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ISOLATION AND CHARACTERIZATION OF THE WATER SOLUBLE ANTINEOPLASTIC PRINCIPLES OF THE COMMON QUAHOG, *MERCENARIA, MERCENARIA*

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ISOLATION AND CHARACTERIZATION OF THE WATER SOLUBLE ANTINEOPLASTIC PRINCIPLES OF THE COMMON QUAHOG, *MERCENARIA MERCENARIA*

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

by

Stanley Stephen Stavinski, B.S.

The Ohio State University

1981

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To Mom, Dad, and my wife, Patuske,

for all their love that has nourished

me through these years.

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CHAPTER 1. INTRODUCTION

Many marine organisms have been shown to exhibit significant biological activity. The majority of natural-product investigations have centered on organic-soluble compounds. However, an increasing number of studies are being focused on the hydrophilic constituents of marine organisms. As early as 1952, Nigrelli demonstrated the inhibitory effect of holothurin, a toxic substance isolated from the se cucumber, on sarcoma 180 in mice.[1] Mollusk extracts have been shown by a number of workers to exhibit significant biological properties, with relatively high activity. Limasset was the first to investigate the antiviral activity of various mollusks. He reported that canned abalone juice and freshly prepared extracts of mussels (*Mytilus edulis*), clams (*Mercenaria mercenaria*), and oysters (*Ostrea virginica*) possessed antiviral activity against tobacco mosaic virus.[2] Li and co-workers have demonstrated the antibiotic and antiviral activity of oysters and abalone.[3-4] Schmeer was the first to illustrate the antineoplastic activity of mollusk extracts.[5] She has shown that the clam (*Mercenaria mercenaria*), oyster (*Ostrea virginica*), whelk (*Busycon*

canaliculatum), snail *Helix* sp. (*land Snail*), and squid (*Loligo* sp.) exhibit significant activity *in vivo* versus sarcoma 180 in Swiss albino mice.[6]

1.1 Choice of the Common Clam, *Mercenaria mercenaria*, as Target of the Investigation

Schmeer's comparative study of the antineoplastic activity of a variety of mollusks demonstrated that the clam (*Mercenaria mercenaria*) exhibited the highest relative activity per unit body weight against sarcoma 180, followed by lower but equal activities of the oyster (*Ostrea virginica*), snail (*Helix* sp.), and squid (*Loligo* sp.), with the whelk (*Busycon canaliculatum*) being the least active.[6] In addition, the low toxicity, availability, inexpensiveness, ease in handling, and stability make the clam an attractive potential source of antineoplastic material and the target of this investigation.

1.2 Activity and Toxicity of the Common Clam, *Mercenaria mercenaria*

A number of workers have investigated the antineoplastic, antiviral and antibiotic activity of water extracts of the clam (*Mercenaria mercenaria*). Schmeer has shown that

the antineoplastic principle, named mercenene, is active versus sarcoma 180 *in vivo*,[5] Krebs 2 carcinoma *in vivo*[5] human HeLa cells *in vitro*,[7-8] the P388 lymphocytic tumor *in vivo*,[9] and the mouse melanoma B16.[9] Li and co-workers demonstrated the therapeutic effect of clam-liver extract against L1210 lymphocytic leukemia *in vivo* and *in vitro*.[10] Lavelle reported that the clam extract contains a general inhibitor of growth and inhibits chemical carcinogenesis.[11]

Lavelle assayed against methylchloanthrene induced tumors in mice. Judge proved the inhibition of Bittner mammary tumor and of Friend Virus Leukemia in Swiss mice.[12] Both Hegyeli and Szent-Györgyi have confirmed the activity versus Krebs 2 solid carcinoma in mice.[13-14] Fishtein has shown that activity is present versus sarcoma 180 in Swiss-Webster mice,[15] squamous cell carcinoma MT-70,[16] and Krebs 2 carcinoma (ip ascitic) in Swiss mice.[17] The antiviral and antibiotic agents have been named by Li as Paolin 2 and Paolin 1, respectively. Li first established the antiviral activity by showing the inhibition of tumor formation in hamsters induced by Adenovirus type 12.[18] Liu established that the liver of the clam possessed most of the antiviral

activity when assayed by the chick embryo influenza virus system.[19] Prescott and co-workers have shown that Paolin 2 is also active versus herpes simplex virus.[20]

The crude water extracts of the clam exhibit no significant toxicity.[7-8] Schmeer has demonstrated that the extracts possess negligible toxicity *in vitro* versus the normal human amnion cell line and no toxicity *in vivo* in experimental animals. This lack of toxicity by crude extracts is surprising in view of the relatively high toxicity normally expressed in animal extracts. The intrinsic toxicity of animal extracts have hindered exploration of biologically significant principles present because of the *in vivo* toxicity expressed. The low toxicity of the clam is also surprising in view of the toxicity expressed by many other marine organisms. Many of the most toxic substances known are derived from marine organisms. Aqueous extracts of the other mollusks also demonstrate no high levels of toxicity *in vivo*. The correlation between the toxicity of the clam, *Mercenaria mercenaria*, and the other mollusks has not been established. A summary of the antineoplastic activity is presented in Table 1.

TABLE 1

MERCENENE ANTINEOPLASTIC ACTIVITY

SYSTEM	INVESTIGATOR	REFERENCE
Sarcoma 180	Schmeer	5
	Fishtein	14
Induced Methylcholanthrene Tumors in Mice	Lavelle	11
Krebs 2 Carcinoma	Schmeer	5
	Hegyeli	12
	Szent-Györgyi	13
P388 Lymphocytic Tumor	Schmeer	9
Mouse Melanoma B16	Schmeer	9
L1210 Lymphocytic Leukemia	Li	10
Bittner Mammory Tumor	Judge	12
Friend Virus Leukemia	Judge	12
Squamous Cell Carcinoma	Fishtein	15

1.3 Attempted Purification of the Antineoplastic Principle

Several laboratories have attempted the isolation and purification of the active antineoplastic principle(s). Schmeer was the first to attempt the purification.[5-7] The fractionation involved aqueous extraction of the activity from the whole clam or liver, a 20% ammonium sulfate precipitation, centrifugation, and gel-permeation column chromatography. A fraction containing repeatable significant activity was obtained.

Prescott and co-workers attempted to purify the antineoplastic agent from clam liver.[21] Their isolation scheme involved extracting the liver with cold water in a blender, centrifugation and retention of the supernatant, precipitation of the soluble extract with 70% alcohol, solubilization of the pellet by 8% sodium chloride solution, and exhaustive dialysis. At this step, activity was lost by further manipulation of this crude liver fraction. It is apparent at this stage that Prescott was dealing with a relatively high molecular weight fraction.

Horton and co-workers in our laboratories have performed a partial purification of the antineoplastic principle.[8] The purification attempt entailed extraction of the crude liver with water in a blender, a 20-30% ammonium sulfate

precipitation, centrifugation, partial dialysis of the resulting supernatant, and fractionation of the retentate by gel-permeation chromatography using Sephadex G-25. The greatest concentration of activity was contained in the second and third void volume collected. The active principle in this fraction was termed Mercenene. These fractions were pooled and lyophilized. At this stage of fractionation, the *in vivo* antineoplastic activity was reproducibly demonstrated in the Krebs 2 carcinoma and by *in vitro* activity against the human HeLa cell line. The Mercenene fraction was desalted by an electric desalter and exhaustively dialyzed. Both the retentate and dialyzate exhibited significant activity. It is apparent that the active principle(s) in this isolation scheme contain a relatively high molecular weight and low molecular weight component.

1.4 Properties of the Antineoplastic Principle(s)

The amount of antineoplastic activity per unit weight of the crude whole clam aqueous extract is a function of the season of harvest.[13] *Mercenaria mercenaria* harvested in August exhibit 8-9 times the antineoplastic activity of quahogs harvested during February as assayed *in vivo* by the Krebs 2 carcinoma. The antineoplastic activity of cold-water-harvested quahogs can be restored to summertime levels by

incubating the quahog in fresh seawater at 15.5°C for 26 days.[13]

The antineoplastic activity is not dependent on the location of harvest. The clam is a notorious concentrator of trace compounds so it has been suggested that the active principle(s) may be ingested from the local environment. Schmeer has established that the antineoplastic activity is present in other species of clams from different locales, such as Mercenaria campechiensis from the Chesapeake Bay area.[7]

Prescott and co-workers obtained an exhaustively dialyzed crude liver fraction which was unstable to further purification attempts.[21] Their clam liver extract was heat stable up to 40°C for 30 minutes as assayed in vivo by the L1210 lymphocytic leukemia tissue culture assay. The activity was gradually lost at higher temperatures. A molecular weight of 8,000-10,000 for the fraction was reported, using the synthetic boundary method. No interferon activity of the clam liver extract could be demonstrated, although the assay technique was not stated. No induction of interferon occurred, again the assay technique was not reported. The active fraction contained carbohydrate and protein. No detectable amounts of purines or pyrimidines were present. Various attempts were made to show antineoplastic activity in the

nuclear and lipid material of the clam livers with negative results. In their hands, the active fraction was unstable during further purification attempts. When fractionated by gel-permeation chromatography on Sephadex G-25, the antineoplastic activity was lost. Other attempted purification methods were not reported. No further progress since 1974 of the isolation has been reported from this laboratory.

The active fraction obtained by Horton and co-workers after fractionation by gel-permeation chromatography on Sephadex G-25 was heat stable to 100°C for 45 minutes.[8] It was heterogeneous, contained salts, organic components of low molecular weight, and material of intermediate molecular weight. This fraction was exhaustively dialyzed. Both the dialyzate and retentate exhibited significant antineoplastic activity. The maximum dilution at which human HeLa cells displayed 4+ degeneration was 0.016 m/mL for the dialyzate and 0.008 mg/mL for the retentate. The retentate contained one-third of the total activity present while the dialyzate contained the remaining two-thirds of the activity. The carbohydrate and amino acid content of both were examined by established procedures.

1.5 Research Plan and Statement of the Problem

It is apparent from the previous attempts to isolate the active principle that a low molecular weight and a relatively high molecular weight antineoplastic principle are present. The exhaustively dialyzed fraction from Prescott's laboratory contains the relatively high molecular weight component. The low molecular weight component was discarded. The Mercenene fraction from our laboratory contains both components, but the low molecular weight fraction in the dialyzate exhibits the major part of the total activity. By the use of the appropriate fractionation and purification techniques, both components were to be isolated. The antineoplastic activity was to be monitored during the purification by the L1210 mouse lymphocytic leukemia tissue culture assay *in vitro* in our laboratory and by the P388 lymphocytic tumor assay *in vivo* in collaboration with the laboratory of Dr. Schmeer. Both components were to be characterized by standard chemical and physical techniques. The low molecular weight component was to be identified and any synergistic relationships were to be demonstrated.

The purpose of this work was to:

1. Account for all the antineoplastic activity present in the water soluble, clam liver extract as assayed by the L1210 Tissue Culture Assay.

2. Isolate, characterize, and identify the major low molecular weight active principle.

3. Isolate, characterize, and identify the remaining low molecular weight components that surpass the chosen criterion of activity, ID_{50} 1.0 X 10^{-4}M, and yield 0.001%.

4. Account for any remaining activity present in components that do not meet the chosen criterion of activity and yield.

5. Isolate and characterize the relatively high molecular weight component(s) that exhibit significant antineoplastic activity.

6. Explain the instability of Prescott's active fraction to further purification attempts.

7. Examine whether the majority of the activity is contained in the high or the low molecular weight component(s) and if it is contained in the high molecular weight component to explain why the Mercenene fraction previously obtained by our laboratory contains the low molecular weight principle(s) as the major active component.

8. Establish if synergism exists between the major active principles.

1.6 Significance of the Proposed Research

The significance of the specific goal of the isolation of new nontoxic antineoplastic agents for the treatment of cancer is readily apparent, but the general overview must also be considered. This research is in a little-explored area of natural product chemistry, namely the hydrophilic constituents of marine organisms. The seas stand as a vast undeveloped resource for new biologically significant natural products. The bulk of the rather sparse literature on fully characterized natural products from marine sources has been concerned with compounds extracted by use of organic solvents.[22] Research in this area has strongly emphasized compounds of this type, but there is no reason to expect a lower incidence of biologically significant hydrophilic compounds. Few laboratories have addressed themselves to this problem of isolation and assay of the hydrophilic constituents. It was hoped that this research would contribute to the expansion of this potentially valuable area of research.

CHAPTER II. MATERIALS AND GENERAL METHODS

2.1 Materials

2.1.1 Reagents

The following reagents were purchased from the Sigma Chemical Company: penicillin K, streptomycin, HEPES, Coomassie Blue, 1,4-dithiothreitol, uridine 5'-phosphate, ninhydrin, tris(hydroxymethyl)aminomethane (Tris), amino acid standards.

Bovine serum,fetal bovine serum, glutamine, RPMI 1640 media, HEPES, phosphate buffered saline, and Trypan Blue were purchased from Flow Laboratories.

Bovine gamma globulin, sodium dodecyl sulfate, electrophoresis protein standards, and protein assay reagent were obtained from Bio-Rad Laboratories.

Gel-permeation protein standards were obtained from Pharmacia Fine Chemicals Company. Potassium metabisulfite was purchased from Mallinckrodt. Periodic acid was obtained from G. Frederick Smith Chemical Company. Alcian Blue was from Eastman Chemical Company. Deuterium oxide was purchased from Stohler Isotope Chemicals. HPLC grade methanol was from Baker Chemicals while HPLC grade acetonitrile and

tetrahydrofuran were from Fisher Chemicals. Dimethylsulfoxide was from Eastman Chemical Company, 5-Fluorouracil from PCR, Lumilux Grün ZS Super from Brinkmann, and the boron trichloride-methanol esterification reagent from Applied Science.

2.1.2 Chromatographic Media

Sephadex G-10, G-15, G-25, and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals. Sigma supplied DEAE-Cellulose and Poly U-Sepharose 4B. Hydroxylapatite and Bio-Gel P-100 were purchased from Bio-Rad Laboratories. Microcrystalline cellulose was obtained from the FMC Corporation and dialysis tubing from Spectrapor.

2.1.3 L1210 Cells

L1210 mouse lymphocytic leukemia cells were obtained from the laboratory of Dr. Alexander Bloch at Roswell Park Memorial Institute in Buffalo, New York.

