ISOLATION AND IDENTIFICATION OF OXOPROLINE IN ACTINOMYCIN X_2 (V) HYDROLYSATES

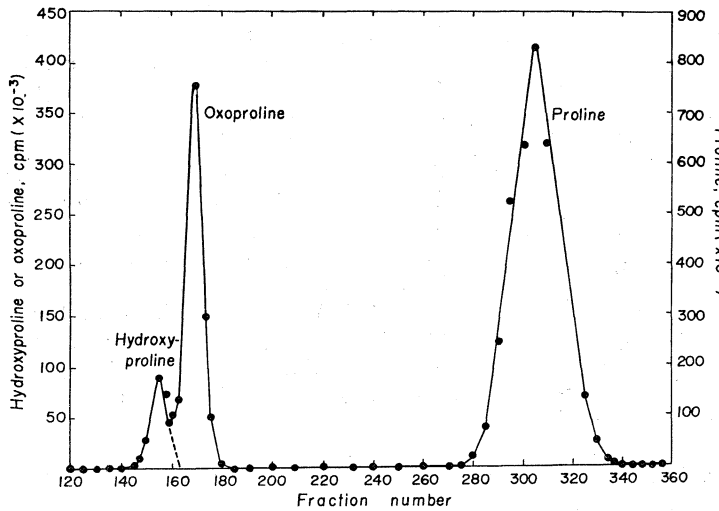
Sir:

The identification of 4-oxo-L-proline in actinomycin X_2 (V) has been based on the behavior of the imino acid *in situ* in comparison with synthetic oxoproline under different conditions of catalytic reduction[1,2]. In the case of actinomycin X_2, such reduction leads to formation of actinomycin C_1 (IV, L-proline) or actinomycin $X_{0\beta}$ (I, threo-hydroxy-L-proline) and $X_{0\delta}$ (allohydroxy-L-proline). Previous reports have suggested that oxoproline was destroyed by vigorous hydrolysis of actinomycin X_2 with hydrochloric acid and that recovery of the imino acid was achieved in low yield after hydrolysis with hydroiodic acid at 100°C[1,2].

During oxygen-18 experiments with *Streptomyces antibioticus*, we prepared ^{14}C-labeled actinomycin mixtures to facilitate the isolation and recovery of hydroxyproline and proline. However, it was established that oxoproline could be isolated also in good yield from actinomycin hydrolysates. L-Proline-^{14}C was administered to a 48-hour old actinomycin producing culture of *S. antibioticus* and, following a 2-hour incubaton, the antibiotic mixture was recovered from the medium with ethyl acetate (1 : 1.5)[3]. After evaporation of the solvent, the actinomycins were hydrolyzed in 6 N HCl for 3.5 hours at 121°C and 15 pounds pressure. The hydrolysate was decolorized with Norit A and evaporated to dryness to remove excess acid.

The imino acids in the hydrolysate were resolved on a Dowex 50-X8 ion-exchange column (1×150 cm, H^+, 200~400 mesh) with 1 N HCl[4]. As seen in Fig. 1, tubes 145~163 contained hydroxyproline and tubes 275~337 contained proline. A large radioactive peak (tubes 163~180) was eluted directly after hydroxyproline. Since previous labeling experiments had revealed that L-proline-^{14}C is incorporated into the imino acids of actinomycin mixtures with virtually no radiolabel in the other amino acids of the antibiotic[3,5], it was considered likely that the unknown radioactive peak was oxoproline. Reduction of an aliquot with sodium borohydride in water yielded hydroxyproline. This was concluded from the fact that the latter compound on ascending paper chromatograms had the same Rf value as authentic hydroxyproline with the following solvent systems: a) 80 % aqueous phenol – ethanol – ammonia (150 : 40 : 10), b) 77 % ethanol, c) *t*-butanol – formic acid – water (150 : 30 : 30), d) phenol – *n*-butanol – acetic acid – water (10 g : 30 : 10 : 50). The Rf values were: a) 0.45, b) 0.35, c) 0.34, d) 0.17. The radioactive peak obtained with the Vanguard 880 chromatogram autoscanner coincided with the isatin-positive spot of hydroxyproline. In the solvent system: e) butanol – acetic acid – water (4 : 1 : 7), the unreduced radioactive compound had an Rf value (0.18) that was identical with authentic oxoproline and gave a red color on

Fig. 1. The elution of ^{14}C-labeled imino acids from a Dowex 50-X8 column (1×150 cm, H^+, 200~400 mesh) with 1.0 N HCl at 25°C. A hydrolysate of an actinomycin mixture was placed on the column; the effluent was collected in 2 ml fractions at 8 ml/hr. One-tenth ml of each sample was counted in a liquid scintillation spectrometer.



treatment with 2 % aqueous triphenyltetrazolium chloride reagent in agreement with the findings of BROCKMANN *et al.*[1,2] With solvent system a), which contains ammonia, the unreduced imino acid failed to move from the origin. Oxoproline is highly unstable at an alkaline pH and polymerizes to a black residue[6].

