MICROBIAL TRANSFORMATION OF PEPTIDE ANTIBIOTICS. VI

PURIFICATION AND PROPERTIES OF A PEPTIDE LACTONASE HYDROLYZING DIHYDROSTAPHYLOMYCIN S

C. T. HOU, D. PERLMAN and M. R. SCHALLOCK

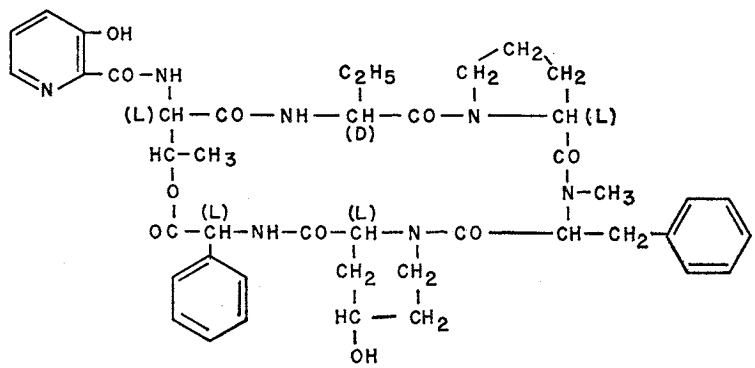
The School of Pharmacy, University of Wisconsin,
Madison, Wisconsin 53706, U. S. A.

(Received for publication November 18, 1969)

A constitutive peptide antibiotic lactonase opening the lactone linkage of dihydrostaphylomycin S was isolated from *Actinoplanes missouriensis*. Approximately 200-fold purification was achieved by ammonium sulfate precipitation followed by chromatography on calcium phosphate-cellulose, DEAE-cellulose, and Sephadex G-200 columns. The molecular weight, as determined by gel filtration on Sephadex G-200, is 35,000. The K_m value for dihydrostaphylomycin S is 3.73×10^{-4} M. This enzyme also hydrolyzed the lactone bond in echinomycin, etamycin, staphylomycin S, stendomycin, and vernamycin B_α. The enzyme content of the cells was increased by addition of these peptides and also actinomycin to the growing culture.

The induction in *Actinoplanes missouriensis* of enzymes capable of hydrolyzing the lactone bonds in actinomycin has been previously reported from this laboratory[1,2,3]. This inducable enzyme showed homogeneity in acrylamide gel electrophoresis of a 100-fold purified preparation[4]. Further study of the crude enzyme preparation showed the presence of a second peptide antibiotic lactonase which hydrolyzed the lactone bond of dihydrostaphylomycin S (Fig. 1) and other peptide antibiotics containing the lactone bond. This second enzyme was found to be constitutive in *A. missouriensis* and has been separated from the inducable enzyme by chromatography on DEAE-cellulose columns.

Fig. 1. Structure of dihydrostaphylomycin S



Materials and Methods

Growth of microorganisms: The culture of *Actinoplanes missouriensis* (IMRU #824) was maintained on slants of BERGER's agar (Contadina tomato paste-Heinz baby oatmeal) and grown in liquid culture in a soybean meal (3 %)-glycerol (3 %) medium as previously described[2]. Induction tests were carried out by adding the antibiotics compounds to 48~72-hour old cultures (to give a final concentration of 10 mcg/ml) and continuing incubation for an additional 18 hours[3]. The cells were then harvested by centrifugation, washed with 0.05 M pH 7.0 phosphate buffer, and recentrifuged. The cell pastes were frozen and stored until needed.

Substrates and enzymes: The dihydrostaphylomycin S-^{3}H prepared by reduction of staphylomycin S with sodium borohydride-^{3}H was obtained from Dr. H. VANDERHAEGHE who noted that dihydrostaphylomycin S has the same antibacterial potency as staphylomycin S (personal communication). The actinomycin D-^{3}H (referred to as actinomycin-^{3}H in this paper) was purchased from Schwartz Bio-Research, Inc. and used without further purification. The other peptide antibiotics including actinomycin D, echinomycin, etamycin, staphylomycin S, stendomycin, thiostrepton, and vernamycin B_α were obtained as gifts and used in our experiments without further purification.

