inversion—a) A solution containing 0.6 g. of the oxazoline (III) and methyl tosylate in 4 cc. of dry toluene was refluxed in an oil bath for 2 hours and two layers formed. After cooling and removing the toluene layer by decantation, a solution prepared by refluxing 5 g. of glacial acetic acid, 0.5 g. of acetic anhydride, and 0.5 g. of fused sodium acetate for 10 minutes was immediately added to the residue. The mixture was refluxed in an oil bath for 3 hours with exclusion of moisture, cooled, and poured into 150 cc. of water, causing precipitation of crystals, m.p. 125~128°. After filtering and recrystallization from benzene-petroleum ether, 0.5 g. of colorless needles, m.p. 130~131°, were obtained. *Anal.* Calcd. for $C_{19}H_{21}O_3N$ (O-acetyl-N-benzoyl-*dl*-ephedrine): N, 4.22. Found: N, 4.25.

A small piece of the crystal in 10% sodium hydroxide was heated in a boiling water bath, for 45 min., the resulting oil product solidifying soon after cooling. After filtering, washing with water, and recrystallizing from benzene-petroleum ether, it melted at 107° and was proved the same as N-benzoyl-*dl*-ephedrine and not with N-benzoyl-*dl*-ψ-ephedrine by admixture.

b) A solution containing 0.7 g. of the oxazoline (III) and 0.45 g. of dimethyl sulfate in 3 cc. of anhydrous benzene was refluxed on a water bath for 40 minutes avoiding moisture. Subsequent treatments were conducted after the manner of (a) and 0.7 g. of O-acetyl-N-benzoyl-*dl*-ephedrine was obtained, from which N-benzoyl-*dl*-ephedrine was formed.

Summary

When the methylating agents are applied to oxazolines, derived from 2-aminoalcohols, N-methyloxazolinium salts are formed. The products seem to be so unstable that addition of one mole of water causes conversion into 2-methylaminoalcohols. If they are resoluble, retention and inversion of configuration simultaneously with the ring opening, may be controlled by some reaction conditions. Based on these suppositions, *dl-trans*-2,5-diphenyl-4-methyloxazoline was reacted with methylating agents and subsequently treated in the presence of either water or dry acetic acid-sodium acetate. Consequently, by the former treatment N-methylation and retention of configuration occured, yielding *dl*-ψ-ephedrine derivatives. On the contrary, by the latter treatment, N-monomethylation and inversion of configuration resulted, forming *dl*-ephedrine derivatives. Thus *dl*-norephedrine and *dl*-ψ-norephedrine can be converted to either *dl*-ephedrine or *dl*-ψ-ephedrine, for the *dl-trans*-oxazoline is derived from either *dl*-norephedrine or *dl*-ψ-norephedrine and N-mono methylation is complete.

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78. **Minoru Kawashima and Shizuko Fujii:** Biochemical Studies on Acidomycin. II.* Increase of Urinary Biotin Excretion of Acidomycin-Treated Rabbits.

(*Research Laboratory, Takeda Pharmaceutical Industries, Ltd.***)

The authors recently reported that intramuscular injection of 200 γ per g. body weight of acidomycin sodium into rabbits increased an anti-acidomycin factor in their urine, and that the factor seemed to be biotin from the result of inhibition analyses with *Lactobacillus caseii, Lactobacillus arabinosus 17-5*, and *Mycobacterium tuberculosis typus avium*, and partition chromatographic analyses of urine samples[1].

Stokes, *et al.*[2] stated that some kind of lactic acid bacteria appear to require for their growth an exogenous supply of both biotin and aspartic acid if the biotin is limited to

* "Studies on Acidomycin VI[1]" is designated as "Biochemical Studies on Acidomycin. I."

** 4-chome, Juso-Nishino-cho, Higashi-yodogawa-ku, Osaka (川島 実, 藤井シズ子).

1) M. Kawashima, *et al.*: This Bulletin., 1, 94(1953).
2) J. L. Stokes: J. Bact., 54, 219(1947).

