FURTHER PURIFICATION AND CHARACTERIZATION OF MACROMOMYCIN

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The antitumor polypeptide, macromomycin (MCR) produced by *Streptomyces macromomyceticus*, was purified by ammonium sulfate precipitation, ultrafiltration and chromatographic techniques using Amberlite IRA-410, DEAE Sephadex A-25 (Cl^- and OH^- type) and Sephadex G-50. Purified MCR was obtained as a white powder by lyophilization. MCR, thus purified, exhibited a single peak on Sephadex G-50 chromatography with no detectable contaminant by ultracentrifugation and polyacrylamide gel electrophoresis. MCR is an acidic polypeptide having an isoelectric point of pH 5.4. It contains no arginine and methionine. The molecular weight was 11,700, 12,500 and 11,400 by amino acid composition, gel filtration and analytical ultracentrifugation, respectively. MCR is labile as a lyophilized powder but is successfully stabilized by the addition of maltose.

Macromomycin (MCR) discovered by CHIMURA *et al.*[1] in 1968 was reported to be a polypeptide antitumor antibiotic produced by *Streptomyces macromomyceticus* and to prolong the life-span of mice bearing L1210 leukemia and EHRLICH carcinoma. LIPPMAN *et al.*[2] reported that it inhibits L1210 leukemia, P388 leukemia, B16 melanoma and LEWIS lung carcinoma. According to KUNIMOTO *et al.*[3,4], MCR binds to mammalian cell surfaces and causes inhibition of DNA synthesis. The study of LIPPMAN *et al.*[5] suggests that it is effective in increasing immunity to tumors.

Though macromomycin has interesting biological activities, it is unstable. Consequently it was necessary to study its physicochemical characters in detail in order to find a method for stabilizing this antibiotic. In this paper, we report further observations on characteristics of MCR and methods to obtain stable MCR powder.

Methods and Materials

Assay of MCR

The cylinder agar plate method using *Micrococcus flavus* reported by CHIMURA *et al.*[1] was employed. *M. flavus* was cultured at 33°C for 48 hours in a medium containing 2.0 % glucose, 1.0 % meat extract, 1.0 % peptone, and 0.3 % sodium chloride (pH 7.0). The agar medium contained 0.1 % glucose, 0.6 % peptone, 0.4 % casamino acid, 0.15 % meat extract, 0.3 % yeast extract and 1.5 % agar; the pH was adjusted to 7.0.

Production of MCR

S. macromomyceticus was cultured at 28°C for about 90 hours with aeration and agitation. The medium contained 1.0 % glucose, 1.0 % soluble starch, 1.5 % soy bean meal, 0.1 % K_2HPO_4, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.3 % NaCl, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 %

$MnCl_2 \cdot 4H_2O$ and 0.0002% $ZnSO_4 \cdot 7H_2O$ at pH 7.2. In jar fermentations 0.05% Disfoam CC-118 was added.

Purification of MCR

Dicalite 4159 was added to the culture broth as filter aid and the broth filtered using a filter press. Dicalite 4159 was purchased from Dicalite Orient Co., Ltd. The filtrate was salted out by saturation with ammonium sulfate. MCR was purified by sequential use of Amberlite IRA-410 (Cl^- type) chromatography, ultrafiltration, DEAE Sephadex A-25 (Cl^- type) chromatography, Sephadex G-50 (fine) chromatography, DEAE Sephadex A-25 (OH^- type) chromatography and Sephadex G-50 (fine) chromatography. Purified MCR was lyophilized and stored.

Homogeneity of MCR

Analysis by ultracentrifugation was carried out on a solution containing 8 mg MCR/ml by the sedimentation velocity method[8] at 72,000 rpm for 90 minutes using a Hitachi Analytical Ultracentrifuge type 282.