The lactic dehydrogenase, alcohol dehydrogenase, sterol dioxygenase, catalase, bovine serum albumin, and trypsin were purchased from Sigma Chemical Company.

Standard assay for enzyme activity: The standard reaction mixture contained enzyme preparation, 22.4 mμmoles dihydrostaphylomycin S-^{3}H (980 cpm per mμmole), 6 μmoles Tris·HCl buffer (pH 7.8), and water to a total volume of 0.20 ml. The reaction mixture was incubated at 38°C for 60 minutes using a Gyrotary shaker. At the end of the incubation period, the hydrolysis was terminated by addition of 0.8 ml of 0.5 N HCl. Four ml of ethylacetate was then added, and the tubes shaken to extract the residual dihydrostaphylomycin S and the hydrolysis product into the organic phase. Three ml of the ethylacetate layer were transferred to another tube containing 1.5 ml of 0.1 M Tris·HCl buffer (pH 7.8), and after thorough mixing, the solvent layer was discarded. Under these conditions the linear, acidic peptide formed by opening the lactone bond of dihydrostaphylomycin S is extracted into the buffer, while the unchanged dihydrostaphylomycin S remains in the ethylacetate. A 1.0 ml aliquot of the aqueous buffer phase was assayed for radioactivity. Essentially no degradation of dihydrostaphylomycin S-^{3}H occurred if the enzyme was ommitted or if boiled enzyme was substituted to the active enzyme. One unit of enzyme activity converts 1 mcg of substrate to acidic product in 60 minutes at 38°C.

Preparation of linear peptide from staphylomycin S: Alkaline hydrolysis of staphylomycin S and dihydrostaphylomycin S to yield the linear antibacterially inactive peptide was carried out according to the procedure of VANDERHAEGHE and PARMENTIER[5] who found that this treatment of staphylomycin S with 0.1 N NaOH for 30 minutes cleaved the lactone bond but did not effect any other change in the molecule. This linear peptide derived from staphylomycin S after purification by chromatography on silica gel G (acetone - water, 98 : 2, as developing solvent) as a standard for comparison with the material produced by enzymatic treatment of dihydrostaphylomycin S and of staphylomycin S.

Radioactive determinations: All radioactive determinations were carried out using a Packard Tri-Carb liquid scintillation counting system (model 2002) with BRAY's naphthalene-dioxane counting fluid[6].

Protein determination: The protein content of the various enzyme preparations was determined by the method of LOWRY *et al.*[7] with bovine serum albumin as reference standard.

Determination of antibiotic activity: Agar diffusion bioassays were used for determination of loss of antibiotic activity when certain peptide antibiotics were hydrolyzed by the *Actinoplanes* enzyme preparations. *Sarcina lutea* was the test organism for the bioassays for echinomycin, etamycin, staphylomycin S, thiostrepton and vernamycin B_α. *Candida albicans* was used for the stendomycin assays. In the latter assay the agar medium was adjusted to pH 8.0 (after autoclaving) and 50 mg per liter of tetracycline was added just before pouring the melted agar. Boiled enzyme controls and an equal number of standards were included in each bioassay. The antibiotic potency of the samples was calculated from the semi-logarithmic plots of the diameter of the inhibition zone *vs.* the log of the concentration of the antibiotic.

Analytical disc electrophoresis: Polyacrylamide gel electrophorsis was performed at pH 9.5 with the Canalco instrument. The protein dissolved in 33 % glycerol was layered above the gels. The Davis procedure[8] was used in these studies with the gels being stained with a solution of 0.05 % of Coomassie Brilliant Blue R 250 in 12 % trichloracetic acid[9].