2.2.1 L1210 Mouse Lymphocytic Leukemia Tissue Culture Assay

The L1210 cells were maintained in 250 mL Spinner flasks or 100 mL T-flasks at 37°C in RPMI 1640 media containing 5% bovine serum, 0.3 mg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were fed when cell

concentration exceeded 2 X 10^6 cells/mL. The medium was withdrawn and fresh medium added to a concentration of 2.0 X 10^5 cells/mL. Cell stocks were frozen within a sterile ampule in a 10% (v/v) dimethylsulfoxide-media solution at a concentration of 2.5-4.0 X 10^6 viable cells/mL and stored at -70°C. Actively growing cell lines were replaced when the viability decreased or the cell line mutated. L1210 cells are sensitive to new glass or T-flasks. The containers were incubated with medium for 48 hours and fresh medium added before introduction of the cells. This was especially important when frozen stocks were revived. Cells were prepared for assay by feeding one part of cell culture to five parts fresh medium 24 hours before the assay or one part of cell culture to ten parts medium 48 hours before the assay. This assured that the cells were in the normal log phase of growth. For the assay, cells were diluted to a final stock concentration of 3.0 X 10^5 cells/mL by buffered RPMI 1640 media. The buffered media contained 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.3 mg/mL glutamine, and 20 mM Hepes buffer pH 7.4. The test component was added to a two dram vial and diluted to 1 mg/mL by phosphate buffered saline pH 7.4. Serial dilutions in the buffered growth medium in sterilized screw-cap culture tubes were performed to the desired concentrations. Three concentrations were routinely assayed. An amount of 0.5 mL of the desired concentration was added to

the screw-cap culture tube, three tubes for each desired concentration, and 0.5 mL of the stock cell inoculum was added. The tubes were flamed, sealed and then incubated at 37°C for 40 hours. 5-Fluorouracil and crude clam liver extract were used as standards for each assay. A set of controls containing no test component were run. Following incubation, the culture tube was vortexed for several seconds and 0.3 mL of the cell culture was added to an equal volume of Trypan Blue in a test tube. The total and viable cell number were determined on a standard hemocytomter by counting eight white-cell sections at a magnification of 100X. Cells that have not degenerated but are dead absorb the blue stain. The results obtained are expressed as % Inhibition as follows:

$$\% \text{ Inhibition} = \frac{(T_c - I) - (T_d - I)}{(T_c - I)}$$

where T_c - total number of cells in the control, I = inoculum, and T_d = total number of cells in the sample.

The % viability is defined as:

$$\% \text{ Viability} = \frac{T-D}{T}$$

where T = total number of cells and D = number of dead cells. For an assay to be valid, the controls must exhibit 95% viability and exceed a final concentration of 9.0×10^5 cells/mL, which is three times the inoculum concentration.

One unit of activity is defined as the weight of sample diluted in 1 mL that exhibits 50% inhibition.

Synergism between two different fractions was investigated by adding a known weight of each fraction together, diluting to the desired concentrations, and determined the ID_{50} value of the combination of the fractions.

ID_{50} is defined as the weight dissolved in 1.0 mL that exhibits 50% inhibition. Unless stated otherwise, the ID_{50} weight is mg.

CHAPTER III. ISOLATION AND IDENTIFICATION OF THE LOW MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE

3.1 Introduction

Previous isolation attempts by Horton and co-workers have indicated the presence of antineoplastic agent(s) that readily pass through a dialysis bag.[8] Prescott did not address the problem of isolating the low molecular weight hydrophilic constituent(s).[21] A preliminary investigation of this fraction in our laboratory indicated the presence of free amino acids and free reducing sugars including glucose and a reducing substance, $R_{Glc} = 0.70$, that did not correlate to any standards employed. No hexosamines were detected. The presence of a 6-deoxyhexose was indicated by the Dische reaction, but free fucose and rhamnose were absent.[8]

Unpublished data from our laboratory includes the analysis of the whole clam tissue for total carbohydrate, lipid, nucleic acid, free carbohydrate, and free amino acid content. The lyophilized tissue was fractionated by solvent extraction and the fractions assayed for tumor-inhibitory activity. Mercenene, the active fraction obtained by Sephadex G-25

gel-permeation chromatography as previously described, was analyzed and its composition compared with the composition of the whole clam tissue.

Mytilitol and taurine were isolated from the whole clam tissue in 0.005% yield based on the weight of wet clam tissue.

The lyophilized whole clam tissue was extracted in sequence by the following solvents with yields in parenthesis: n-hexane (0.5%), acetone (13.8%), methanol (21.7%), water (19.3%). Activity versus human HeLa cells *in vitro* could be demonstrated for the methanol and water extracts.

The proportion of total lipid, nucleic acid, protein, carbohydrate, and free amino acids in the whole clam tissue and mercenene fraction were established. For the whole clam tissue, the composition was 4.3% lipid, 41.0% protein, 14.9% nucleic acid, 14.4% carbohydrate, 2.1% free amino acids. For the mercenene fraction, the composition was 3.28% lipid, 13.6% protein, 2.5% nucleic acid, 19.5% carbohydrate, 2.5% free amino acids.

The lyophilized whole clam tissue and mercenene fractions were extracted with water and exhaustively dialyzed. The water insoluble portion of the lyophilized whole clam tissue contained 2.0% polysaccharide and 15.6% protein. After hydrolysis with 2N HCl at 100°C for 4 h, 3.4% free carbohydrate, 10% free basic amino acids, and 16.4% acidic amino

acids were detected. The water insoluble mercenene fraction contained 2.3% polysaccharide and 4.1% protein. After acid hydrolysis under the same conditions, the fraction contained 12% free carbohydrate, 7.5% free basic amino acids, and 15.0% free acidic amino acids. The whole clam retentate contained 8.72% carbohydrate, 6.0% protein, 0.4% uronic acid, and 17.9% insoluble material. The mercenene retentate was composed of 20.0% carbohydrate, 74% protein, 2.6% uronic acid, and 6.5% insoluble material.

The whole clam tissue dialyzate contained 4.73% free carbohydrate, 1.8% free basic amino acids, and 0.4% free acidic amino acids.

The mercenene dialyzate was comprised of 11.9% free carbohydrate, 2.0% free basic amino acids, and 0.6% free acidic amino acids.

The fractionation procedure employed to yield the dialyzate was not adaptable to large-scale isolation. Electric desalting of the pooled mercenene fractions from the Sephadex G-25 gel-permeation column was limited to 10 mL fractions and exposed the sample directly to a mercury surface. Mercury can exhibit toxicity toward both the *in vitro* assay system employed and *in vivo* assay systems. An

alternative way of separating the salts from the active fraction was needed. Preparative TLC was considered as a fast, economical means. Due to the complexity of the fraction, an efficient, fast, and nondestructive method was needed for the final resolution of the active component(s). It was hoped that HPLC would satisfy these requirements.

3.2 Methods

3.2.1 HPLC

Samples were prepared for HPLC by dissolving the sample in HPLC-grade water or in the eluant used in the separation. The dissolved sample was filtered through a sintered-glass funnel to remove any particulate matter. HPLC-grade water was prepared by passing demineralized, distilled water through a Waters Bondapak C18/Porasil B Reverse Phase Column to remove organic impurities. The organic impurities retained on the column were eluted by 99% methanol. The prepared water was stored in clean glass containers. All solvent mixtures were degassed under diminished pressure before use. Sample concentrations of 5-50mg/mL were employed. The initial analytical separations were performed on a Waters μBondapak C18

Reverse Phase Column using eluant combinations of acetonitrile, methanol, and water. A Waters HPLC 440 with gradient capabilities was used to establish the initial separation conditions. Detection was by UV 254nm and RI. For each run, 1-10 μL of sample was injected. Preparative separations employed a Waters semipreparative μBondapak C18 Reverse Phase Column with isocratic elution at ambient temperature. Detection was by UV 254 nm and RI. For each run, 0.1-1.0 mL of sample were injected. Sample fractions were collected manually and evaporated under diminished pressure on a rotary evaporator at room temperature to remove methanol and acetonitrile. Samples were then lyophilized and stored at $-20^{o}C$. The column was flushed after use by 99% methanol and the eluate collected. The methanol was evaporated under diminished pressure on a rotary evaporator and 5 mL of distilled, demineralized water added to concentrate the residue. The residue was lyophilized and stored at $-20^{o}C$. This material adhering to the column was assayed to ascertain that no activity adhered to the column during the separation. Binding of relatively hydrophobic consistuents to the column support is often observed in reverse phase liquid chromatography. The phenomenon is usually observed at low organic compositions in the mobile phase.

The Capacity factor, k', is defined as:

$$k = \frac{T_r - T_o}{T_o}$$

Where: T_r = Elution time of individual component.

T_o = Elution time of unretained standard.

3.2.2 Preparative TLC

Preparative TLC fractionations were performed using microcrystalline cellulose, Avicel TG-101 from FMC Corporation, as the solid support. The plates were prepared by adding 100 grams of the dry powder, volume of 430 mL, to a blender. The microcrystalline cellulose was slurried by adding 630 mL of distilled water. Several mg of fluorescent indicator, Lumilus Grün Z3 Super, were added to the slurry. The slurry was then blended for 25-35 seconds to yield the proper consistency for plate preparation. The slurry was then transferred to a two-liter round-bottom flask and degassed under diminished pressure for one hour. Glass plates, 20 X 20 cm, were cleaned and dried. The microcrystalline cellulose was applied to the plates at a thickness of 1.75 mm by a Desaga spreader. The plates were air dried overnight to yield a dry layer thickness of

approximately 0.8 mm. The plates were stored and used without activation. Samples were dissolved in distilled water to a concentration of 50 mg/0.1mL. To each plate 50-70 mg of sample were applied by capillary tube. Each place was air dried to remove water. Plates were developed in a saturated solvent chamber by 3:1:1 1-butanol-acetic acid-water for 6-7 h. After detection by UV, the desired fractions were scraped off the glass plate, pooled and suspended in distilled water. The fractions were subsequently sonicated for 5 min and vacuum filtered. The filtrate was lyophilized and stored at -20°C.

3.2.3. Determination of Carbohydrate Content

Carbohydrate content was determined by the phenol-sulfuric acid colorimetric method.[23] Amounts of reagents were proportionately scaled down. Into a matched cuvette, 0.4 mL of an aqueous solution containing 0.08 to 0.25 mg of sample was pipetted. An equal volume of 5% phenol solution was added and mixed. Blanks were prepared using 0.4 mL of distilled, demineralized water. To the cuvette, 2.0 mL of sulfuric acid was added by a fast-flowing pipet. The cuvette was agitated during addition of the sulfuric acid to ensure good mixing and an even heat-distribution. The cuvettes were placed in a water bath for 20 min at 25°C. Within one

h, absorbances were measured at 490 nm with a Beckman DU spectrophotometer. Background absorbance determined by the blank was subtracted from the reading. The carbohydrate concentration is determined by comparison with a standard curve for glucose. Determinations were made in triplicate.

3.2.4. GLC Determination of Amino Acids

Determination of amino acids was performed by a revised procedure of the method of Gehrke.[24]

Inorganic salts were removed by preparative TLC on microcrystalline cellulose as previously described.

The salt-free sample was dissolved in demineralized, distilled water and passed through a 25 X 1.6 cm cation exchange column of Amberlite IR-120 H^+. The effluent was discarded. The column was washed with five void volumes of demineralized, distilled water and the effluent was discarded. The column was then eluted with 7 N NH_4OH and the effluent collected and evaporated in a sand bath at 100°C under a stream of nitrogen. The sample was then dissolved in a minimum amount of distilled water to concentrate it, lyophilized, and stored at -20°C.

The samples were derivatized in the following manner: An aqueous aliquot containing 1.0 mg of amino acids or 1.0 mg

dry weight was added to a 5-mL Reacti-Vial. Ornithine internal standard, 0.05 mg, was added and the solution was evaporated to dryness by placing the vial in a sand bath at 100°C while directing a stream of dry, filtered nitrogen into the vial. Dichloromethane (1.0 mL) was added and evaporated to dryness under a stream of nitrogen to remove water azeotropically. This step was repeated twice. To the Reacti-Vial, 5 mL of a 10% BCl_3-MeOH solution was added. The vial was agitated by hand, followed by ultrasonic mixing for 15 seconds. Esterification was allowed to take place for 30 min at room temperature. After esterification, the sample was evaporated as previously described. To the vial, 1.8 mL of 1M $NaHCO_3$-Na_2CO_3 (1:1) solution and 0.2 mL of Ac_2O were added. The vial was heated for 30 min at 100°C in the sand bath. The solution was extracted with an equal volume of chloroform, washed, and dried with Na_2SO_4. The sample was evaporated under diminished pressure to dryness and stored at -20°C or used immediately.

The sample was dissolved in $CHCl_3$ to a concentration of 2.0 mg/mL. 5-10 μL of sample were chromatographed on properly conditioned OV-17 or ECNSS columns. The oven temperature was programmed from 75°C at 4°C or 8°C per min. The detector

temperature was 250°C and the injection port was 200°C. The carrier gas flow-rate was 30 mL/min. The columns were calibrated with a series of amino acid standards at each temperature program. Assignments were checked by adding the standard to the sample and chromatographing the mixture.

3.2.5 Paper Chromatography

Amino acid samples were prepared as previously described for GLC. The sample was examined by descending paper chromatography on Whatman 1 or Whatman 3 paper in 3:1:1 (v/v) 1-butanol-acetic acid-water. Free amino acids were detected by spraying with a 0.2% solution of ninhydrin.

3.3 Instruments and Equipment

The following instruments and equipment were used:

NMR Bruker WP 200

IR Perkin-Elmer 137 Spectrophotometer
Perkin-Elmer 457 Grating Infrared Spectrophotometer

UV-VIS Beckman DU Spectrophotometer
Perkin-Elmer 202 Spectrophotometer

O.R. Perkin-Elmer 140 Polarimeter

GC Hewlett-Packard 5720A

RI Pharmacia 300L

HPLC Waters 440 RI Detector R401

HPLC Columns Waters 27324 μBondapak C_{18} 10u
ID=3.9mm L=30cm
Waters 84167 μBondapak C_{18} 10u
ID=7.8mm L-30cm

GC Columns Applied Science 3% OV-17 100/120 GCQ
Applied Science 3% ECNSS-M 100/120 GCQ
HP5720X10.2

UV Pharmacia UV

Centrifuge Servall Refrigerated-Automatic
Rotor SS-34

Figure 1. Flow Chart of the Isolation Scheme for the Major Low Molecular Weight Antineoplastic Principle

The isolation scheme for the low molecular weight antineoplastic principles is summarized. The section of the text for each individual fractionation and characterization step is indicated next to that step.