Analytical polyacrylamide gel electrophoresis was done following the ORSTEIN-DAVIS system[7,8]. The gel type was pH 9.4, the concentration of the acrylamide monomer was 15% and the buffer solution was tris-glycine at pH 8.3. Polyacrylamide gel was made in a glass tube (5 mm of diameter, 60 mm of length). A sample of MCR was placed on the top of the gel with 1 M sucrose solution. Initially, a current of 3 mA per tube was applied, and then the current was increased to 5 mA per tube when the sample entered the gel. Exposure of MCR to light was carefully avoided during the treatment to avoid destruction. After electrophoresis for about 40 minutes, the gel was stained with amino black 10B for 1 hour and the excess dye was rinsed away with a 7% solution of acetic acid.

Determination of Isoelectric Point of MCR[9]

The isoelectric point of MCR was determined by electrofocusing with an LKB Ampholine 8101 using a carrier ampholyte with a pH range of 5 to 8. Seven mg of MCR was applied with an initial voltage of 300 V. After 12.5 hours, the voltage was increased to 700 V. Electrofocusing continued for 17.5 hours and then the carrier ampholyte in the focusing column was fractionated and the MCR in each fraction was determined by measuring the UV-absorption and the microbial activity.

Amino Acid Analysis of MCR

The amino acid composition was analyzed using a Hitachi Amino Acid Analyzer KLA-5. MCR was hydrolyzed at 110°C for 22 hours in 6 N HCl or 4 N $Ba(OH)_2$. Cysteine and methionine were analyzed by the method of performic acid oxidation. To ensure analysis of representative hydrolysates, various conditions for the hydrolysis of MCR were examined. Accordingly MCR was hydrolyzed in 3 N or 6 N HCl for 3, 6, 12, 22, 48 and 72 hours.

Estimation of Molecular Weight of MCR

A Sephadex G-50 column (15 mm×900 mm) was treated with 0.1 M phosphate buffer at pH 7.0 containing 0.1 M KCl[10]. Purified MCR was chromatographed with four different reference compounds. Three-ml fractions were collected at a flow rate of 35 ml/hour. The reference compounds, blue dextran 2000, human albumin (M.W. 45,000), chymotrypsinogen A (M.W. 25,000), and cytochrome C (M.W. 12,500) were purchased from Boehringer Mannheim Gmbh.

Analytical ultracentrifugation of a solution of MCR containing 0.4 mg/ml was carried out at 20,000 rpm for 16.5 hours by the sedimentation equilibrium method. Results were recorded by an ultraviolet absorption system.

Acute Toxicity of MCR

MCR was dissolved in 0.9% NaCl solution and given to mice intravenously, intraperitoneally and orally. After administration, the mice were observed for two weeks.

Stability of MCR

Compounds were screened for ability to stabilize MCR by the following method:

An aqueous solution of MCR and an aqueous solution of the compounds to be tested, selected from saccharides, amino acids, organic acids, inorganic salts and chelating agents, were mixed and adjusted to pH 5.0 to 7.5. Two ml of each resulting solution were placed in a vial of 10 ml volume and lyophilized with heating below 50°C. The vial was sealed and kept at 65°C for 55 hours.

The stability of a lyophilized powder containing 2 mg of MCR and 78 mg of maltose was examined. After lyophilization, the vials were sealed and stored at 35°, 45° and 50°C for 3, 6, 9, 12 and 15 days in the dark. The activity immediately after lyophilization was taken as 100 %.

Results

I. Purification of MCR

Fermentation and Precipitation of MCR

Streptomyces macromomyceticus was cultured at 28°C for 80 to 90 hours. Production of MCR started in the middle of the logarithmic growth phase and reached the maximum shortly after the growth entered the stationary phase. Thereafter, the MCR concentration in the culture broth decreased rapidly. The fermentation was terminated and the culture broth (12 liters) was cooled to 5°C. Dicalite 4159 (200 g) was added and filtered by a small filter press at 5° to 10°C. For example, 8.0 liters of the filtrate contained 119 g of dry material. The cold broth filtrate was saturated with ammonium sulfate and salting out allowed to proceed for 5 hours at 10°C. The precipitate was collected by centrifugation and 120 g of wet crude precipitate was obtained.

The wet precipitate was dissolved in 2 liters of deionized water and the insoluble material discarded. The salting out process was applied again and after the second precipitation, 68.6 g (24 g dry weight) of wet precipitate was obtained. The recoveries of potency in the first and second precipitations were 55.8 % and 72.9 %, respectively.