Results and Discussion

Origin of Peptide Antibiotic Lactonases

The origin of the peptide lactonases hydrolyzing actinomycin and dihydrostaphylomycin S was determined by examining the enzyme content of crushed *Actinoplanes* cells. Actinomycin and vernamycin B_α (a close chemical relative of dihydrostaphylomycin S and staphylomycin S) were added to flasks of 2 day old cultures of *Actinoplanes missouriensis* growing in the soybean meal-glycerol medium so that the final antibiotic concentration was 10 mcg/ml (control flasks without antibiotic were also included in the experiment). After an additional 18-hour incubation the cells were collected by centrifugation, resuspended in pH 7.0 (0.05 M) phosphate buffer and collected again by centrifugation. The collected cells were then frozen until assayed. They were then thawed and mixed with about an equal weight of the phosphate buffer and crushed in a French pressure cell. The supernatant liquid was assayed for lactonase using the actinomycin-^{3}H assay (see ref. 2) and the dihydrostaphylomycin S-^{3}H assay. Data collected in this study are summarized in Table 1. It is obvious that the lactonase hydrolyzing the dihydrostaphylomycin S is a constitutive enzyme while that hydrolyzing the actinomycin is induced. Actinomycin acts as an inducer for both of the lactonases, while vernamycin B_α does not act as an inducer for the lactonase hydrolyzing the actinomycin but does increase the synthesis of the constitutive lactonase hydrolyzing dihydrostaphylomycin S.

Identification of the Enzymatically Formed Degradation Product from Staphylomycin S

The enzymatically produced degradation product formed from staphylomycin S by washed cells of *A. missouriensis* was compared with that prepared by alakaline degradation in four solvent systems by thin-layer chromatography. The results of this comparison are summarized in Table 2. It is obvious that the two degradation products are closely related.

These two degradation products were studied further by determining their amino acid compositions. Both were hydrolyzed in 6 N HCl at 110°C for 18 hours. Chro-

Table 1. Induction of peptide antibiotic lactonases

Inducer	Enzyme activity (units/mg protein)	
	Actinomycin substrate	Dihydrostaphylomycin S as substrate
None	0.02	10.7
Actinomycin	14.4	22.1
Vernamycin B_α	0.36	25.6

Inducer was added to 3-day *Actinoplanes* culture to give concentration of 10 mcg/ml and incuation was continued for 18 hours at 37°C. The enzyme preparations obtained by French press extrusion of the cells followed by freezing-thawing were used in this study.

Table 2. Comparison of enzymatic degradation product of staphylomycin S with linear peptide formed by alkali treatment

Substance	Rf in solvent systems used for chromatography			
	System A	System B	System C	System D
Staphylomycin S	0.85	0.90	0.37	0.68
Enzyme product	0.31	0.84	0.25	0.38
Alkali product	0.31	0.84	0.25	0.38

Composition of solvent mixtures:
System A: acetone - acetic acid - water, 97 : 1 : 2
System B: chloroform - methanol - acetic acid - water, 85 : 15 : 3 : 2
System C: acetone - water, 98 : 2
System D: chloroform - methanol - acetic acid - water, 90 : 8 : 1 : 1

matographic analysis using butanol - acetic acid - water systems showed the presence of 6 ninhydrin positive spots in the acid hydrolyzates from both compounds and the same spots in the hydrolyzate of staphylomycin S. Hydroxypicolinic acid was detected in all three hydrolyzates when the chromatograms were examined under ultraviolet light. This study showed that both the enzymatic degradation and the chemical degradation resulted in opening the lactone linkage and the resulting linear peptide contained all of the amino acids originally present in the staphylomycin S.

Purification of Dihydrostaphylomycin S Hydrolyzing Enzyme

Step 1—Extraction of enzyme from cells

Frozen *Actinoplanes* cells were thawed and suspended in an equal weight of 0.05 M phosphate buffer (pH 7.0) at 4°C. The suspension was passed through a French pressure cell, and then contrifuged at 10,000×*g* for 20 minutes at 4°C. The supernatant solution was retained for further study.

Step 2—Freeze treatment

The enzyme solution from Step 1 was frozen in a −20°C freezer and then thawed and centrifuged for 20 minutes at 10,000×*g*. The precipitate was discarded and the supernatant retained for further study.