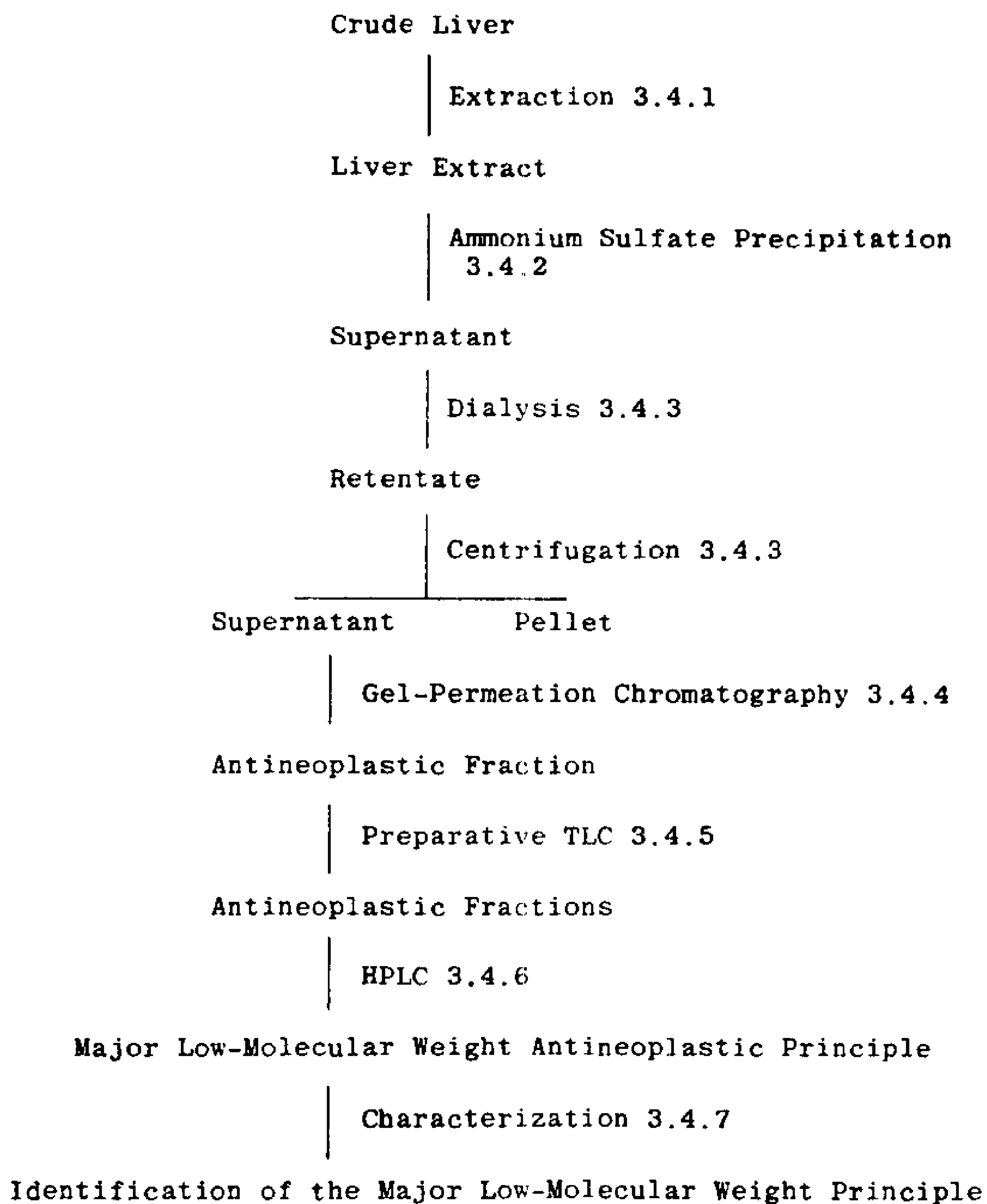


Figure 1.

3.4 Isolation and Characterization of the Low Molecular Weight Antineoplastic Principles

The isolation scheme for the low molecular weight antineoplastic principles is summarized in Figure 1.

3.4.1 Extraction of the Antineoplastic Principles

One hundred and forty g of frozen whole clam livers were added to 800 mL of distilled water at 4°C in a Sears Insta-Blend blender. One liver weighs approximately 1 mg dry weight. The livers were homogenized for 2 min, divided into 4-200 mL portions, and centrifuged at 4000 RPM at 4°C for 30 min. The resulting supernatant was lyophilized and stored at -70°C. The pellet was inactive and was discarded. The yield was 50 g of crude liver extract. The ID_{50} 5.6 mg, corresponding to 8930 units of total activity.

3.4.2. Ammonium Sulfate Precipitation

The crude liver extract was suspended in 180 mL of 0.10M potassium phosphate buffer pH 6.8 within a 400 mL beaker at 4°C. The appropriate volume of saturated ammonium sulfate was added dropwise with gentle stirring to bring the solution to 16% saturation. The supernatant fluid was collected after centrifugation at 4°C, 4000 RPM, for 25 min. The pellet

weight was 10 g. It was inactive and discarded. Contrary to previously published reports,[8] 20-25% ammonium sulfate saturation was found to precipitate a significant portion of the activity.

3.4.3. Partial Dialysis of the Supernatant

The supernatant solution resulting from the ammonium sulfate precipitation was dialyzed in a Spectrapor 3500 molecular weight-cutoff dialysis membrane at 4°C versus 4 L of distilled, demineralized water for 6 h. After 4 h, a precipitate formed in the dialysis membrane. After dialysis, the dialyzate was collected and lyophilized. Salts were removed by preparative TLC on microcrystalline cellulose as previously described. The organic fraction yielded 5 g of a material after purification by preparative TLC. The ID_{50} was 33 mg, which corresponds to 150 units of total activity. The amino acid content of the dialyzate was determined by GLC paper chromatography as previously described. The retentate was centrifuged at 5000 RPM at 4°C for 30 min. The supernatant was lyophilized and stored at -20°C. It weighed 25.8 g. The ID_{50} of the supernatant was 7, which is 3680 units of total activity. The retentate pellet weighed 7.5 g after lyophilization. The pellet ID_{50} was 2.0, which corresponds to 3750 units of total activity. The pellet was stored

at $-70^{\circ}C$. The amino acid profile of the retentate supernatant was determined as before. Free amino acids were present in a 0.5% yield based on the weight of crude clam liver. After partial dialysis, 55% of the free amino acids had passed through the dialysis membrane. Assuming that the maximum loss of low molecular weight antineoplastic compounds through the dialysis membrane would approach the rate loss of the free amino acids, 50% of the low molecular weight antineoplastic principles could be lost. The actual loss was determined by assay of the dialyzate. Moreover, this provides an estimate of the maximum loss of any cofactor that may be associated with the high molecular weight principle. The cofactor may not express activity by itself, if it is present. Therefore, the loss of the cofactor may not be detected by an assay of the dialyzate but its maximum loss may be estimated by the rate loss of the free amino acids.

Figure 2. GLC Chromatography of the Dialyzate Amino Acids

A 5-μL sample of the derivatized dialyzate amino acids at a concentration of 2.0 mg/mL was injected on a preconditioned 3% OV-17 column coated on 3.2 g of 100/120 mesh Gas-Chrom Q support. The initial temperature was 75°C. The injector temperature was 200°C and the detector temperature was 250°C. A temperature program of 4°C/min was employed for the oven.

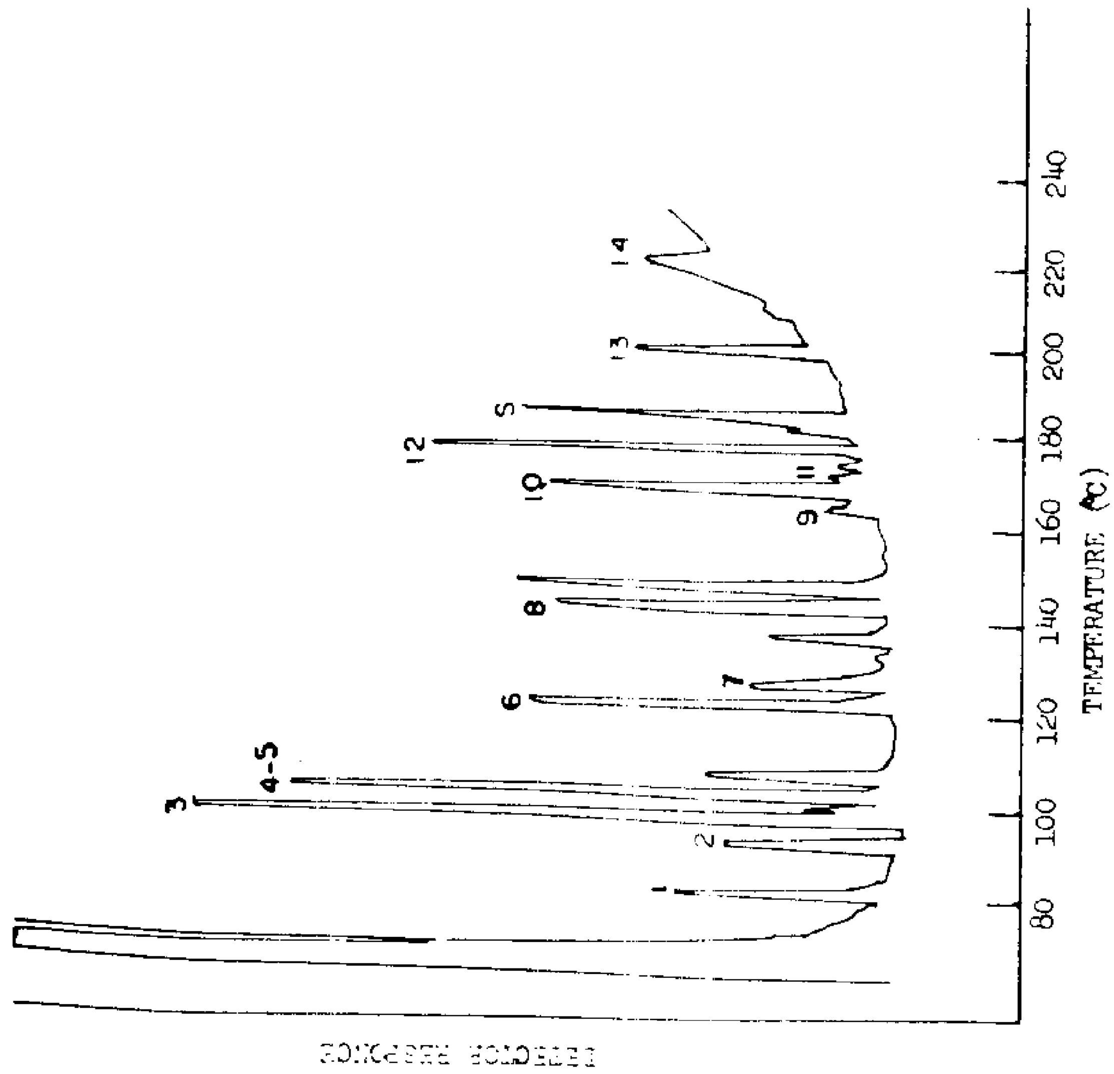


Figure 2.

TABLE 2

FREE AMINO ACID COMPOSITION

IN THE DIALYZATE

Amino Acid	GLC Temp C[a]	R_f[b]
1. Alanine	83	0.22
2. Valine	91	0.41
3. Threonine	93	0.18
4. Leucine	98	0.80
5. Isoleucine	98	0.90
6. Glycine	122	0.19
7. Aspartic Acid	131	0.08
8. Methionine	142	0.40
9. Serine	162	0.11
10. Asparagine	166	0.07
11. Lysine	167	0.06
12. Glutamic Acid	182	0.15
13. Arginine	198	0.13
14. Histidine	232	0.13

[a]GLC on OV-17 with $4^{o}C$/min temperature program.

[b]Paper chromatography with 3:1:1 1-butanol-acetic acid-water.

3.4.4 Gel-Permeation Chromatography of the Retentate Supernatant

The retentate supernatant was fractionated by gel-permeation chromatography on Sephadex G-25 and G-15. For the preparative work Sephadex G-25 was employed. The elution profile of a 300 mg sample, ID_{50} 7 corresponding to 43 units of total activity, is shown in Figure 2. Fractions 1 and 2 were dialyzed versus demineralized, distilled water at 40°C for 4 h. After lyophilization, the weight was 40 mg for fraction 1. Fraction 1 was eluted at the void volume and was not significantly active. The ID_{50} was 20, which is 2 units of total activity. Fraction 2 yielded 250 mg with the ID_{50} 7 or 36 units of total activity. After dialysis at 4°C versus distilled, demineralized water for 24 h, fraction 3 yielded 1 mg with ID_{50} 0.2 or 5 units of total activity. Increased resolution was obtained by Sephadex G-15, as shown in Figure 3. The same elution profile was obtained when the buffer contained 0.1M NaCl or 0.1M pyridine-acetic acid pH 5.1. The carbohydrate content was determined by the phenol-sulfuric colorimetric assay as previously described. The elution profile is shown in Figure 4. Preparative gel-permeation chromatography on Sephadex G-25 provided a baseline resolution of the low molecular weight antineoplastic principles

from the high molecular weight fraction. Sephadex G-15 gel-permeation chromatography indicated the presence of three low molecular weight antineoplastic principles of less than 1500 molecular weight. An active principle is eluted from the column after the salts. The elution and activity profiles are not affected by 0.1M NaCl or 0.1M pyridine-acetic acid pH 5.1, suggesting that this component was not adhering to the support. During a preparative run when up to 1.0 g of retentate supernatant is loaded on the Sephadex G-25 column, 3 to 5 mg of the material in colloidal form in 120 mL of eluant can be obtained after elution of the salts. Apparently this active principle precipitates when the material is loaded onto the Sephadex column. The ID_{50} value and the slope of the % inhibition versus dose concentration curve is similar to those of the retentate pellet extract. The properties of this principle are identical to those exhibited by the high molecular weight principle during its purification in our laboratory. Prescott reported that the high molecular weight principle was unstable in their isolation matrix.[21] He stated that the principle degraded into a series of components of lower molecular weight during gel-permeation chromatography on Sephadex G-25. This observed precipitation can account for the instability observed by Prescott. The high molecular weight principle proved to be stable in our

isolation matrix. Contrary to our present work, previous isolation attempts in our laboratory had indicated that two-thirds of the total activity is present in the low molecular weight fraction that is contained in the dialyzate after a 24 h dialysis.[8] This mercenene fraction was obtained by Sephadex G-25 gel-permeation chromatography as previously described. The major portion of the high molecular weight principle presumably had precipitated during the chromatographic run and was thus not recovered. Therefore, the low molecular weight fraction would appear to contain most of the activity.