The imino acids and amino acids in actinomycin X_2 were identified also by gas chromatography after their conversion to N-trifluoroacetyl methyl esters. For comparison, N-trifluoroacetyl-4-oxoproline methyl ester was prepared both from 4-oxoproline hydrobromide and by chromic acid oxidation of N-trifluoroacetyl-4-hydroxyproline methyl ester. On one column (1.8 % EGSP-z on 100~120 mesh Gas Chrom Q, argon flow 70 ml/min) at 115°C, the 4-oxoproline derivative both from the hydrolysate and in the reference sample had a retention time of 7.0 min (proline, 2.4 min). On a second column (5 % EGS on 100~120 mesh Gas Chrom Q, argon flow 40 ml/min) at 180°C, the derivative of authentic oxoproline gave a peak at 9.5 min. The hydrolysate (actinomycin X_2) gave peaks at 3.0 min (proline), 6.1 min (monoacylated threonine) and at 9.5 min (4-oxoproline). At 105°C on the latter column, the other amino acids (N-methylvaline, 7.8 min; valine, 9.6 min; sarcosine, 13.0 min) in actinomycin X_2 were also identified.

The radioactive hydroxyproline formed by reduction of oxoproline with sodium borohydride was resolved into *allo* and *threo*-hydroxyproline (3 : 1) on a Dowex 50 column (1×133 cm, 200~400 mesh) developed with a citrate-sodium citrate buffer of pH 2.91[7,8]. The column also proved useful for separating sarcosine from hydroxyproline fractions*.

Recovery of the imino acids (μmoles) isolated from hydrolysates of actinomycin mixtures in three experiments was: L-proline (190~240), 4-hydroxy-L-proline (6.0~6.6) and 4-oxo-L-proline (14~17.3). In a subsequent experiment, 28.3 μmoles of actinomycin X_2 were isolated by thin-layer chromatography[9]. The oxoproline was reduced *in situ* to hydroxyproline with sodium borohydride and the actinomycin was then hydrolyzed. Analyses[10,11] revealed that the hydrolysate contained 23.6 μmoles of hydroxyproline and 30.8 μmoles of proline for a recovery of 0.84 and 1.1 μmoles, respectively, per μmole of actinomycin X_2. These data establish the 1 : 1 molar ratio of oxoproline to proline in the antibiotic molecule. Similar observations were reported by FURUKAWA *et al.*[12] on the direct isolation of oxoproline from actinomycin S_3.

Acknowledgements

This investigation was supported by grants: CA 06926 (E.K.) from the National Cancer Institute; AI 07662 (A.B.M.) and TI-AI 298 from the National Institute of Allergy and Infectious Diseases, N. I. H., U.S.A.

ROBERT DIEGELMANN
ANTHONY MAUGER*
EDWARD KATZ

Department of Microbiology, Georgetown University Schools of Medicine and Dentistry and the Research Foundation of the Washington Hospital Center*
Washington, D. C., U. S. A.

(Received December 25, 1968)

References

1) BROCKMANN, H. & J. H. MANEGOLD: Überführung von Actinomycin X_2 in Actinomycin X_1. Naturwiss. 45 : 310~311, 1958
2) BROCKMANN, H. & J. H. MANEGOLD: Überführung von Actinomycin X_2 in die Actinomycine C_1, $X_{0\beta}$ und $X_{0\delta}$. Chem. Ber. 93 : 2971~2982, 1960
3) SALZMAN, L. A.; H. WEISSBACH & E. KATZ: Conversion of C^{14}- and *cis*- or *trans*-4-H^3--L-proline to 4-hydroxy-L-proline and 4-oxo-L-proline by *Streptomyces antibioticus*. Proc. Nat. Acad. Sci. 54 : 542~546, 1965
4) HIRS, C. H. W.; S. MOORE & W. H. STEIN: The chromatography of amino acids on ion exchange resins. Use of volatile acids for elution. J. Am. Chem. Soc. 76 : 6063~6065, 1954

* The authors wish to thank Dr. E. J. MILLER for his suggestions concerning the chromatographic separation of sarcosine and hydroxyproline.

5) KATZ, E.; D. J. PROCKOP & S. UDENFRIEND : Precursors of the hydroxyproline and ketoproline in actinomycin. J. Biol. Chem. 237 : 1585～1588, 1962
6) PATCHETT, A. A. & B. WITKOP : Studies on hydroxyproline. J. Am. Chem. Soc. 79 : 185～192, 1957
7) PIEZ, K. A. & L. MORRIS : A modified procedure for the automatic analysis of amino acids. Anal. Biochem. 1 : 187～201, 1960
8) PIEZ, K. A.; F. IRREVERRE & H. L. WOLFF : The separation and determination of cyclic imino acids. J. Biol. Chem. 223 : 687～697, 1956
9) KATZ, E.; A. B. MAUGER & H. WEISSBACH : Biosynthesis of highly labeled actinomycins. Mol. Pharm. 1 : 107～109, 1965
10) PROCKOP, D. J. & S. UDENFRIEND : A specific method for the analysis of hydroxyproline in tissues and urine. Anal. Biochem. 1 : 228～239, 1960
11) TROLL, W. & J. LINDSLEY : A photometric method for the determination of proline. J. Biol. Chem. 215 : 655～660, 1955
12) FURUKAWA, M.; A. INOUE, K. ASANO & J. KAWAMATA: Chemical studies of actinomycin S. II. Chemical structures of actinomycin S_2 and S_3. J. Antibiotics 21 : 568～570, 1968