0.001 μg. or less per 10 cc. medium. On the other hand, Lardy, *et al.*[3] showed that oxalacetic acid is also capable of promoting growth of *L. arabinosus 17-5* on media deficient in both biotin and aspartic acid, although it is not so effective as aspartic acid.

The purpose of the present work is to establish the influence of these compounds on biotin assay values, and to elaborate to some extent the previous work on increase of urinary biotin excretion by acidomycin administration.

Methods

The microörganism used in the experiments was *Lactobacillus arabinosus 17-5*, and the medium employed was completely synthetic in composition[1].

Effect of Aspartic Acid and Sodium Ethyloxalacetate on Biotin Assay Values—i) Two mg. of aspartic acid was added to 5 cc. of medium containing 50 mγ per 100 cc. of biotin in the initial tube, and the contents of the tube were diluted by consecutive 6-fold dilution. The same amount of sodium ethyloxalacetate was also processed as above. Both series were sterilized by autoclaving at 15 lbs. for 10 minutes and, after inoculation with *L. arabinosus*, incubated at 30° for 24 hours, and the resulting turbidities were measured electrophotometrically (Fig. 1).

ii) Bacterial growth in the biotin deficient medium was inhibited by the addition of 40 γ per cc. of acidomycin sodium. One mg. per cc. of aspartic acid was added to 5 cc. of the above solution in the initial tube and the contents of the tube were diluted by consecutive 5-fold dilution. An equal amount of sodium ethyloxalacetate was treated in the same manner. Another series containing no acidomycin sodium was prepared as a contrast. The resulting turbidities of the three series were measured as in (i) (Fig. 2).

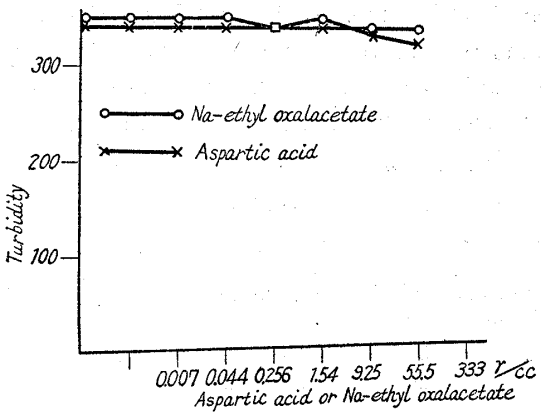

Fig. 1. Effect of aspartate and Na ethyloxalacetate on synthetic medium containing 50 mγ/100 cc. of biotin. *L. arabinosus 17—5*, 30°, 24 hrs.

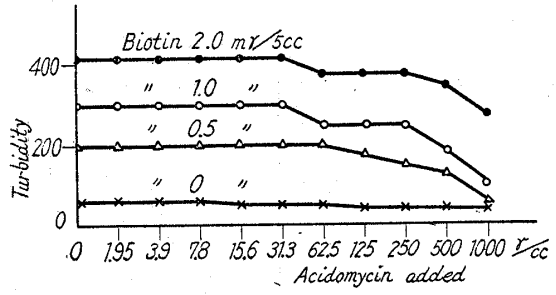

Fig. 3. Effect of acidomycin on synthetic medium having various concentration of biotin

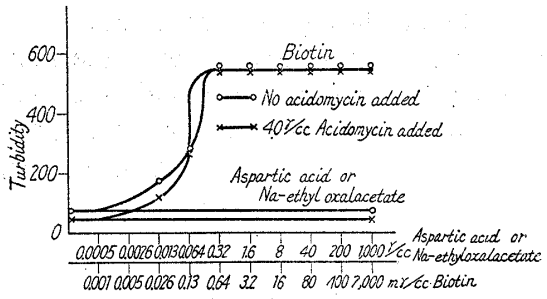

Fig. 2. Effect of aspartate and Na ethyloxalacetate on biotin deficient medium. *L. arabinosus 17—5*, 30°, 24 hrs.