Step 3—Ammonium sulfate precipitation

Ammonium sulfate was added gradually to the enzyme solution to obtain 25% saturation. After 15-minute stirring at 4°C, the precipitate was removed by centrifugation at 10,000×*g* for 15 minutes. The supernatant solution containing most of the lactonase activity was mixed with additional ammonium sulfate until 50% saturation was achieved. After 15-minute stirring at 4°C, the suspension was centrifuged at 10,000×*g* for 15 minutes. The enzyme-containing pellet was dissolved in a small amount of 0.0005 M pH 7.0 potassium phosphate buffer and dialyzed overnight against this buffer.

Step 4—Calcium phosphate-cellulose column chromatography

Calcium phosphate gel (prepared by the method described by TSUBOI and HUDSON[10]) was mixed with cellulose powder at a ratio of 4 g of gel to 60 g of powder. After equilibration with 0.0005 M pH 7.0 phosphate buffer the cell was packed in a glass column at atmospheric pressure. The enzyme from Step 3 was applied to the column

and the protein eluted with phosphate buffer using stepwise increments increasing the concentration from 0.001 M to 0.1 M. The antibiotic peptide lactonase activity was eluted with the 0.001 M buffer fraction. The fractions eluted by 0.1 M buffer have high optical absorbancy but showed no enzyme activity.

Step 5—DEAE-cellulose column chromatography

DEAE-cellulose (medium mesh) was equilibrated with 0.01 M pH 7.0 potassium phosphate buffer and packed into a 4.2×14 cm column. The enzyme preparation from Step 4 was applied directly to the column. The elution rate was 70 ml per hour, and 7 ml fractions were collected. The column was then washed with an additional 600 ml of 0.01 M phosphate buffer. The actinomycin specific peptide lactonase was eluted when the phosphate buffer was 0.07~0.08 M, and the lactonase hydrolyzing dihydrostaphylomycin S was eluted when the concentration of buffer was between 0.1 and 0.2 M. The enzymatically active fractions obtained were used for the kinetic studies summarized below.

Step 6—Sephadex G-200 column chromatography

The pooled active fractions obtained from Step 5 were mixed with ammonium sulfate (to give 75 % saturated ammonium sulfate) and the precipitate collected by centrifugation at 10,000×*g*. After dialysis against 0.01 M phosphate buffer (pH 7.0), the enzyme solution was concentrated further by adding Lyphogel[R] (Gelman Instrument Company, Ann Arbor, Michigan) to a final fluid volume of 2 ml. This solution was applied to a 2×80 cm column of Sephadex G-200 previously equilibrated with 0.01 M phosphate pH 7.0 buffer. The enzyme activity was eluted with this buffer at a rate of 16 ml per hour. Two ml fractions were collected and the dihydrostaphylomycin S hydrolyzing activity was found in the buffer after 180 ml had passed through the column.

Purification of the dihydrosphylomycin S hydrolyzing activity from 4 kg of *Actinoplanes* cells is summarized in Table 3. The over-all recovery to 120-fold purification was 28 % representing about 0.2 % of the protein present in the French pressure cell exudate.

Table 3. Purification of constitutive peptide antibiotic lactonase

Purification step	Protein recovery (mg)	Specific enzyme activity (units)	Recovery of enzyme activity (%)
Extrusion through French Pressure Cell	11,134	11.4	100
Freeze-thaw	6,247	14.6	78
$(NH_4)_2SO_4$ precipitation (25~50% saturation)	2,108	28.3	47
Calcium phosphate gel treatment	623	68	33
DEAE-cellulose column chromatography	27	1,328	28
Sephadex G-200 (best fraction)	5	2,360	9.3

The dihydrostaphylomycin S hydrolyzing enzyme was found to be less stable than the actinomycin hydrolyzing lactonase[4]. About half of the enzyme activity purified through Step 5 was lost durig 30 days' storage in 0.1 M pH 7.0 phosphate buffer at −20°C.

Characterization of Enzyme by Gel Electrophoresis

Several protein bands were found when material from purification Step 6 was examined by gel electrophoresis. Fig. 2 is a picture of one of the studies by this method.