3.4.5 Preparative TLC on Mircrocrystalline Cellulose of the Low Moelcular Weight Antineoplastic Fraction

Fraction 2 of the Sephadex G-25 gel-permeation fractionation was fractionated by preparative TLC on microcrystalline cellulose as previously described. The fractions were assayed at a concentration of 0.250 mg/mL. The high assay concentration was used to ensure the detection of all trace components. The results are presented in Table 3. Significant activity is defined as 50% inhibition at 0.25 mg/mL. Preparative TLC fraction 13 was pooled and rechromatographed because it was too complex for subsequent purification by HPLC.

Figure 3. Sephadex G-25 Gel-Permeation Chromatography of the Retentate Supernatant

A 300 mg sample of the retentate supernatant was dissolved in 3 mL of 0.01M sodium phosphate buffer pH 6.8. The sample was loaded onto a 2.6 X 70 cm Sephadex G-25 column previously equilibrated with 0.01M sodium phosphate buffer pH 6.8. The column was eluted at 20°C with the equilibration buffer. The flow rate was 25 mL/h. RI was used to monitor the effluent. Fractions were collected every 15 min, lyophilized, and the antineoplastic activity determined. The % inhibition of each fraction was determined at 0.250 mg/mL. For routine preparative work, fractions from 0 to 50 mL elution volume were pooled into fraction 1. Fractions from 50 to 200 mL elution volume were pooled into fraction 2. Fraction 3 was from 200 to 350 mL elution volume.

Symbols: ———— RI

—●— —●— —●— % inhibition at 0.250 mg/mL

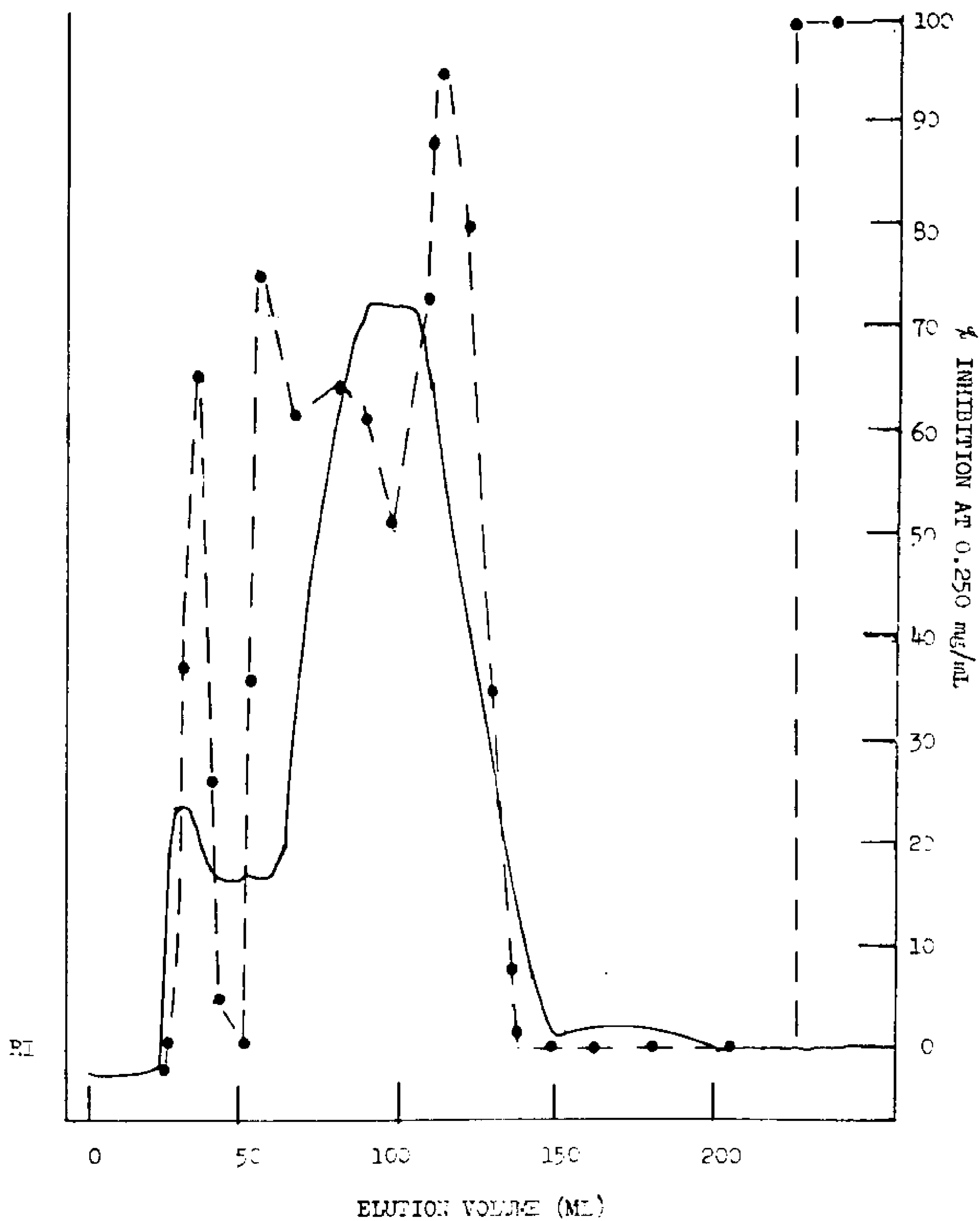


Figure 3.

Figure 4. Sephadex G-15 Gel-Permeation Chromatography of the Retentate Supernatant

A 200 mg sample of the retentate supernatant was dissolved in 2.0 mL of 0.01M sodium phosphate buffer pH 6.8. The sample was loaded onto a 1.6 X 70 cm Sephadex G-15 column equilibrated with 0.01M sodium phosphate buffer pH 6.8. The column was eluted at 20°C with the same buffer at a flow rate of 25 mL/h. RI was used to monitor the column. Fractions were collected every 18 min, lyophilized, and assayed for antineoplastic activity at a concentration of 0.250 mg/mL.

Symbols: ——— RI

●— ●— ●—% inhibition at 0.250 mg/mL

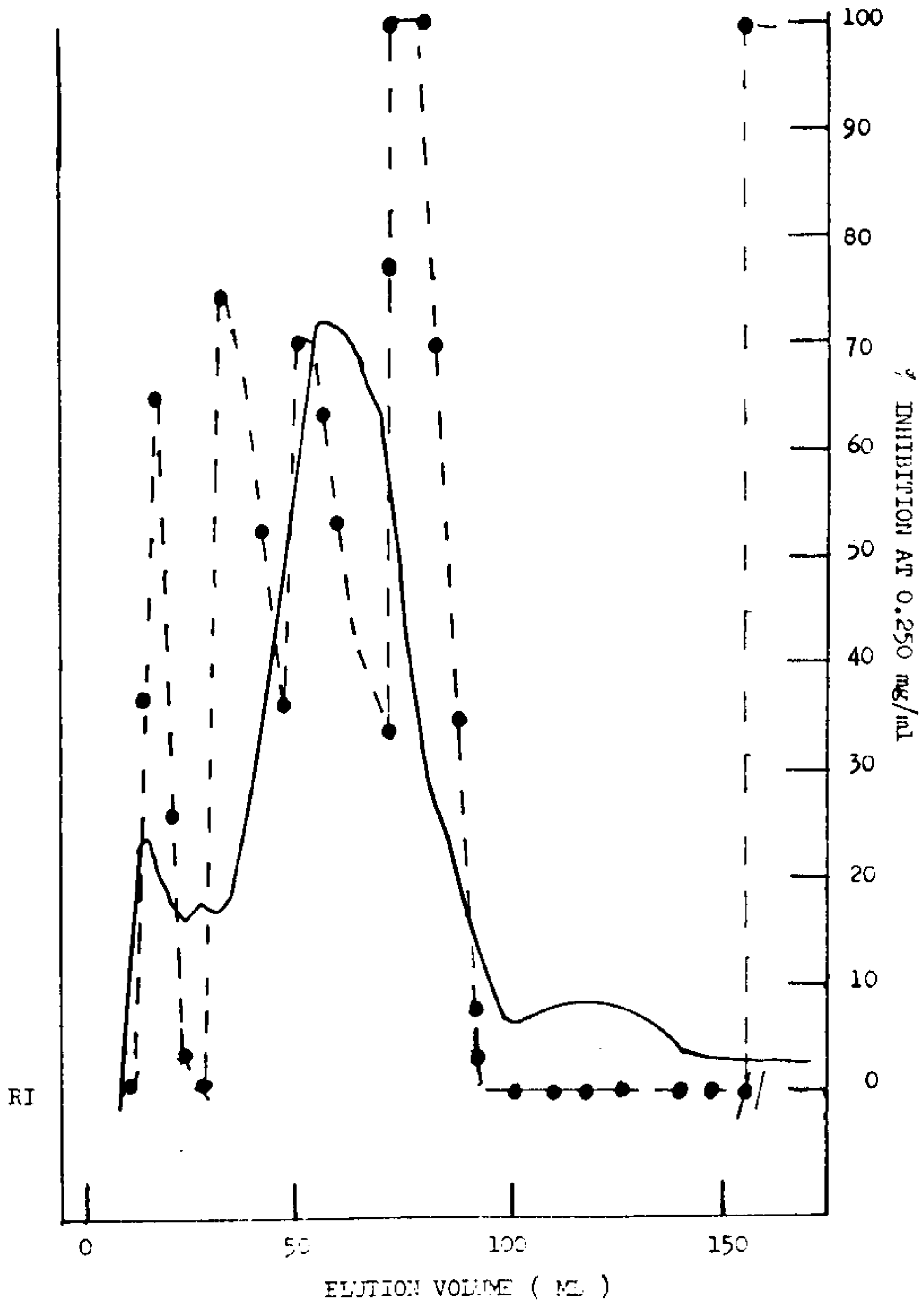


Figure 4.

Figure 5. Sephadex G-15 Gel-Permeation Chromatography of the Retentate Supernatant

A 200 mg sample of the retentate supernatant was dissolved in 2.0 mL of 0.01M sodium phosphate buffer pH 6.8. The sample was loaded onto a 1.6 X 70 cm Sephadex G-25 column equilibrated with 0.01M sodium phosphate buffer pH 6.8. The column was eluted at 20°C with the equilibration buffer at a flow rate of 25 mL/h. RI was used to monitor the column. Fractions were collected every 18 min, lyophilized, and assayed for carbohydrate content by the phenol-sulfuric acid colorimetric assay.

Symbols: ——— RI

●— ●— ●—% carbohydrate

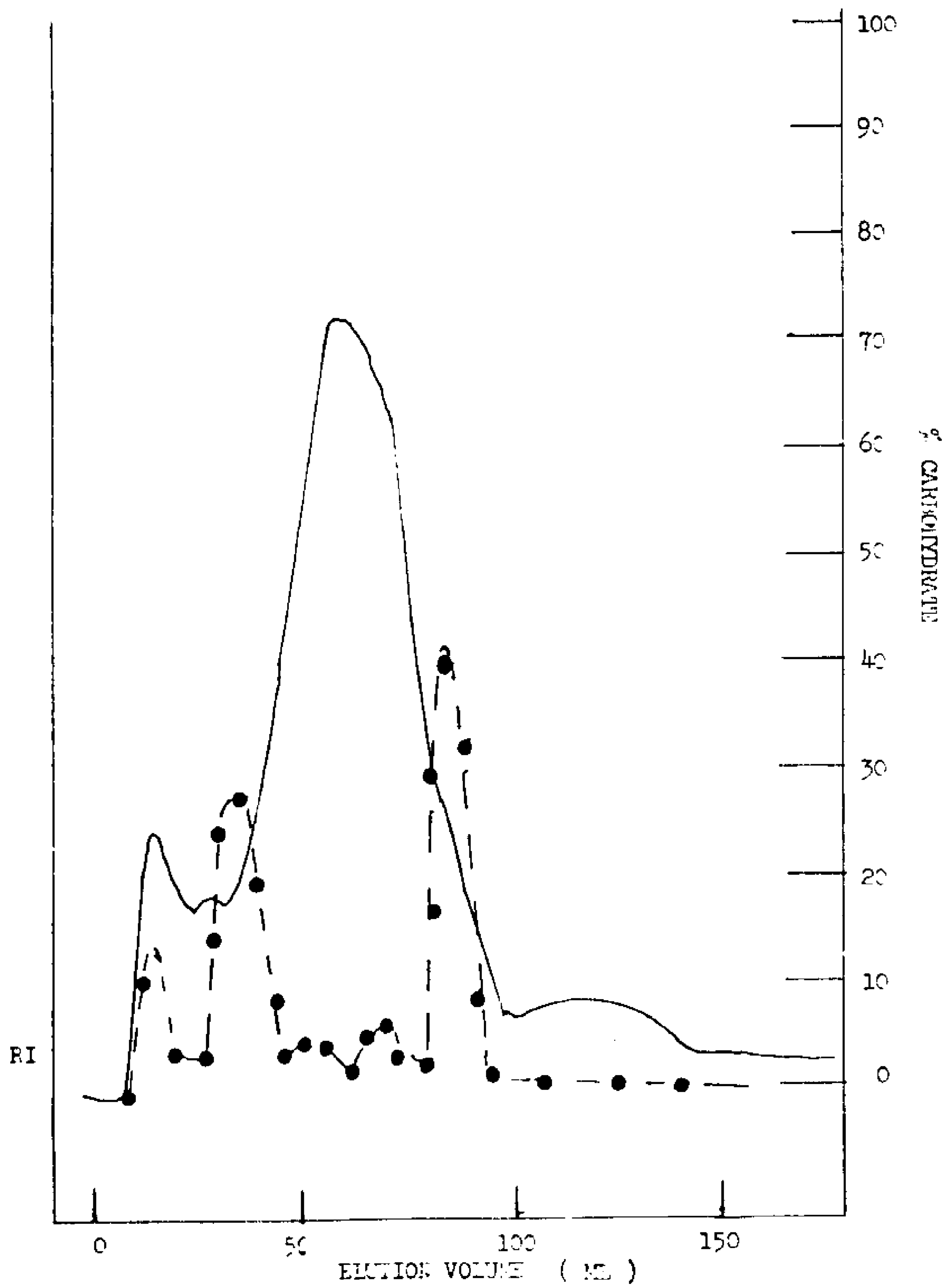


Figure 5.