Effect of Acidomycin Concentration on Biotin Assay Values—A thousand γ per cc. of acidomycin sodium was added to 5 cc. of four media containing 0.0, 0.5, 1.0, and 2.0 mγ per 5 cc. of biotin, respectively, and then diluted by consecutive 2-fold dilution. The four series were autoclaved, inoculated, and incubated as described above, and the resulting turbidities were measured electrophotometrically (Fig. 3).

Microbiological Assay of Urinary Biotin of Rabbits—Three normal rabbits (ca. 2,500 g., male) were kept under the same conditions for ten days before experiment, and from each of them urine samples were collected in the following manner. The animal was fixed on its back, and total urine was discharged by the urethral catheter, thereafter 3 urine samples were collected at 2-hour intervals. Next day, 2 hours after total urine had been discharged the first urine sample was collected, and immediately 200 γ per g. body weight of acidomycin sodium was injected intramuscularly, after which 2 urine samples were collected every 2 hours in the same manner as on the previous day.

3) H. A. Lardy: J. Biol. Chem., 169, 451(1947).

One-half cc. aliquot of a 1:100 dilution of the urine samples was brought into the tubes containing 2.5 cc. of medium doubly strengthened for biotin assay, and the contents of the tubes were diluted to 5 cc. with water. At the same time, standard series containing 0.0~2.0 mγ per tube of biotin were prepared. All the tubes were sterilized by autoclaving at 15 lbs. for 10 minutes and, after inoculation with *L. arabinosus*, incubated at 30° for 24 hours, and the resulting turbidities were compared with that of the standard series by electrophotometrical measurement.

Results and Discussion

The medium employed contained hydrolyzed casein as the main nitrogen source. It was anticipated that in this medium neither aspartic acid nor sodium ethyloxalacetate had any effect on biological assay values of biotin. Experimental results showed, as expected, that both compounds were not effective on the bacterial growth supported by biotin (50 mγ per 100 cc.) in the synthetic medium (Fig. 1), and also could not reverse the inhibition of bacterial growth by 40 γ per cc. of acidomycin sodium added (Fig. 2). Therefore, the anti-acidomycin factor, which we recently reported to be biotin from the agreement of Rf values and results of inhibition analyses, was more positively proved by the present observations which showed that neither aspartic acid nor sodium ethyloxalacetate had biotin-like activity in the synthetic medium employed.

Determination of minimal effective concentration of acidomycin sodium in the synthetic medium for biotin assay showed that acidomycin sodium had no influence in an amount less than 30 γ per cc., so that this effect was negligible under the conditions used (Fig. 3).

Acidomycin concentrations in the urine samples of which the biotin had been measured by microbiological assay, were determined by means of cup plate method with *M. tuberculosis typus avium* and found to be 11.1~4.26 mg. and 2.156~2.000 mg. per cc. of the urine collected at 2 hours and 4 hours respectively after acidomycin treatment, (Table I).

TABLE I. Urinary Biotin Excretion

	Not Treated						Treated					
	1		2		3		1		2*		3*	
	mg./cc.	Vol.	mg./cc.	Vol.	mg./cc.	Vol.	mg./cc.	Vol.	mg./cc.	Vol.	mg./cc.	Vol.
R_1	24	32	20	10	30	10	20	28	184	10	152	9
R_2	24	23	20	24	24	21	16	35	96	23	200	12
R_3	12	23	12	27	28	10	16	20	96	20	112	21

*Acidomycin concentration in urine

	after 2 hrs.	after 4 hrs.
R_1	11.1 mg./cc.	2.0 mg./cc.
R_2	6.4 „	2.07 „
R_3	4.26 „	2.15 „

Biotin levels in urine increased more remarkably after intramuscular injection of 200 γ per g. body weight of acidomycin sodium than those not treated with the compound. It was found that the disparity between the urinary biotin excretion levels before and after the acidomycin administration was significant from the viewpoint of statistical analysis, and this increases of biotin excreted in urine was undoubtedly due to the treatment with acidomycin sodium (Fig. 4, Table I).