Amberlite IRA-410 (Cl^- type) Process

The MCR solution (455 ml), obtained by dissolving the second precipitate (described above) in deionized water, was fed to the Amberlite IRA-410 column. MCR was not adsorbed to the resin and 92.1 % of potency was recovered in the effluent. The effluent (656 ml) contained 21 g of dry material.

Concentration and Elimination of Low Molecular Contaminants by Ultrafiltration

By Dia-filter MC-6 using a GT-10 membrane (M.W. 10,000, Bio-engineering Co., Ltd.), the effluent (656 ml) obtained above was concentrated to 104 ml and ten volumes of deionized water were added. The filtration process was repeated yielding 100 ml of concentrate. Thus contaminants with molecular weights below 10,000 were eliminated and 2.3 g of high molecular weight compounds remained in the concentrate. Recovery of potency was 58.1 %.

Decolorization of MCR by DEAE Sephadex A-25 (Cl^- type)

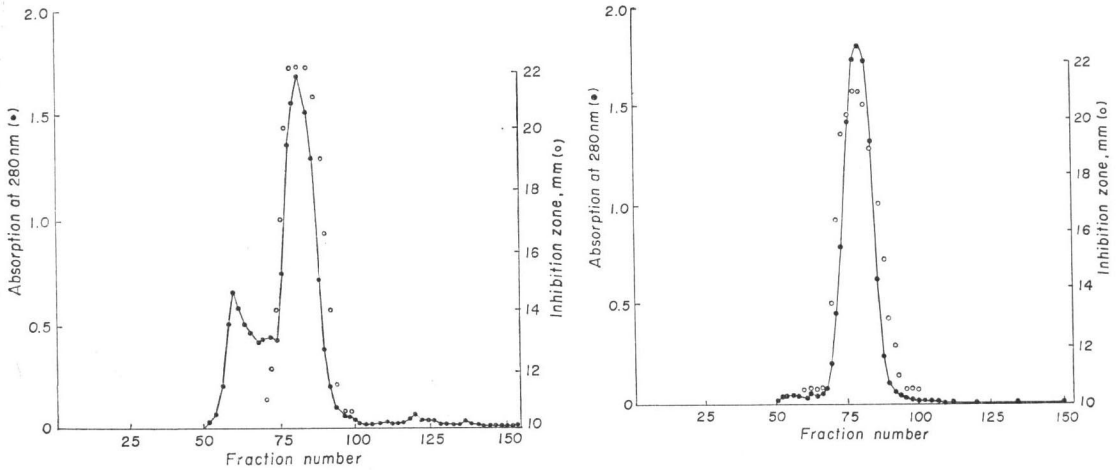
Cl^- type of DEAE Sephadex A-25 was used for decolorization of the MCR solution. DEAE Sephadex A-25 (5.0 g) was packed in a column. The concentrated solution (100 ml) obtained above was applied to the column followed by deionized water. A dark brown pigment was adsorbed to the column and 142.2 % of the MCR potency was found in the effluent (160 ml).

Fig. 1. First gel filtration of MCR on Sephadex G-50

Sephadex G-50 column of 60 mm×850 mm was treated with 0.1 M of phosphate buffer at pH 7.0. Sixteen ml fractions were collected at a flow rate of 140 ml/hour. The inhibition zone is for *M. flavus*.

Fig. 2. Second gel filtration of MCR on Sephadex G-50

The second gel filtration was carried out with the same column as previously described but the buffer solution was not used. The column was developed using deionized water only. The inhibiton zone is for *M. flavus*.



The effluent contained 1.85 g dry material with a small quantity of yellowish pigment still remaining.

