 by using AcONa and AcOH buffer solution.

This color followed Beer's law over the range of 5 to 40 γ/cc. of Aureomycin Hydrochloride. The interfering substances are PO_4'', SO_4'', and F'. In the presence of such anions it was found necessary to extract Aureomycin with butanol in slightly acidic medium.

Reagent Solutions—1% Solution of $Th(NO_3)_4$ was prepared in a buffer solution of pH 4.0. One gram of reagent grade $Th(NO_3)_4$ was dissolved in 20 cc. of distilled water, adjusted to pH 4.0 with 0.1 *N* NaOH solution and brought to 100 cc. with the following AcONa and AcOH buffer solution.

Buffer Solution—Prepared by adding 35 cc. of 0.2 *M* AcONa solution into 165 cc. of 0.2 *M* AcOH solution.

Standard Solution—Exactly 50 mg. of standard Aureomycin Hydrochloride was dissolved in 100 cc. of distilled water.

Apparatus—All absorption measurements were made with Hitachi Spectrophotometer, Model EPB-V, using 1-cm. cells.

Procedure—For the determination of Aureomycin with Th, an aliquot (0.25~2.0 cc.) of the unknown solution containing ca. 50 mg% Aureomycin Hydrochloride, which was within the concentration range illustrated by the Beer's law curve (Fig. 3), was transferred to a 25-cc. volumetric flask, and the volume brought to 20 cc. with distilled water. The resulting solution was neutralized with 0.05 *N* NaOH solution, followed by adding 2 cc. of the AcONa–AcOH buffer solution and 1.0 cc. of 1% $Th(NO_3)_4$ solution. Its volume was brought up to 25 cc. with distilled water and left standing until the yellow color of Th–Aureomycin chelate appeared to remain constant. The absorbance of the resulting solution was taken at 405 mμ about 20 mins. later. The results are shown in Tables I and II.

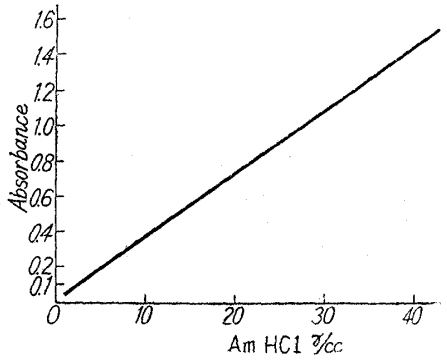


Fig. 3. Aureomycin–$Th(NO_3)_4$ Calibration Curve

Results

The conformity of the colored solution of Th–Aureomycin chelate to the Beer's law was seen over a concentration from 5 to about 40 γ of Aureomycin Hydrochloride per cc. (Fig. 3). From results given in Table I a regression line was established. The equation for the line was $y=0.0364x+0.0262$, where x represents γ of Aureomycin–HCl and y is the absorbance. The correlation coefficient was +1.000.

Table I.

Aureomycin-HCl γ/cc.	5	10	20	30	40
Absorbance	0.200	0.392	0.761	1.12	1.47
〃	0.203	0.392	0.764	1.13	1.46
〃	0.200	0.390	0.762	1.13	1.49

Results of Analysis—Experiments were run on the concentrations of 6, 14, and 24 γ of Aureomycin-HCl per cc. The standard deviation and mean value are shown in Table II.

Table II.

Aureomycin-HCl γ/cc.	6	14	24
Absorbance	0.250	0.548	0.900
〃	0.250	0.550	0.910
〃	0.260	0.552	0.900
〃	0.255	0.550	0.900
〃	0.245	0.549	0.890
Mean value	0.2520	0.5499	0.9000
Standard deviation	0.00161	0.00133	0.002

Effect of pH—Several measurements were taken on the chelates from 390 to 405 mμ at various pH values of 3.5 to 4.8 (Table III and Fig. 2). The absorbance increased with pH and the maximum was at pH 4.0. However, the density reading remained constant in the pH range of 3.5 to 4.5 giving maximum absorbancy by using AcONa-AcOH solution. Above pH 5.5 a slightly yellow turbidity or precipitate was noted and it could be maintained in the basic medium.