TABLE 3

MICROCRYSTALLINE CELLULOSE PREPARATIVE TLC OF THE LOW MOLECULAR WEIGHT ANTINEOPLASTIC FRACTION [a]

Fraction	R_f [a]	Sign. Activity (0.250 mg/mL)	UV Active	% Wt. Recovery
1	0.00	NO	YES	29
2	0.07	NO	YES	10
3	0.15	NO	YES	4
4	0.21	NO	YES	3
5	0.46	YES	YES	10
6	0.53	YES	YES	3
7	0.62	YES	YES	3
8	0.07-0.15	NO	NO	5
9	0.15-0.21	NO	NO	4
10	0.22-0.45	NO	NO	5
11	0.47-0.52	NO	NO	2
12	0.54-0.61	NO	NO	3
13	0.63-1.00	YES	YES	11
13a	0.62-0.70	NO	NO	1
13b	0.70-0.80	NO	NO	5
13c	0.80-0.95	YES	YES	3
13d	0.95-1.00	NO	NO	4

[a]Preparative TLC with 3:1:1 1-butanol-acetic acid-water.

3.4.6 HPLC Purification of the Active Preparative TLC Low Molecular Weight Fractions

The significantly active fractions obtained by preparative TLC on microcrystalline cellulose were fractionated and purified by HPLC as previously described. The distribution of the antineoplastic activity for each fraction was determined. The HPLC purification of the individual fractions is presented in Figures 6 through 17. A summary of the results is presented in Table 4.

The major low molecular weight antineoplastic principle was located in microcrystalline cellulose preparative TLC fraction 7. The HPLC purification to homogeneity is shown in Figures 11-14. The ID_{50} was $1.6 \times 10^{-5}M$. The overall yield was 0.006% based on g of wet liver.

The other two antineoplastic principles were located in microcrystalline cellulose preparative TLC fraction 13C. Their HPLC fractionation is shown in Figures 16-17. At this stage of fractionation, the yields of both fractions were below the chosen criterion of 0.001%. The attempted purification of both components was halted at this step.

Figure 6. HPLC of Preparative TLC Fraction 5

A 0.05 mL volume of a 100 mg/mL solution of preparative TLC fraction 5 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 5% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Fraction 1 was contained between 0.0-0.3 capacity factor. Fraction 2 was contained between 0.3-0.5 capacity factor. Fractions 1 and 2 were rechromatographed as described in Figures 7 and 8. Fraction 5D had an elution time of 12.8 min with a capacity factor, k', 2.2. Fraction 5E had an elution time of 17 min with k' 3.3. Fraction 5F had an elution time of 22 min with k' 4.5. Fraction 5G had an elution time of 28 min with k' 6.0.

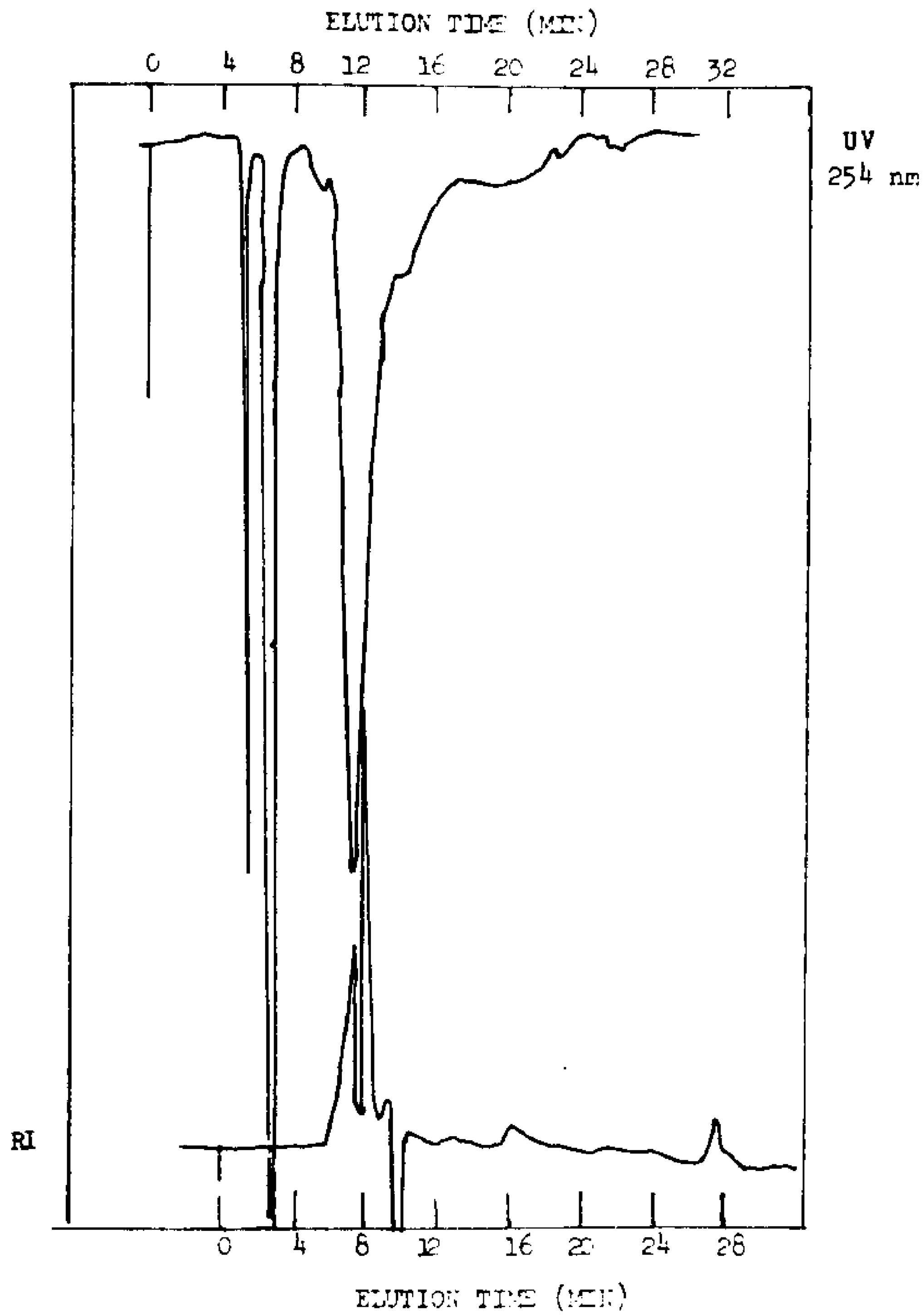


Figure 6.

Figure 7. HPLC of Preparative TLC Fraction 5 HPLC Fraction 1

A 0.10 mL volume of a 20 mg/mL aqueous solution of preparative TLC fraction 5 HPLC fraction 1 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 1% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Fraction 5A had an elution time of 4-7 min with k' 0.08. Fraction 5B had an elution time of 9 min with k' 1.3.

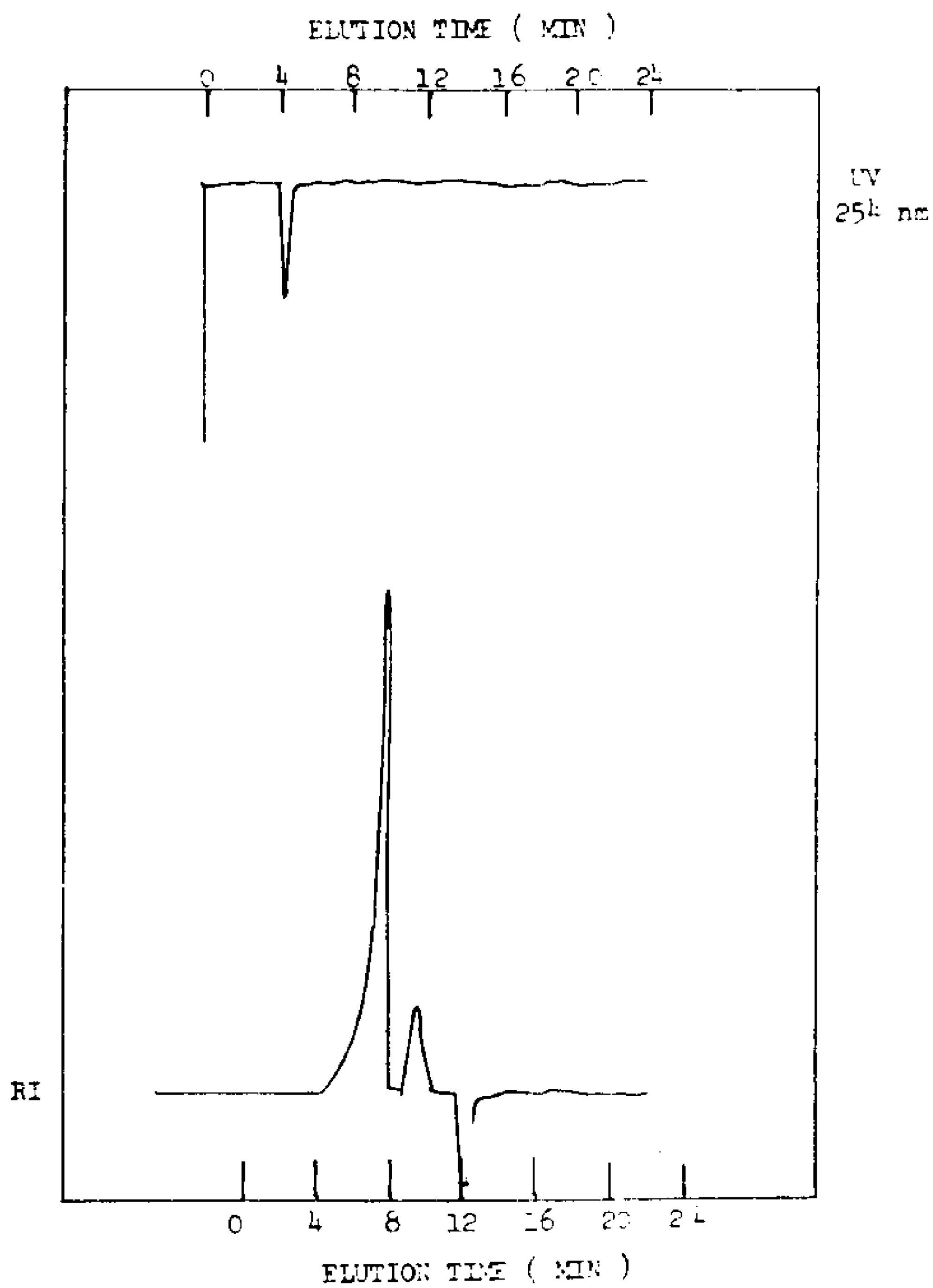


Figure 7.

Figure 8. HPLC Preparative TLC Fraction 5 HPLC Fraction 2

A 0.10 mL volume of a 15 mg/mL 1% acetonitrile in water solution of preparative TLC fraction 5 HPCL fraction 2 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 1% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components were collected and assayed. Fraction 5C exhibited an elution time of 11.5 min with k' 1.9.

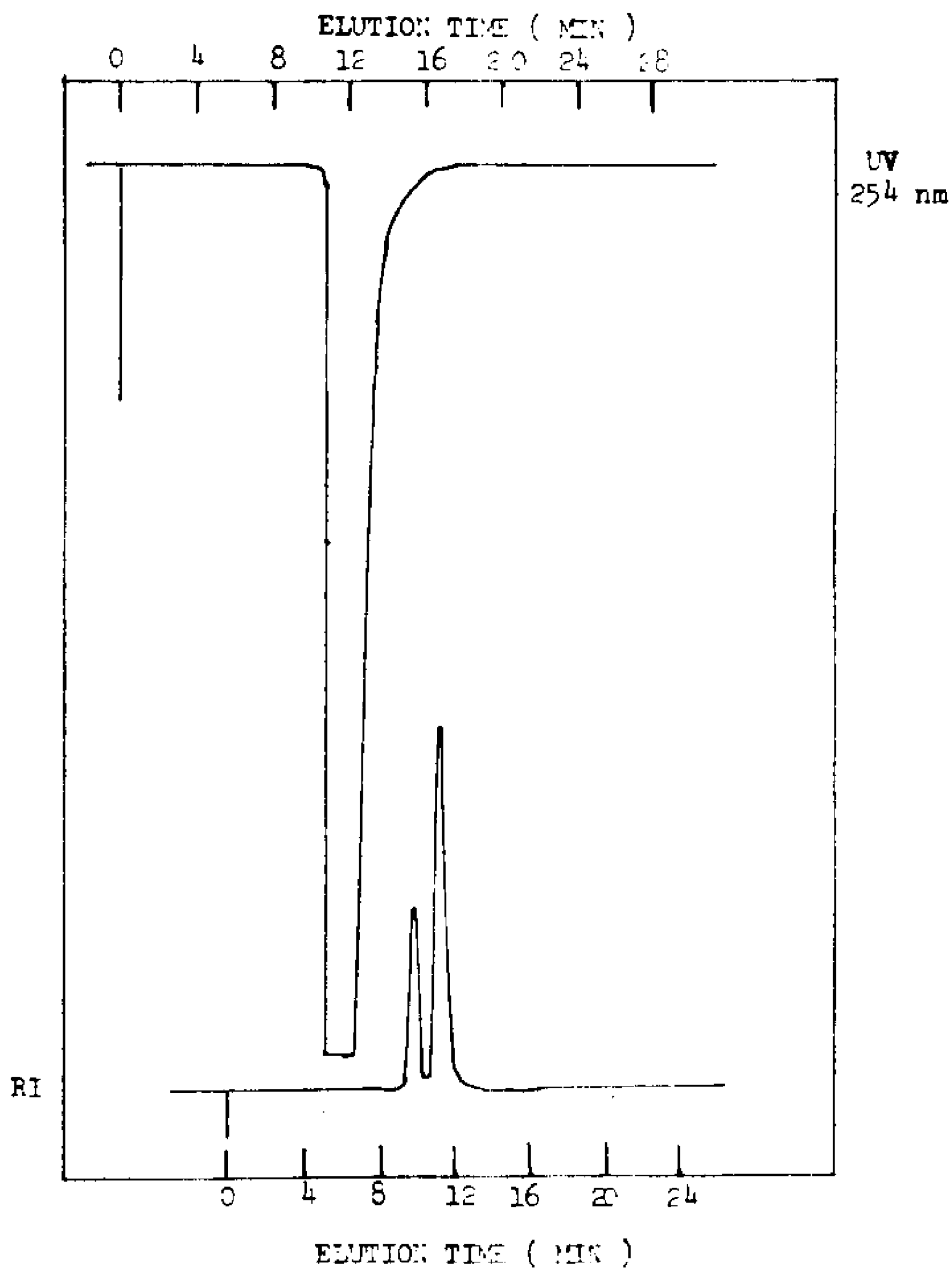


Figure 8.