Fig. 2. Analytical disc electrophoresis of enzyme from Step 6 of purification procedure

Acrylamide gel electrophoresis (pH 9.5) was carried out on enzyme purified by Sephadex G-200 treatment. Ten mcg protein were placed on gel. Migration is toward the anode (at the bottom of figure).

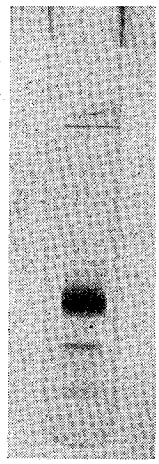
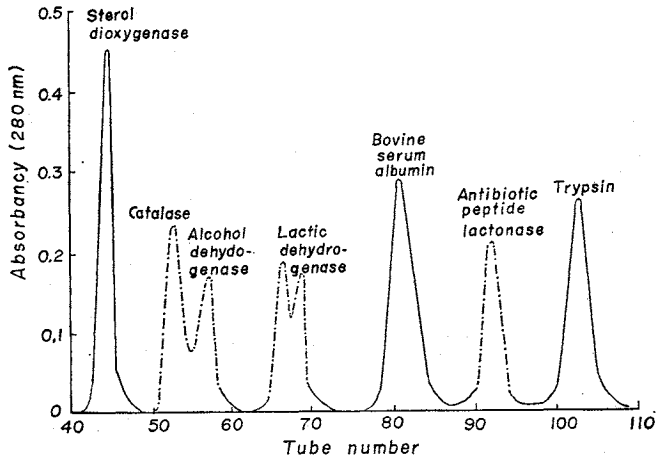


Fig. 3. Molecular weight determination of constitutive peptide antibiotic lactonase using Sephadex G-200 chromatography.

1.5 ml of a solution containing (1) sterol dehydrogenase (mol. wt. 300,000), (2) catalase (mol. wt. 250,000), (3) alcohol dehydrogenase (mol. wt. 151,000), (4) lactic dehydrogenase (mol. wt. 140,000), (5) bovine serum albumin (mol. wt. 75,000), (6) trypsin (mol. wt. 24,000) and (7) peptide antibiotic lactonase was applied to a 2×80 cm column of Sephadex G-200 previousiy equilibrated with 0.01 M potassium phosphate buffer (pH 7.0) at 4℃. Two ml fractions were collected with a flow rate of 6 ml per hour. ·—· enzyme activity.



Determination of Molecular Weight

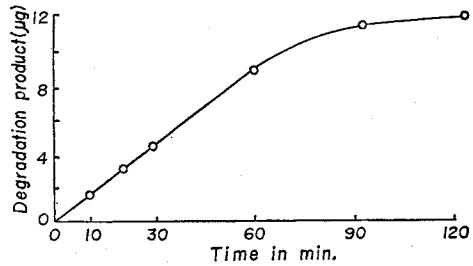
A column of Sephadex G-200 (2×80 cm) was washed with 0.01 M pH 7.0 phosphate buffer. The column was calibrated by using a 1.5 ml solution of a mixture of the dihydrostaphylomycin S hydrolyzing enzyme and proteins of known molecular weight. A constant flow rate of 8 ml per hour was maintained during the elution, and 2 ml fractions were collected. The elution volume of blue dextrin, 88 ml, was taken as the void volume (V_0) of the column. The presence in the eluate fractions of the various proteins was established either by measuring the absorbance of the eluate of 280 nm or by enzymatic activity. The pattern shown in Fig. 3 was obtained in this study. The molecular weight of the dihydrostaphylomycin lactonase determined by reference to a plot of V/V_0 *vs.* log molecuclar weight of the standard proteins was 35,000.

Kinetic Studies

Effect of time of incubation: The standard reaction mixture was incubated for various times at 38°C and the amount of hydrolysis product formed determined. As is shown in Fig. 4, the degradation of dihydrostaphylomycin S increased linearly with time up to about 60 minutes.

Fig. 4. Effect of time on enzymatic degradation of dihydrostaphylomycin S

30 mcg of enzyme protein (from DEAE-cellulose chromatography) were used in the standard assay system with incubation at 38℃.