Gel Filtration of MCR on Sephadex G-50 (fine)

Chromatography was carried out using a Sephadex G-50 column (60 mm×850 mm) treated with 0.1 M phosphate buffer at pH 7.0. The effluent (160 ml) obtained above was applied to this column. The column was eluted in order to obtain the fraction of about M.W. 10,000 representing the MCR. Low molecular contaminants had been eliminated already by ultrafiltration and consequently only high molecular contaminants appeared just before the MCR peak (Fig. 1). The high molecular contaminants were removed in this step and the active fraction (244 ml) was collected. This step yielded material with a dry weight of 0.45 g representing 56% recovery of the potency. The active fraction was concentrated to 100 ml by ultrafiltration simultaneously removing phosphate. During this last concentration, about 10% of the potency and the dry weight was lost.

Chromatography on DEAE Sephadex A-25 (OH^- type)

The concentrate (100 ml) obtained above was charged to a DEAE Sephadex A-25 (OH^- type, 10 g) column. MCR was not adsorbed to Cl^- type of DEAE Sephadex but was adsorbed to OH^- type of DEAE Sephadex. The adsorbed MCR was eluted with 0.1 M NaCl and a yellowish pigment remained on the resin. The eluate (415 ml) contained 0.15 g MCR representing a 28.6% recovery of the potency. The pigment and other contaminants were removed completely in this step and the eluate was shown by polyacrylamide gel electrophoresis to contain pure MCR.

Rechromatography on Sephadex G-50 (fine)

Gel filtration on Sephadex G-50 was carried out again to completely remove salt (Fig. 2).

The technique was applied to the eluate (415 ml) described above, using deionized water. The step yielded 0.11 g MCR (dry weight) in 175 ml effluent. Recovery of the potency was 83.3%.

Lyophilization of MCR

By lyophilization of the MCR solution, 98 mg of a white powder was obtained and stored in a desiccator in the freezer.

As described in Section II, the homogeneity of the lyophilized MCR powder was established by the described methods. Thus, the purification procedures were confirmed to remove all contaminants.

II. Characteristics of MCR

Homogeneity of MCR

The purified MCR was confirmed to be homogeneous by gel filtration on Sephadex G-50, by analytical ultracentrifugation and by polyacrylamide gel electrophoresis.

Gel filtration was carried out on a Sephadex G-50 column (60 mm×850 mm) treated with 0.1 M phosphate buffer containing 0.1 M KCl at pH 7.0. Sixteen-ml fractions were collected at a flow rate of 140 ml/hour. A sharp, single peak of MCR was observed. No other peak representing a contaminant was found. MCR gave maximum absorption between 270 nm and 280 nm with a shoulder at 290 nm (Fig. 3).

Analytical ultracentrifugation by the sedimentation velocity method was carried out on a solution containing 8 mg of MCR per ml at 72,000 rpm for 90 minutes. The sedimentation pattern of MCR showed a single peak. The sedimentation coefficient, $S_{20,w}$ was 1.5.

Polyacrylamide gel was made in a glass tube according to the method of ORSTEIN-DAVIS. The concentration of the acrylamide monomer was 15%. About 40~50 mcg MCR in 1 M sucrose solution were added to each tube. After electrophoresis, MCR was stained with amino black 10B. After removing the excess

Fig. 3. UV spectrum of macromomycin

UV absorption of MCR was measured on a Hitachi Recording Spectrophotometer 323. MCR demonstrated absorption at 270~280 nm with a shoulder at 290 nm.

$E^{1\%}_{1cm}$ at 280 nm was 11.6.

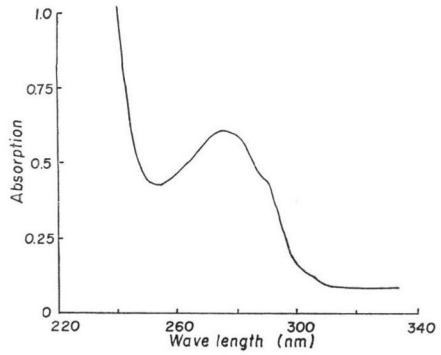
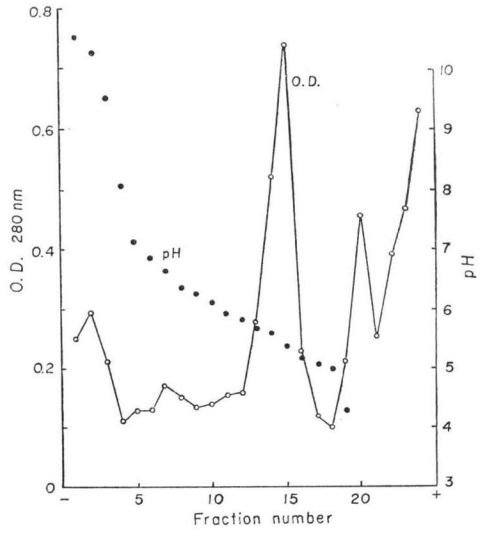