Figure 9. HPLC of Preparative TLC Fraction 6

A 0.20 mL volume of a 60 mg/mL aqueous solution of preparative TLC fraction 6 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 20% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Fraction 1 was between 0.0-0.5 capacity factor. Fraction 2 was between 0.5-1.1 capacity factor. Fraction 2 was rechromatographed as described in Figure 10. Fraction 6G had an elution time of 19 min with k' 3.8. Fraction 6F had an elution time of 14 min with k' 2.5.

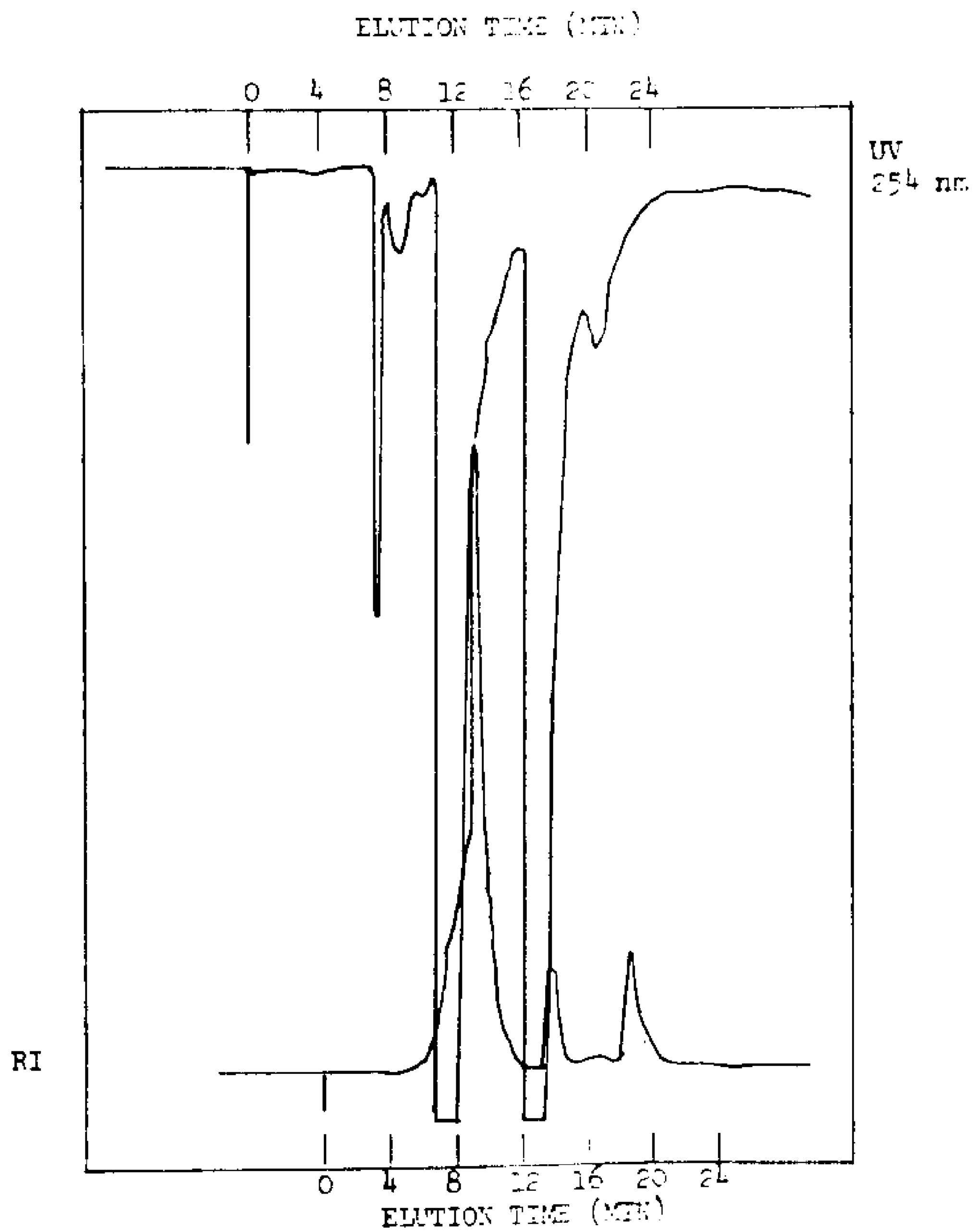


Figure 9.

Figure 10. HPLC of Preparative TLC Fraction 6 HPLC Fraction 2

A 0.10-mL vclume of a 50 mg/mL 2% acetonitrile in water solution of preparative TLC fraction 6 HPLC fraction 2 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 2% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Fraction 6B had an elution time of 7 min with k' 0.8. Fraction 6C had an elution time of 9 min with k' 1.3. Fraction 6D had an elution time of 11 min with k' 1.8. Fraction 6E had an elution time of 12-16 min with k' 2.0-3.0.

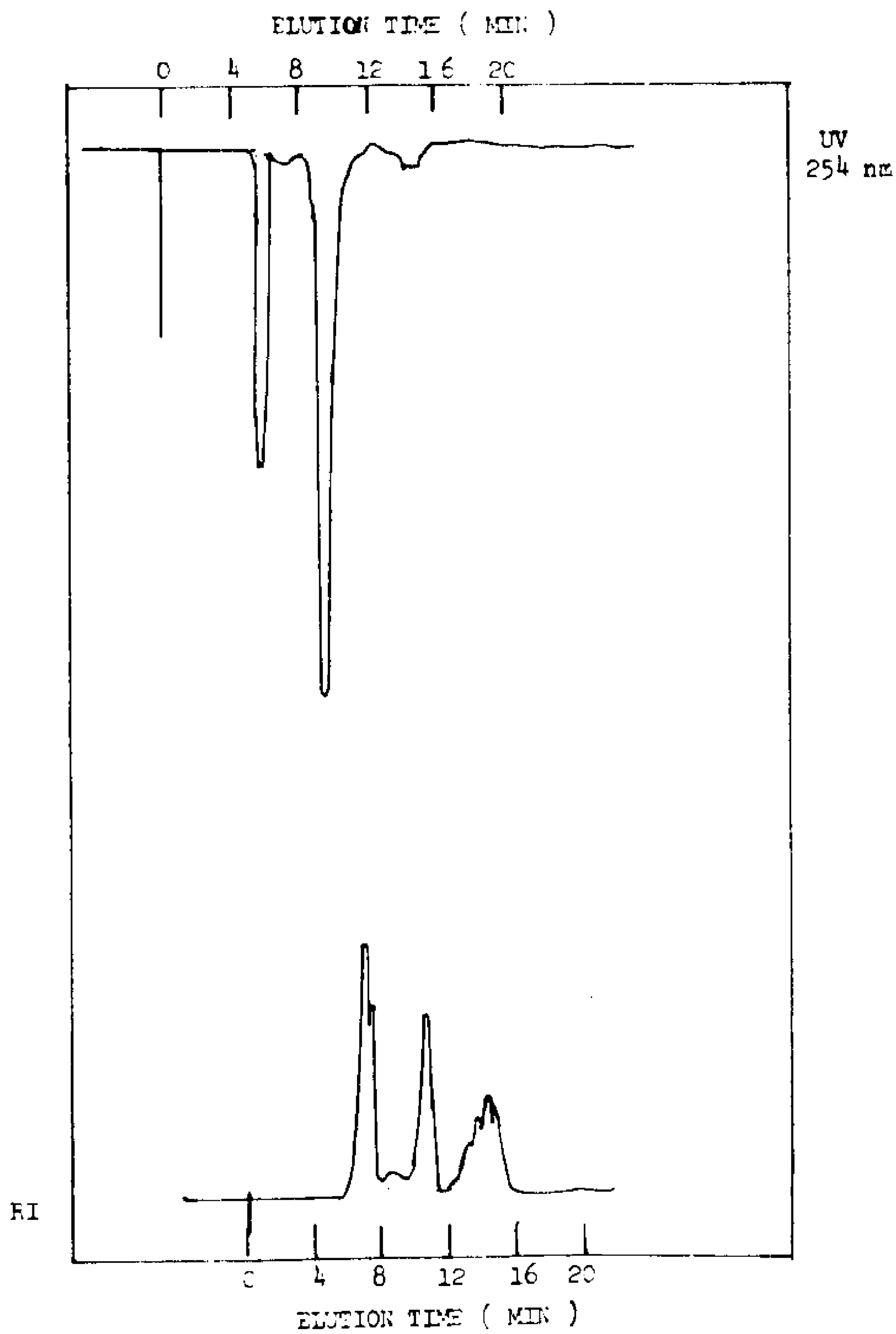


Figure 10.

Figures 11-12. HPLC of Preparative TLC Fraction 7

A 0.10 mL volume of a 50 mg/mL aqueous solution of preparative TLC fraction 7 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 5% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Figure 10 is the elution profile with RI detection. Figure 11 is the elution profile with detection by UV 254 nm. Fraction 7F had an elution time of 19 min, k' 3.8. Fraction 7E had an elution time of 17 min, k' 3.3. Fraction 7D had an elution time of 14 min. k' 2.5. Fraction 7C had an elution time of 9 min, k' 1.3. Fraction 7B had an elution time of 6-8 min, k' 0.5-1.0. Fraction 7A had an elution time of 0-6 min, k' 0.0-0.5.

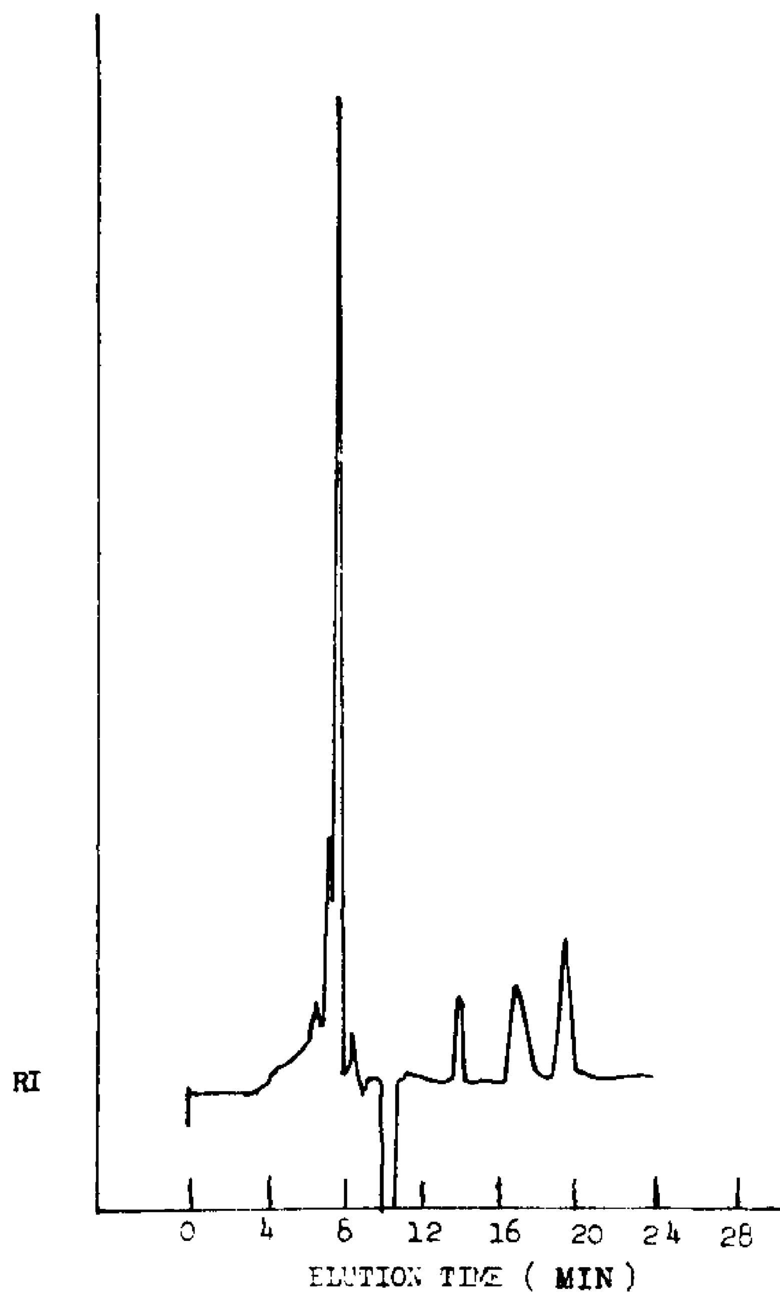


Figure 11.