Table III. Aureomycin-HCl 20 γ/cc., 1% $Th(NO_3)_4$ 1 cc./25 cc. by Using Acetate Buffer

Wave length (mμ) \ pH	3.5	3.7	4.1	4.4	4.8
390	0.680	0.680	0.680	0.670	0.650
395	0.730	0.731	0.729	0.720	0.690
400	0.760	0.750	0.755	0.752	0.717
405	0.760	0.750	0.757	0.745	0.710

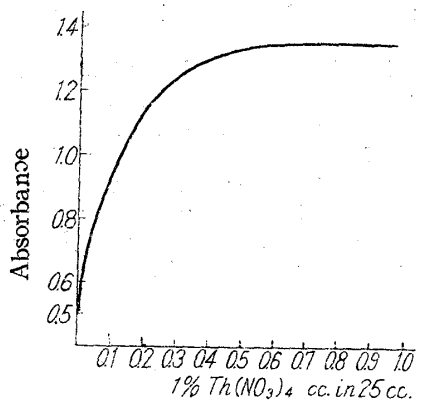


Fig. 4. Aureomycin-HCl 40 γ/cc., pH 4.0

Effect of Th-Reagent Concentration—In Fig. 4, an attempt has been made to show the effect of the various concentrations of Th ion on the color developement, using AcONa-AcOH buffer solution. As can be seen from this curve, it is necessary to have 0.5 cc. of 1% $Th(NO_3)_4$ per 1 mg. of Aureomycin-HCl. A greater excess of reagent over this value has no effect upon the absorbance readings. Hence, the use of 1.0 cc. of 1% Th-reagent was adopted as previously described.

Effect of Time—Absorption measurements were run on an aqueous solution of Aureomycin-HCl (20 γ/cc.) after standing for some time. Table IV indicates that constant absorbance is obtained after 15 mins. of standing.

Table IV.

Time (min.)	6	10	15	20	30	60	48 (hrs.)
Absorbance	0.720	0.750	0.760	0.761	0.761	0.762	0.761

Interferences—There are essentially no serious problems, but some interference was noted in the proposed procedure. Of the common anions, phosphate and sulfate ions would interfere, since they might cause precipitation or form very stable complex with Th(IV). The absorbances, at the same concentration of 40 γ/cc. by the official method of Levine and by the present Th method, are 0.57 and

1.5, respectively at 440 and 405 mμ. Hence, the Th method gives higher sensitivity and more specificity than the known methods.

Avoidance of Interferences—Will be reported in the next paper.

II. Procedure by Th(IV) and HCl

Aureomycin solution gives yellow color with Th in an acid medium and it was converted to an orange yellow chelate having a peak at 450 mμ on heating with dil. HCl. This orange yellow compound was found to be identical with Th-Anhydroaureomycin from the absorption curve.

Reagents—$Th(NO_3)_4$ solution (0.5%) : One-half gram of $Th(NO_3)_4 \cdot H_2O$ was dissolved in 10 cc. of distilled water.

To 1 cc. of 50 mg% Aureomycin-HCl solution was added 0.5 cc. of 0.5% $Th(NO_3)_4$ solution and the mixture was diluted exactly to 3 cc. with distilled water. To this solution was added 5 cc. of 2 *N* HCl and it was heated on a boiling water bath for 5 mins. The resulting solution was transfered to a 25-cc. volumetric flask and brought up to the mark by the addition of sufficient distilled water. The absorbance of the solution was measured at 450 mμ against a distilled water blank after 15 mins. of standing (Table V).

Results

Standard Curve and Regression Line (Fig. 5)—The equation for the line was $y=0.011x+0.0388$, where x represents γ of Aureomycin-HCl and y is the absorbance. The correlation coefficient was + 0.9946.

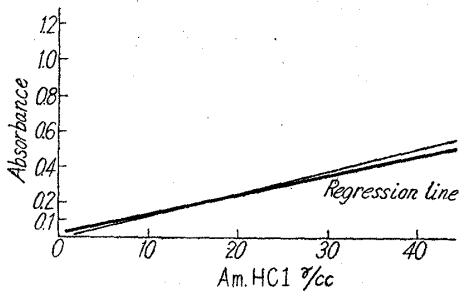
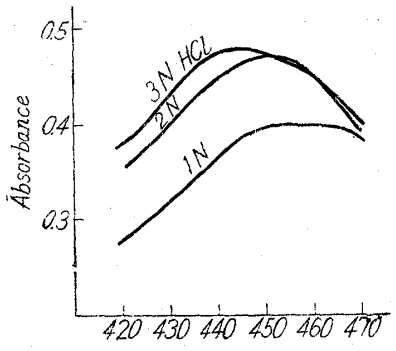


Fig. 5.