Fig. 4. Isoelectric point of macromomycin

Isoelectric point was determined with LKB Ampholine 8101, using a carrier Ampholyte of pH 5.0~8.0. Electrofocusing was carried out 17.5 hours (12.5 hours at 300 V, 5 hours at 700 V). Activity of MCR was focused at pH 5.4.



dye electrically, only one band was observed.

NH_2-terminal of MCR was shown to be alanine by dinitrophenylation[11] and dancylation[12].

Determination of Isoelectric Point

The isoelectric point was determined by the electrofocusing procedure using LKB Ampholine 8101. The carrier ampholyte was in the range of pH 5.0 to 8.0. The MCR activity was detected in the fraction of pH 5.4. Thus, MCR was confirmed to be an acidic polypeptide having isoelectric point of pH 5.4 (Fig. 4).

Amino Acid Analysis of MCR

MCR was hydrolyzed at 110°C for 22 hours in 6 N HCl or 4 N $Ba(OH)_2$. The result of the analysis is shown in Table 1. From the analysis, it became clear that arginine and methionine were not present in the MCR molecule. On hydrolysis of macromomycin, the amount of valine was markedly dependent on the period of hydrolysis; therefore we studied the results of hydrolysis for 3, 6, 12, 22, 48 and 72 hours. The amount of valine decreased after 48-hour hydrolysis. The results are shown in Fig. 5. The amino acid composition obtained after 22-hour hydrolysis and corrected values determined from the data obtained by varying the hours of hydrolysis are given in Table 1.

Estimation of Molecular Weight

The molecular weight of MCR was determined by gel filtration, amino acid analysis and analytical ultracentrifugation. Gel filtration was carried out on a Sephadex G-50 column

Fig. 5. Hydrolysis of macromomycin on various conditions

MCR was hydrolyzed at 110°C for 3, 6, 12, 22, 48 and 72 hours in 3 N and 6 N HCl. In the figure, the results of the hydrolysis in 6 N HCl are shown. Cys. and Met. were measured by the method of performic acid oxidation and Trp. was measured by UV absorption. Arg. and Met. were not detected in MCR.

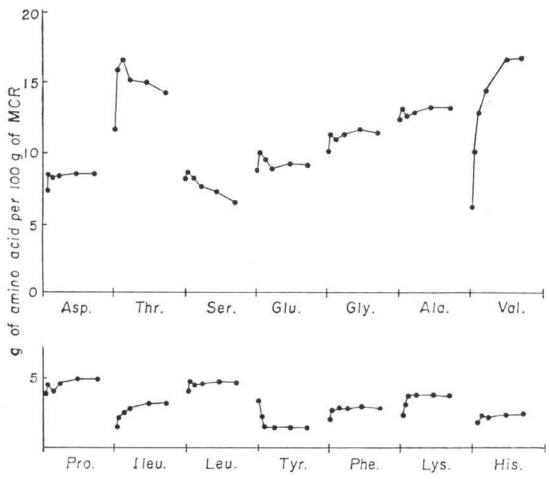


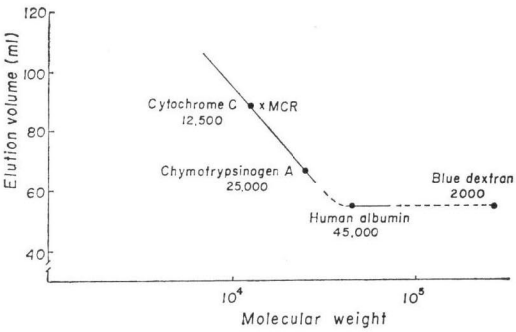
Table 1. The amino acid composition of macromomycin