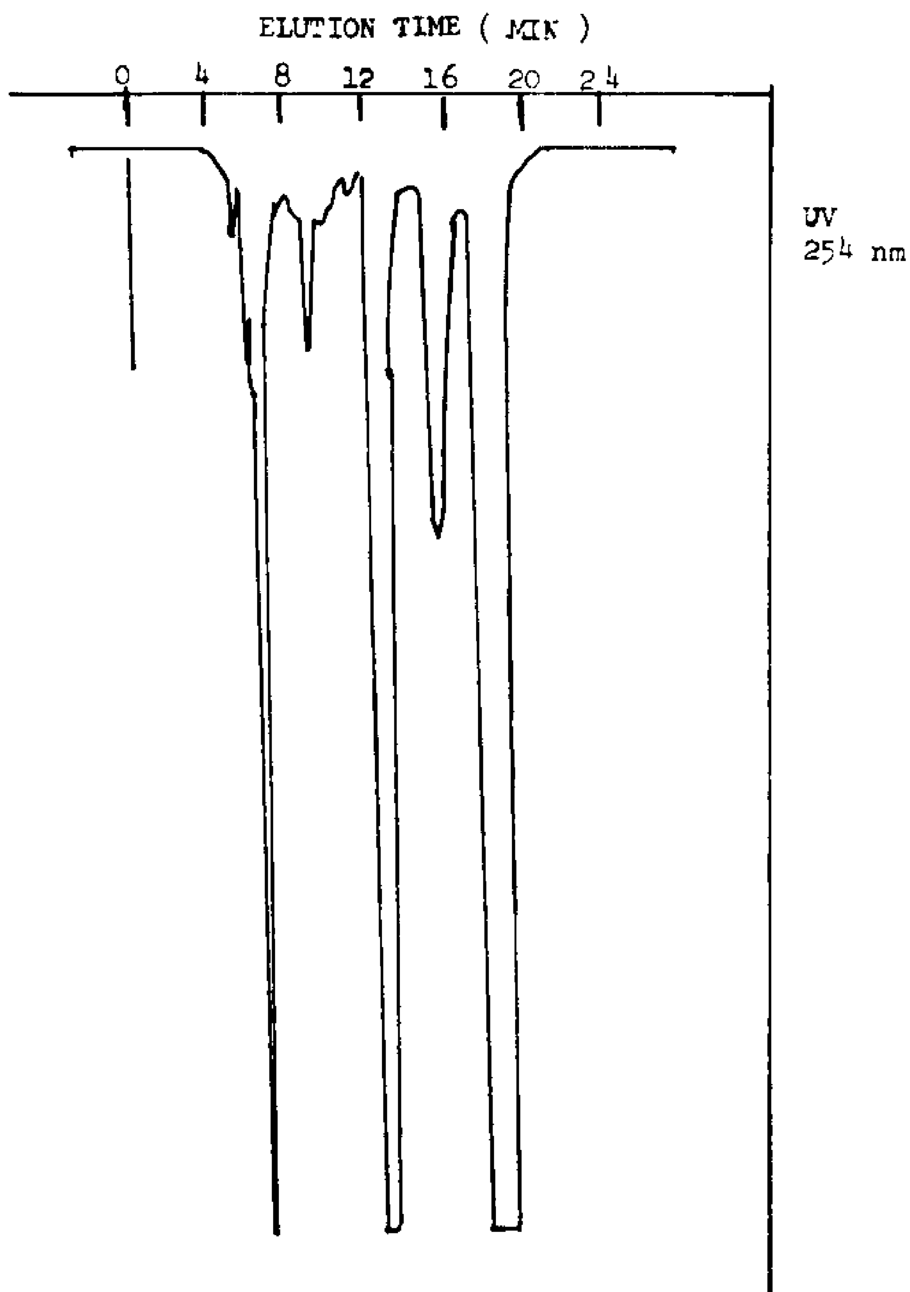


Figure 12.

Figures 13-14. HPLC Preparative TLC Fraction 7

A 0.10 mL volume of a 50 mg/mL aqueous solution of preparative TLC fraction 7 was injected on a 7.8 X 30 cm Waters µBondapak C18 column. The mobile phase was 3% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Figure 13 is the elution profile with RI detection. Figure 14 is the elution profile with detection by UV 254 nm. Fraction 7E had an elution time of 22 min with k' 4.5. Fraction 7F had an elution time of 19 min with k' 3.8. Fraction 7D had an elution time of 14 min with k' 2.5. Fraction 7C had an elution time of 9 min with k' 1.3. Fraction 7B had an elution time of 6-8 min with k' 0.5-1.0. Fraction 7A had an elution time of 0-6 min with k' 0.0-0.5. Fraction 7G had an elution time of 10-12 min with k' 2.3.

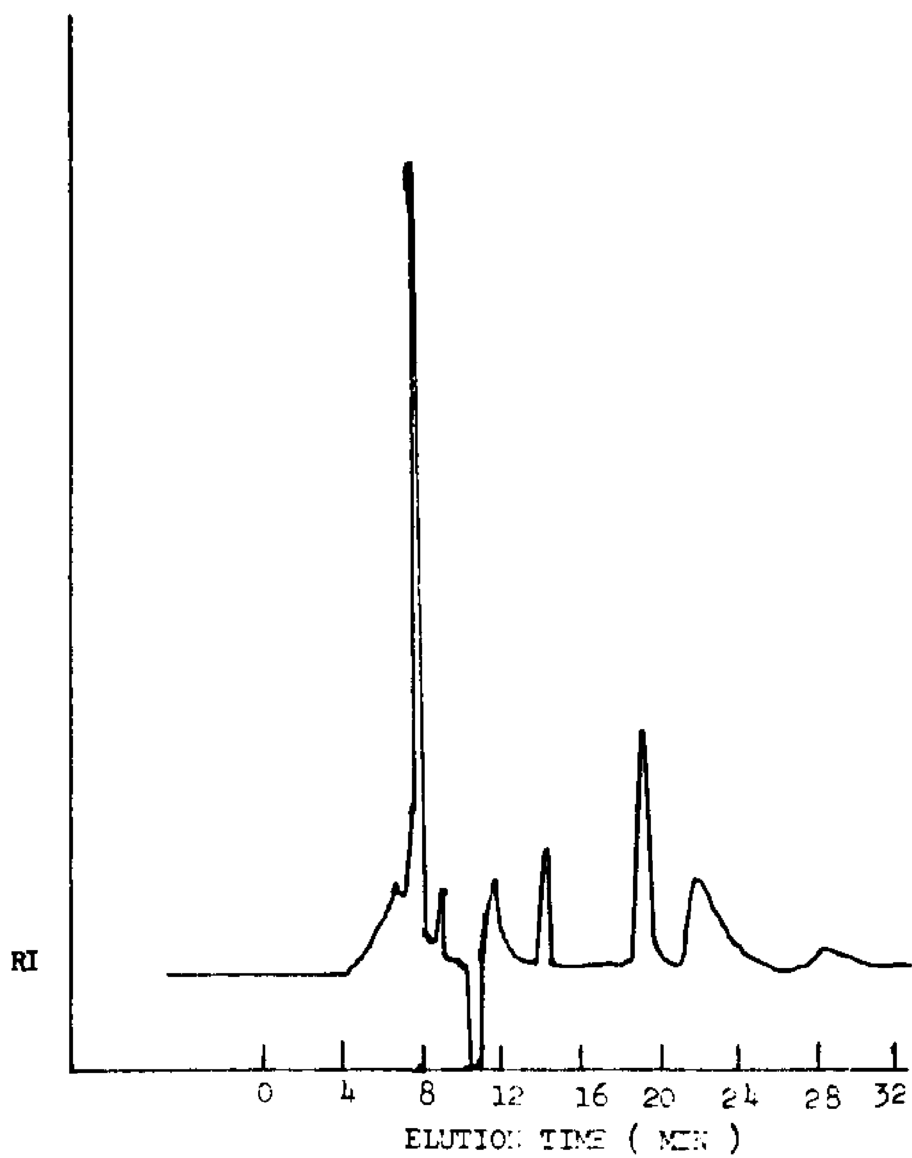


Figure 13.

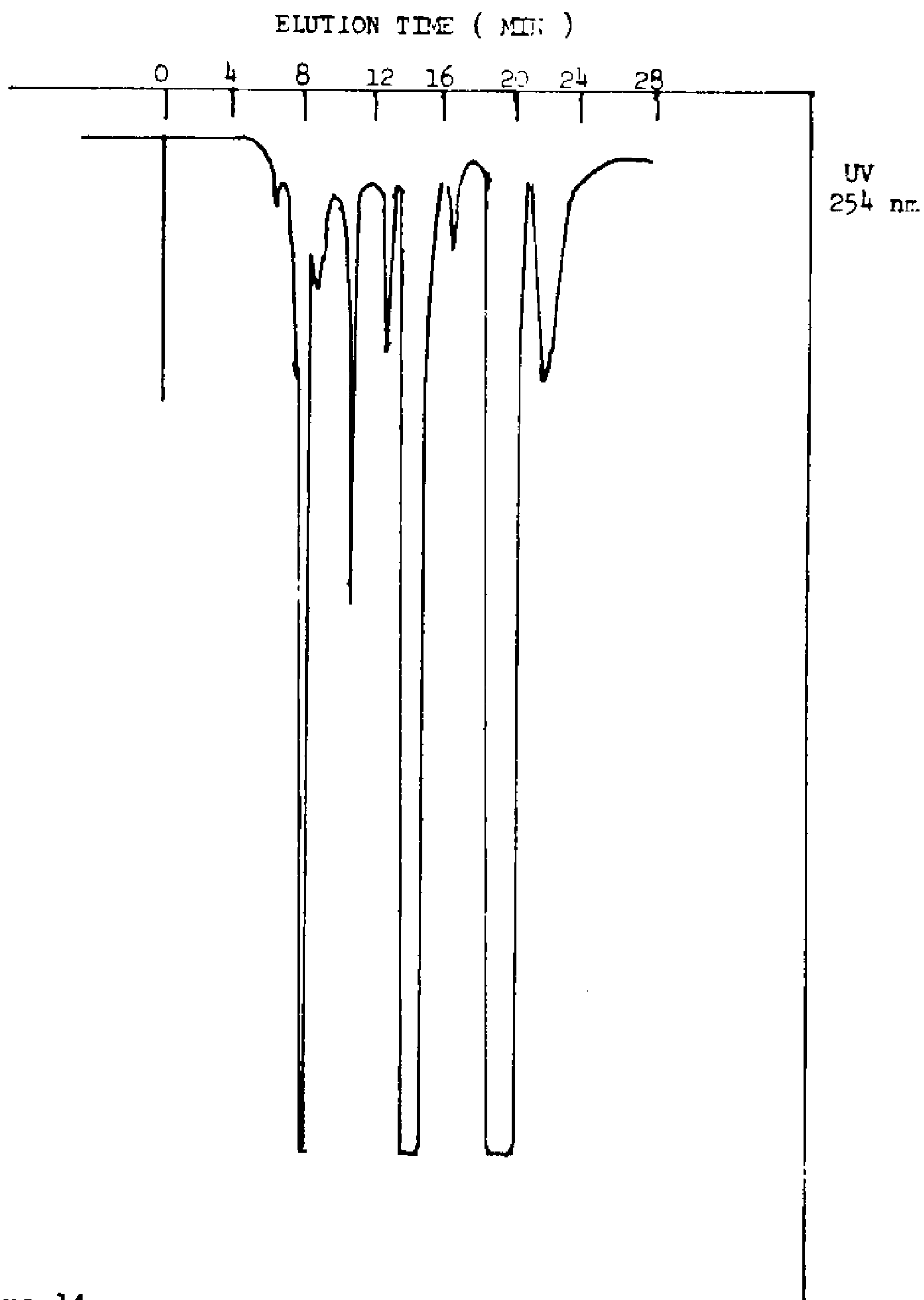


Figure 14.

Figure 15. HPLC of Preparative TLC Fraction 13C

A 0.30 mL volume of a 100 mg/mL aqueous solution of preparative TLC fraction 13C was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 25% methanol in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Fraction 1 had a capacity factor between 0.0-0.5 Fraction 2 was between 0.5-1.2 capacity factor. Fraction 1 and 2 were rechromatographed as described in Figures 16 and 17. Fraction 13C-I had an elution time of 39 min with k' 8.8. Fraction 13C-H had an elution time of 35 min with k' 7.8. Fraction 13C-G had an elution time of 32 min with k' 7.2. Fraction 13C-F had an elution time of 23 min with k' 4.8. Fraction 13C-E had an elution time of 20 min with k' 4.0

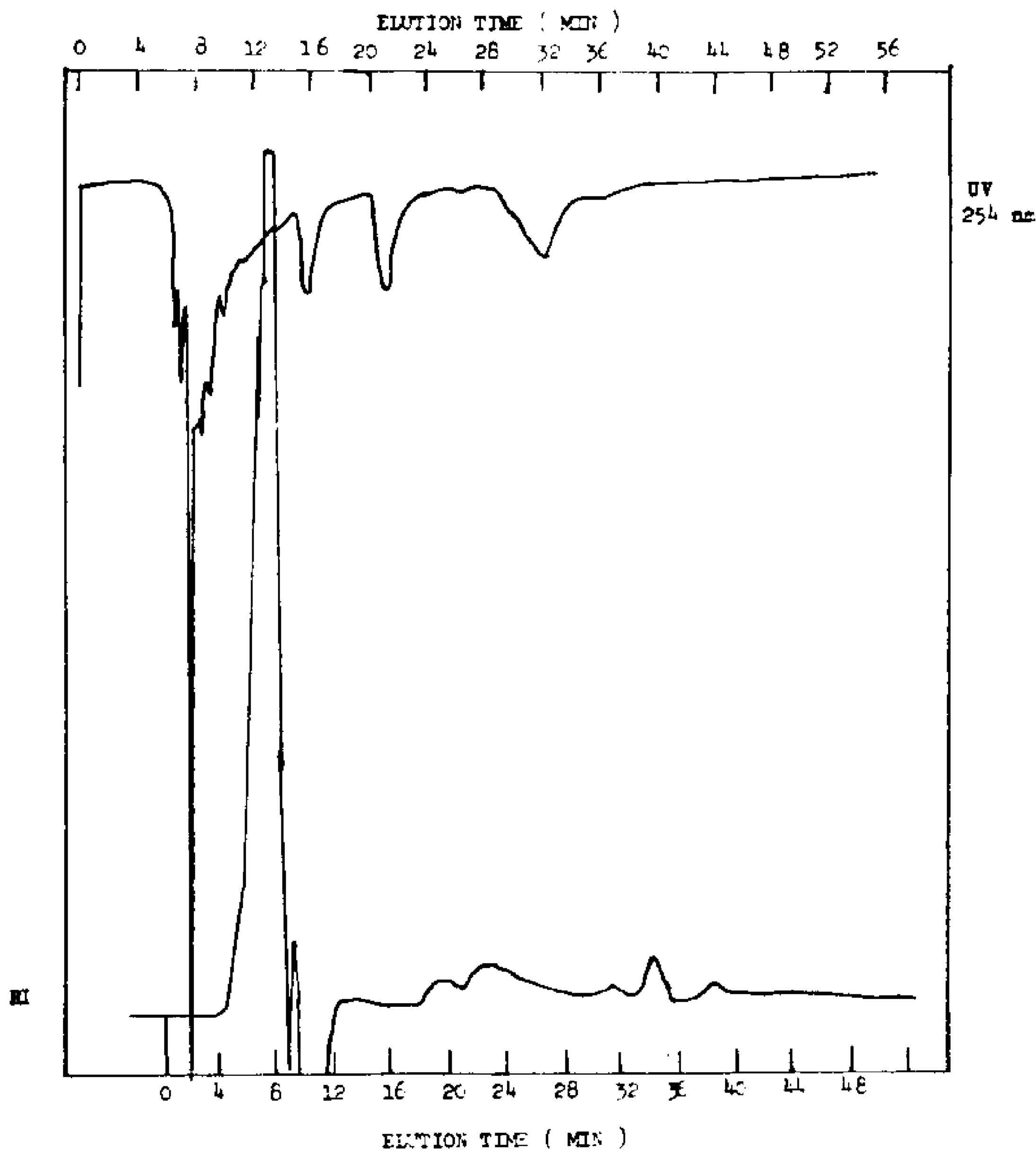


Figure 15.