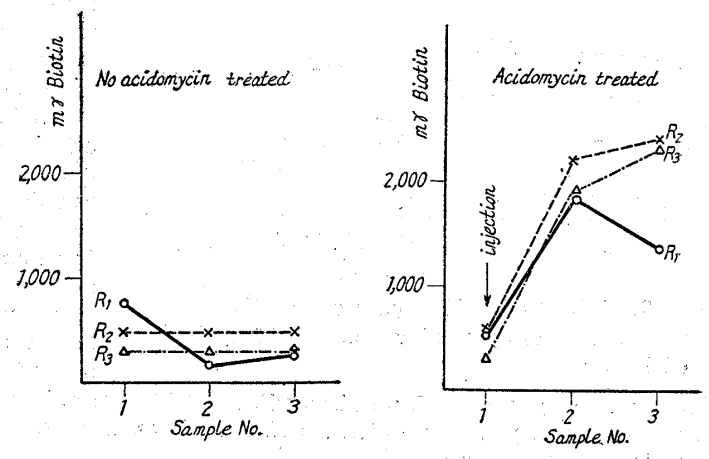

Fig. 4. Urinary biotin excretion in rabbits.

The mechanism of this biotin increase is not yet clear. Wright, *et al.*[4] stated that the biotin antagonists were capable of liberating biotin from the biotin-avidin complex *in vitro*. We believe that the phenomenon observed in the urine of acidomycin-treated rabbits is due to the biotin liberation from the biological protein-biotin complex by the function of acidomycin administered. Wright's observation seems to be the model case of our phenomenon and we intend to study this phenomenon by using avidin preparation.

We are deeply indebted to Mr. A. Miyake of this laboratory for his generous contribution of acidomycin sodium, to Mr. Y. Hamada for acidomycin assay in urine samples, and to Mr. S. Shintani for his statistical treatments. We also express our grateful thanks to Dr. S. Kuwada, Director of this laboratory, for his permission for this investigation and publication.

Summary

In our synthetic medium, we found that neither aspartic acid nor sodium ethyloxalacetate had any influence on biotin assay values, and acidomycin sodium also had no influence in an amount less than 30 γ per cc. concentration. Biotin level in the urine of rabbits increased remarkably after acidomycin treatment. The present observations proved more positively that the anti-acidomycin factor, recently reported, was biotin.

(Received August 5, 1953)

4) L. D. Wright: Arch. Biochem., 12, 27(1947).

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79. Kiichi Arakawa: Antibacterial Activity of Compounds Possessing a Tricarbonylmethane Group. X[1]. Observations on the Acetylation of 4-Hydroxycoumarin and Synthesis of 3-(α-Aminophenylacetyl)-4-hydroxycoumarins.

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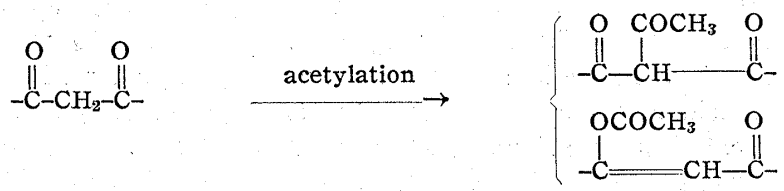
A series of studies have been carried out by Claisen[2] and Dieckmann[3] on the acetylation of 1,3-dicarbonyl compounds as to the formation of C-acetylated compound by the condensation of the acetyl group with the activated methylene between the two carbonyls or of O-acetylated compounds by the substitution of the acetyl group with one of the enolized carbonyl group.



For example, when ethyl acetoacetate and acetyl chloride are reacted, the use of pyridine or other tertiary amines gives an O-acetylated compound, while the reaction of sodium ethyl acetoacetate and acetyl chloride under the same conditions gives a C-acetyl compound[2]. When the original material is a cyclic diketone, such as hydroresorcinol, acetylation with acetic anhydride alone will give an O-acetyl compound whereas the use of organic bases such as pyridine or tripropylamine will give a C-acetyl compound, but not

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1) Part IX: This Bulletin, 1, 255(1953).
2) L. Claisen, H. Haase: Ber., 33, 1242(1900).
3) W. Dieckmann, R. Stein: *Ibid.*, 37, 3370(1904).