Effect of pH: The effect of pH on the enzymatic degradation of dihydrostaphylomycin S was studied over the range 5.0～9.0 using acetate buffer (pH 5.0), phosphate

Fig. 5. Effect of temperature on the enzymatic degradation of dihydrostaphylomycin S

Enzyme from DEAE-cellulose chromatography (30 mcg protein) used in standard assay system and incubated at indicated temperatures for 60 minutes. The ARRHENIUS plot of the data is shown at the right.

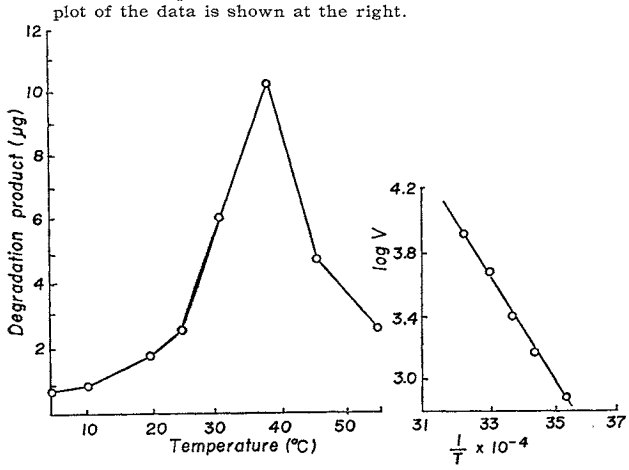
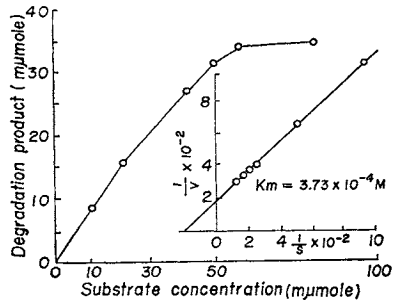


Fig. 6. Effect of substrate cencentration on the rate of enzymatic degradation of dihydrostaphylomycin S



buffer (pH 6.0 and 7.0), and Tris·HCl buffer (pH 7.8 and 9.0). The optimum pH for degradation of dihydrostaphylomycin S by the *Actinoplanes* lactonase appears to be pH 7.8.

Effect of temperature: The effect of incubation temperature on the degradation dihydrostaphylomycin S was determined. Maximum rate of degradation occurred at 38°C as shown in the data plotted in Fig. 5. An ARRHENIUS plot of this data (shown in the right hand side of Fig. 5) permitted the calculation of the activation energy for this reaction, 15,600 cal.

Effect of substrate concentration: The rates of the enzymatic reaction were studied over the range of 107~80 mμmoles per 0.20 ml, *e.g.* 53.5~400 μM, of dihydrostaphylomycin S. A plot of the data obtained is shown in Fig. 6. The MICHAELIS constant calculated from the LINEWEAVER-BURK plot is 3.73×10^{-4} M. The V_{max} value is 1.09 μmoles/hr/mg protein.

Table 4. Effect of metal ions on the enzymatic degradation of dihydrostaphylomycin S

Salt added	Enzyme activity (%)
$AgNO_3$	47
$NaAsO_2$	132
$BaCl_2$	131
$CaCl_2$	131
$CoCl_2$	33
$Cr_2(SO_4)_3$	7
$CuSO_4$	5
$FeSO_4$	38
$HgCl_2$	32
KI	116
KCl	109
KCN	105
$MgSO_4$	127
$MnSO_4$	102
NaCl	59
$NiCl_2$	6
$ZnSO_4$	44

Test system contained 30 mcg of enzyme from DEAE-cellulose chromatography step of purification, 22 4 mμmoles dihydrostaphylomycin S (980 cpm/mμmole), 6 μmoles Tris-HCl buffer (pH 7.8), and metal ions (final concentration 0 04 M) in a total volume of 0 2 ml. Incubation was for 60 minutes at 38°C.