Aureomycin-Th-2 *N* HCl Method



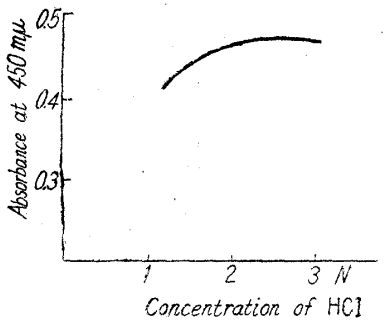


Fig. 6

Fig. 5 shows the calibration curve plotting the mean values of absorbances and regression line. Values for the absorbance obtained from a graphical plot in the usual manner were compared with the ones from the regression line. The difference in the values of absorbance for calibration curve and regression line are given in Table VI.

Effect of HCl—As shown in Fig. 6 the recommended concentration of HCl is 2 *N*.

Effect of Th-Reagent—It may be used three times the amount of Th-reagent as previously described (Table VII).

Effect of Heating Time—Effect of heating time on color was examined and it was found that heating time of 5 mins. is to be recommended (Table VIII).

Table V.

Concn. of Aureomycin-HCl (γ/cc.)	10	20	30	40	50
Absorbance	0.140	0.264	0.370	0.470	0.590
〃	0.140	0.262	0.373	0.472	0.590
〃	0.142	0.270	0.380	0.473	0.591
Mean value	0.141	0.265	0.376	0.472	0.590

Table VI.

Aureomycin-HCl (γ/cc.)	14	24	44
Absorbance	0.183	0.298	0.524
〃	0.191	0.291	0.534
〃	0.185	0.299	0.529
〃	0.178	0.290	0.520
〃	0.190	0.310	0.533
Standard deviation	0.0058	0.0030	0.0079

Aureomycin-HCl (γ/cc.)	14	24	44
Mean value of absorbance	0.185	0.289	0.528
Calculated concentration from regression line (γ/cc.)	9.75	22.1	47.5
Deviation from known amount (%)	30.4	7.90	7.95
Concn. obtained from calibration curve (γ/cc.)	13.7	22.5	44.4
Deviation from known amount (%)	2.14	6.3	0.9

TABLE VII.

Multiple value	1	2	3
Absorbance	0.463	0.461	0.461

TABLE VIII.

Time of heating (min.)	3	5	10
Absorbance	0.409	0.463	0.449
〃	0.401	0.460	0.451

The authors wish to express their thanks to Prof. M. Ishidate of the Pharmaceutical Institute, University of Tokyo, for his valuable advices and instruction. Thanks are also due Dr. Y. Suzuki of the Ophthalmic Department, Chiba University Hospital, for the use of the spectrophotometer.

Summary

New colorimetric assay for Aureomycin with Th(IV) is described. A yellow complex has a maximum at 405 mμ at pH 4.0. It converts to an orange yellow complex on heating with dilute hydrochloric acid. These two color reactions conform to the Beer's law and can be applied to the colorimetric determination of Aureomycin.

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31. **Takeichi Sakaguchi**: Metal Chelate Compounds with Tetracycline Derivatives. III.* Colorimetric Determination of Aureomycin with Boric Acid.

(*Pharmaceutical Institute, University of Chiba***)

Current interest in Aureomycin and related compounds has increased the need for accurate means of assay. There are a few chemical methods available for the quantitative estimation of these antibiotics. Production of color by constituent functional groups of the tetracycline is the basis for the method presented here.

A solution (10 cc.) of concentrated sulfuric acid (sp. gr., 1.84) was added to a solution of Aureomycin Hydrochloride (about 5 cc.) to produce a somewhat stable orange red color (absorption maximum about 455 mμ), which turned brown.[1]

Addition of 1 cc. of 1% boric acid to a few cc. aliquot of Aureomycin Hydrochloride solution and made up to 5 cc. with concentrated sulfuric acid, produced a red violet color (absorption maximum 515~530 mμ) which was stable over a longer period (Fig. 1).

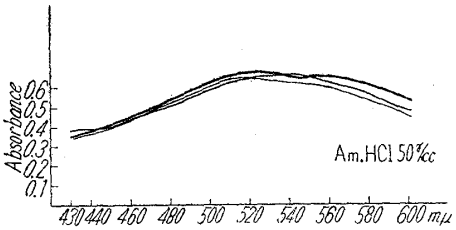

Fig. 1. Aureomycin-H_3BO_3-conc. H_2SO_4

* Part II: This Bulletin, **3**, 166 (1955).

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1) C. G. Van Arkel, A. M. Weshoff: Pharm. Weekblad, **86**, 389 (1951) (C. A., **46**, 5276).