	% (w/w)	
	Experimental data	Corrected value
Asp.	7.72	7.29
Thr.	14.02	14.22
Ser.	7.06	7.24
Glu.	8.28	8.46
Pro.	4.32	4.14
Gly.	10.47	9.82
Ala.	12.04	11.33
Cys.*	3.79	3.48
Val.	13.50	14.35
Met.	—	—
Ileu.	2.68	2.80
Leu.	4.40	4.08
Tyr.**	1.36	2.94
Phe.	2.63	2.48
Trp.	2.11	1.94
Lys.	3.50	3.33
His.	2.08	2.10
Arg.	—	—

* Oxidation with performic acid
** Alkaline hydrolysis

Fig. 6. Determination of molecular weight by Sephadex G-50 gel filtration

Purified MCR was filtered on Sephadex G-50 column (15×900 mm) treated with 0.1 M phosphate buffer at pH 7.0 containing 0.1 M KCl. Three ml fractions were collected at a flow rate of 35 ml/hour. Simultaneously, four reference compounds were chromatographed with MCR.



(15 mm×900 mm) treated with 0.1 M phosphate buffer at pH 7.0. Three-ml fractions were collected at a flow rate of 35 ml/hour. The four reference compounds were chromatographed simultaneously and the activity of MCR was detected in the same fraction as cytochrome C (M.W. 12,500, Fig. 6). Thus, the molecular weight of MCR was shown to be 12,500.

By amino acid analysis and chemical modification using a sulfhydryl reagent such as *p*-chloromercuribenzoic acid, it was found that MCR had four cysteine molecules. Based on the amount of cysteine using the corrected value in Table 1, the molecular weight was calculated to be 11,700.

Table 2. Effects of saccharides, amino acids, organic acids, inorganic salts and chelating agents on the stability of macromomycin (MCR)

Compound	mg/mg of MCR	Residual activity
None		28.3 (%)
Glucose	10	46.5
Mannose	10	45.2
Galactose	10	38.9
Fructose	10	37.1
Maltose	10	92.5
Lactose	10	95.7
Sucrose	10	88.5
Cellobiose	10	83.6
Dextran	10	53.5
L-Glutamine	10	48.7
Sodium glutamate	10	64.9
L-Asparagine	10	53.9
L-Lysine hydrochloride	10	52.6
L-Histidine hydrochloride	10	23.2
Sodium citrate	10	74.0
Sodium tartarate	10	72.0
Sodium oxalate	10	53.4
Sodium fumalate	10	55.4
KCl	0.1	53.8
$MgSO_4 \cdot 7H_2O$	0.1	50.2
$MnCl_2 \cdot 4H_2O$	0.1	48.9
EDTA-disodium salt	0.01	67.0

The solution containing MCR and a test compound was adjusted to pH 5.0~7.5 and 2.0 ml of the resulting solution was lyophilized in a 10-ml vial. The concentration of MCR was 2.5 mg/ml, in the solutions containing a saccharide. In other solutions, the concentration of MCR was 5.0 mg/ml. The vial was sealed and left at 65°C for 55 hours.

Ultracentrifugation by the sedimentation equilibrium method was performed at 20,000 rpm for 16.5 hours. The molecular weight was shown to be 11,400.

Toxicity of MCR

The intraperitoneal LD_{50} was 5.1 mg/kg, and the intravenous LD_{50} was 6.4 mg/kg. Orally, 1.5 g/kg could be administered to mice without any sign of toxicity.

Stability of MCR

Various compounds were screened for their abilities to stabilize MCR. As shown in Table 2, we found that sugars, amino acids and many other compounds were effective in stabilizing MCR, with disaccharides the most effective.

Among disaccharides, we selected maltose for study in detail. As shown in Fig. 7, the

Fig. 7. Time course of thermal inactivation of MCR lyophilized with maltose

MCR lyophilized with maltose was left at 35°C, 45°C and 50°C for 15 days. Log % of residual activity was plotted on a straight line. And the inactivation showed first-order kinetics.