Effects of metal ions: The effects of various metal ions on the hydrolysis of dihydrostaphylomycin S by the purified lactonase was studied by adding various salts to the standard reaction mixture. Some of the data collected are summarized in Table 4. Significant inhibition was noted when 0.04 M cobalt, copper, chromium, iron, mercury, nickel, silver, sodium, or zinc salts were added to the enzyme-substrate mixture.

Substrate Specificity

Study of the substrate specificity of the lactonase hydrolyzing dihydrostaphylomycin

S showed that this enzyme hydrolyzed echinomycin, etamycin, staphylomycin S, stendomycin, and vernamycin B. Some of the data collected are summarized in Table 5. Only slight activity was noted when thiostrepton was the substrate but the extreme insolubility of this material may account for the limited inactivation. The dihydrostaphylomycin S hydrolyzing enzyme had no activity on the actinomycin substrate.

Table 5. Substrate specificity of peptide antibiotic lactonase

Substrate	Assay system	Enzyme activity units/mg protein
Actinomycin-^{3}H	1	0
Echinomycin	2	6.2
Etamycin	2	440
Staphylomycin S	2	185
Stendomycin	3	87
Thiostrepton	2	0.3
Vernamycin B_{α}	2	164

Assay system : 1—radioactivity (see ref. 2)
2—Agar diffusion bioassay using *Sarcina lutea*
2—Agar diffusion bioassay using *Candida albicans*

The enzyme reaction mixture contained 142 mcg enzyme purified by DEAE-cellulose chromatography, 24 μmoles of Tris·HCl buffer (pH 7.8) and antibiotics (64 mcg each for etamycin and thiostrepton, 16 mcg for echinomycin, and 150 mcg for stendomycin, staphylomycin S and vernamycin B_{α}) in a total volume of 0.8 ml. The reaction was terminated by 3 minute immersion of the tube in boiling water.

Acknowledgements

We are indebted to Dr. J. BERGER (Hoffmann-La Roche, Inc.) for the actinomycin and echinomycin, to Dr. G. A. MALLETT (Eli Lilly and Co.) for the stendomycin, to Miss B. STEARNS (Squibb Institute for Medical Research) for the vernamycin B and thiostrepton, and to Dr. H. VANDERHAEGHE (Rega Institute, Belgium) for staphylomycin S and dihydrostaphylomycin S-^{3}H.

This investigation was supported by the Graduate School Research Fund, University of Wisconsin, by Eli Lilly and Co., and by Public Health Service research grant AI-08237-02 from the National Institute of Allergy and Infectious Diseases.

References

1) PERLMAN, D.; A. B. MAUGER & H. WEISSBACH : Microbial metabolism of actinomycins and other heterodetic antibiotic peptides. Biochem. Biophys. Res. Comm. 24 : 513~518, 1966
2) PERLMAN, D.; A. B. MAUGER & H. WEISSBACH : Microbial transformations of peptide antibiotics. I. Degradation of actinomycins by *Actinoplanes* species. Antimicr. Agents & Chemoth.-1966 : 581~586, 1967
3) PERLMAN, D. & A. CAPEK : Microbial transformations of peptide antibiotics. II. Site of actinomycin degradation in *Actinoplanes missouriensis*. Hindustan Antibiot. Bull. 10 : 269~273, 1968
4) HOU, C. T. & D. PERLMAN : (submitted for publication), 1969
5) VANDERHAEGHE, H. & G. PARMENTIER : The structure of factor S of staphylomycin. J. Am. Chem. Soc. 82 : 4414~4422, 1960
6) BRAY, G. A. : A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Chem. 1 : 279~285, 1960
7) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL : Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 193 : 265~275, 1951
8) DAVIS, B. J. : Disk electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121 : 404~427, 1964
9) CHRAMBACH, A.; R. A. REISFELD, M. WYCKOFF & J. ZACCARI : A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. 20 : 150~154, 1967
10) TSUBOI, K. K. & P. B. HUDSON : Enzymes of the human erythrocyte. I. Purine nucleoside phosphorylase; isolation procedure. J. Biol. Chem. 224 : 879~887, 1957