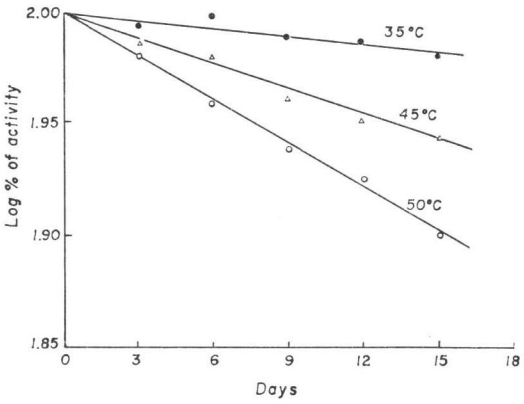
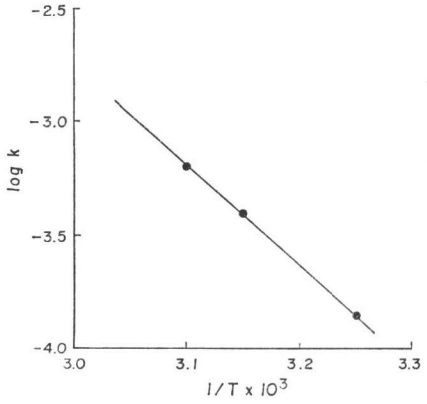


Fig. 8. ARRHENIUS plots of MCR lyophilized with maltose

k was calculated from Fig. 7. Plots of log k to $1/T$ gave a straight line. The period for preservation was calculated by ARRHENIUS equation. k is inactivation rate constant and T is absolute temperature.



plot of the logarithm of the residual activity of MCR yields straight lines as a function of time for each of the three temperatures, 35°, 45° and 50°C. A first-order reaction was shown for the inactivation. The ARRHENIUS plot calculated from the data in Fig. 7 is given in Fig. 8. The period for preserving at least 90% of the activity was calculated by the ARRHENIUS equation as follows: 37 months at 4°C, 17 months at 10°C, 3 months at 25°C and 31 days at 35°C. Experimentally, the residual activity after 30 days at 35°C was confirmed to be 92.8%. The MCR without maltose lost almost all of its activity during storage under these conditions.

Discussion

Extraction and purification of MCR presented a number of problems because of its sensitivity to temperature, light, ultraviolet and reductant. For best results the temperature of the culture broth and of broth filtrate should be kept below 10°C. In addition, careful, rapid filtration is required. Without these precautions, most of the activity is lost during filtration. Also in the case of the subsequent purification procedure, it should be handled carefully. In addition, exposure of the MCR solution or the powder to light should be avoided.

Exacting chromatography by DEAE Sephadex A-25 is the most important step to obtain pure MCR; all remaining contaminants can be removed if this step is handled with precision.

MCR thus purified was confirmed to be homogeneous with the characteristics agreeing fairly well with those in the original paper. MCR is an acidic polypeptide antibiotic having an isoelectric point of pH 5.4. It has the maximum UV-absorption between 270 nm and 280 nm and a shoulder at 290 nm. $E^{1\%}_{1cm}$ is 11.6.

The molecular weight of MCR was shown to be about 12,000 by three different methods but the exact value must wait the detailed structural study. As reported in the next paper, the NH_2-terminal was confirmed to be alanine; two disulfide bonds are present in the molecule.

Up to this time the labile character of MCR has prevented biological study of this antibiotic in detail. Fortunately, we have found that disaccharides, amino acids, as well as other organic and inorganic compounds were effective in stabilizing this antibiotic. Maltose was particularly effective for this purpose. The loss of MCR activity in a maltose preparation was only 7.2% after 30 days at 35°C.

Neocarzinostatin, actinoxanthin and MCR, all of which are acid peptides, belong to the same class of antibiotics. However, when their properties are compared in detail, differences are noted. For example, the isoelectric point of neocarzinostatin (pH 3.5[13]) differs from that of MCR (pH 5.4). Unlike MCR, neocarzinostatin[14] and actinoxanthin[15] contain arginine, neocarzinostatin does not contain histidine and actinoxanthin does not contain tryptophan.

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