Figure 16. HPLC Preparative TLC Fraction 13C HPLC Fraction 1

A 0.10 mL volume of a 50 mg/mL 1% acetonitrile in water solution of preparative TLC fraction 13C HPLC fraction 1 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 1% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Homogeneous components and fractions were collected and assayed. Fraction 13C-C had an elution time of 13 min with k' 2.3. Fraction 13C-B had an elution time of 9 min with k' 1.3. Fraction 13C-A had an elution time of 4-8 min with k' 0.0-2.0.

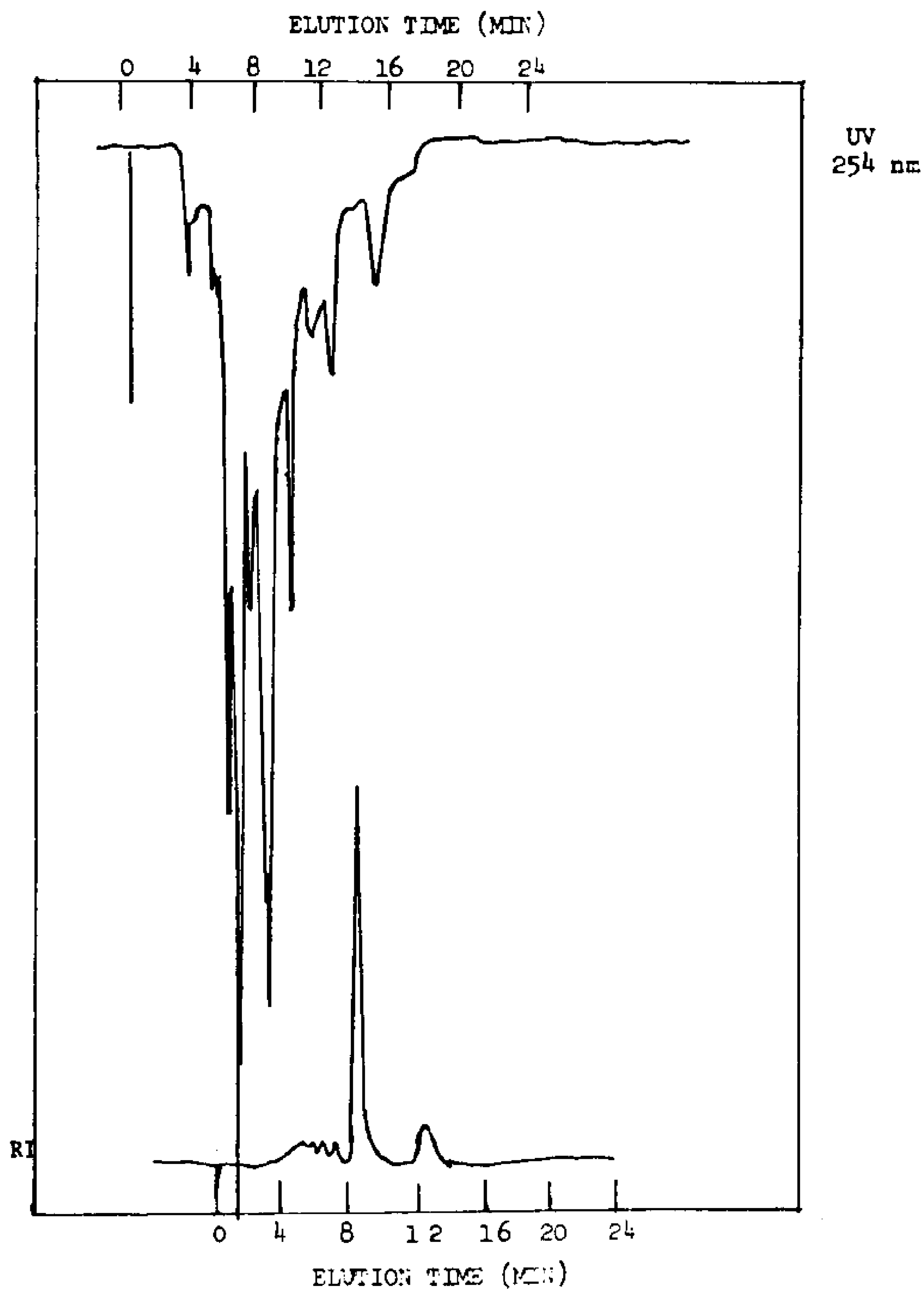


Figure 16.

Figure 17. HPLC of Preparative TLC Fraction 13C HPLC Fraction 2

A 0.10 mL volume of a 30 mg/mL 1% acetonitrile in water solution of preparative TLC fraction 13C HPLC fraction 2 was injected on a 7.8 X 30 cm Waters μBondapak C18 column. The mobile phase was 1% acetonitrile in water at ambient temperature. Flow rate was 1.5 mL/min. Detection was by RI and UV 254 nm. Fractions were collected and assayed. Fraction 13C-D had an elution time of 6-14 min, k' 0.5-2.5.

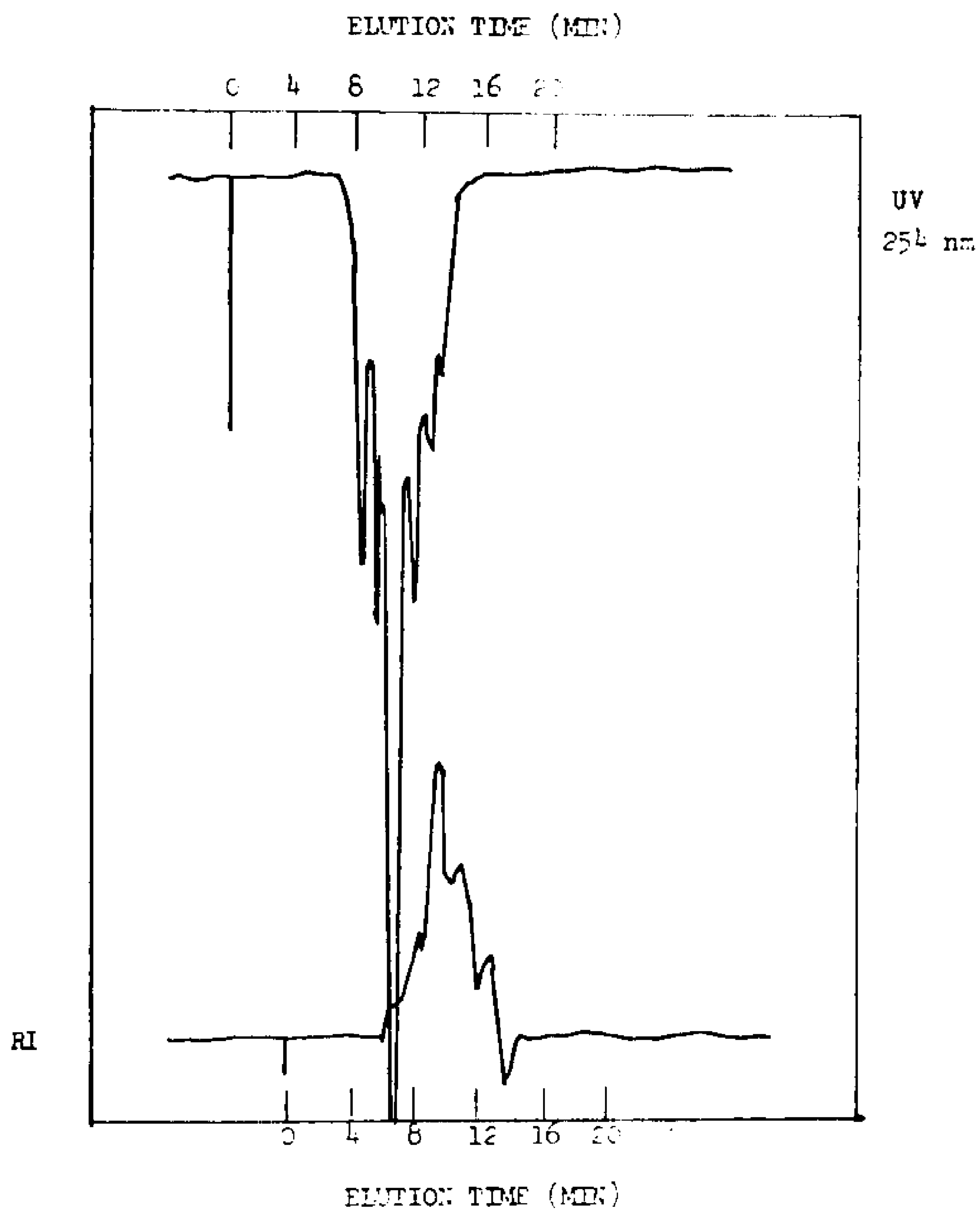


Figure 17.

TABLE 4

DISTRIBUTION OF ANTINEOPLASTIC ACTIVITY IN THE LOW MOLECULAR WEIGHT FRACTION[a]

Fraction	Capacity Factor[a]	% Inh. at Assay Conc. 0.100	0.020 (mg/mL)	0.004	Homogeneous
5A	0.0-0.8	0	0	0	-
5B	1.3	60	35	10	+
5C	1.9	0	0	0	+
5D	2.2	0	0	0	+
5E	3.3	35	5	0	+
5F	4.5	0	0	0	+
5G	6.0	0	0	0	+
6A	0.0-0.5	0	0	0	-
6B	0.8	0	0	0	-
6C	1.3	100	20	0	+
6D	1.8	30	10	0	+
6E	2.0-3.0	0	0	0	-
6F	2.5	0	0	0	+
6G	3.8	20	5	0	+
7A	0.0-0.5	0	0	0	-
7B	0.5-1.0	0	0	0	-
7C	1.3	0	0	0	+
7D	2.5	35	10	0	+
7E	3.3	45	20	10	+
7F	3.8	90	70	45	+
13C-A	0.0-2.0	100	75	30	-
13C-B	1.3	0	0	0	+
13C-C	2.3	0	0	0	+
13C-D	0.5-2.5	100	100	25	-
13C-E	4.0	0	0	0	+
13C-F	4.8	0	0	0	+
13C-G	7.2	0	0	0	+
13C-H	7.8	50	25	10	+
13C-I	8.8	0	0	0	+

[a]The capacity factor is calculated for eluate compositions where the homogeneous compound was obtained or for the last fractionation as shown in Figures 5-16.

3.4.7 Characterization of the Major Low Molecular Weight Antineoplastic Principle

The major low-molecular weight, active principle was identified as thymidine. The yield was 0.006%, based on g of wet liver. The following physical properties agreed with literature values:[25] m.p. 185°C $[\alpha]_{D}^{25}$ +30.5^{o} UV max (pH 7.2 0.01M phosphate buffer) was at 206 nm ε=9.8 X 10^{3}; 267 nm ε = 9.7 X 10^{3}. The ID_{50} of 1.6 X 10^{-5}M is identical to that of standard thymidine assayed in our laboratory in the L1210 lymphocytic leukemia tissue culture assay, and agrees with the literature value.[26]

The ^{1}H-NMR spectrum at 200 MHz in D_2O is shown in Figure 18 with the values listed in Table 5.

The H-coupled and H-decoupled ^{13}C-NMR spectra are shown in Figures 19 and 20 respectively. The values are listed in Table 6. The electron impact mass spectral data are presented in Table 7. The infrared spectra (KBr disc) showed a strong, broad peak at 2.9-3.4 μm, a strong intensity peak at 5.9 μm, and peaks of moderate intensity at 6.5 μm, 7.1 μm 7.8 μm, 9.2 μm, 9.5 μm, 10.4 μm, and 13.1 μm.

The ^{1}H-NMR, ^{13}C-NMR, and electron impact mass spectra are in agreement with the literature.[27-29] The ^{1}H-NMR assignments are based on literature values and were confirmed by off-resonance decoupling when applicable.[27] The ^{13}C-NMR was

assigned by correlation with the published values.[28] The major ions in the mass spectrum arise from the fragmentation pattern exhibited in Figure 21.[29]

Figure 18. Thymidine ^{1}H-NMR

The ^{1}H-NMR spectrum at 200 MHz of a 2.0 mg/mL D_2O solution of thymidine was determined. The sample was scanned 40 times at a scan width of 3521 Hz. The reference was external Me_4Si.

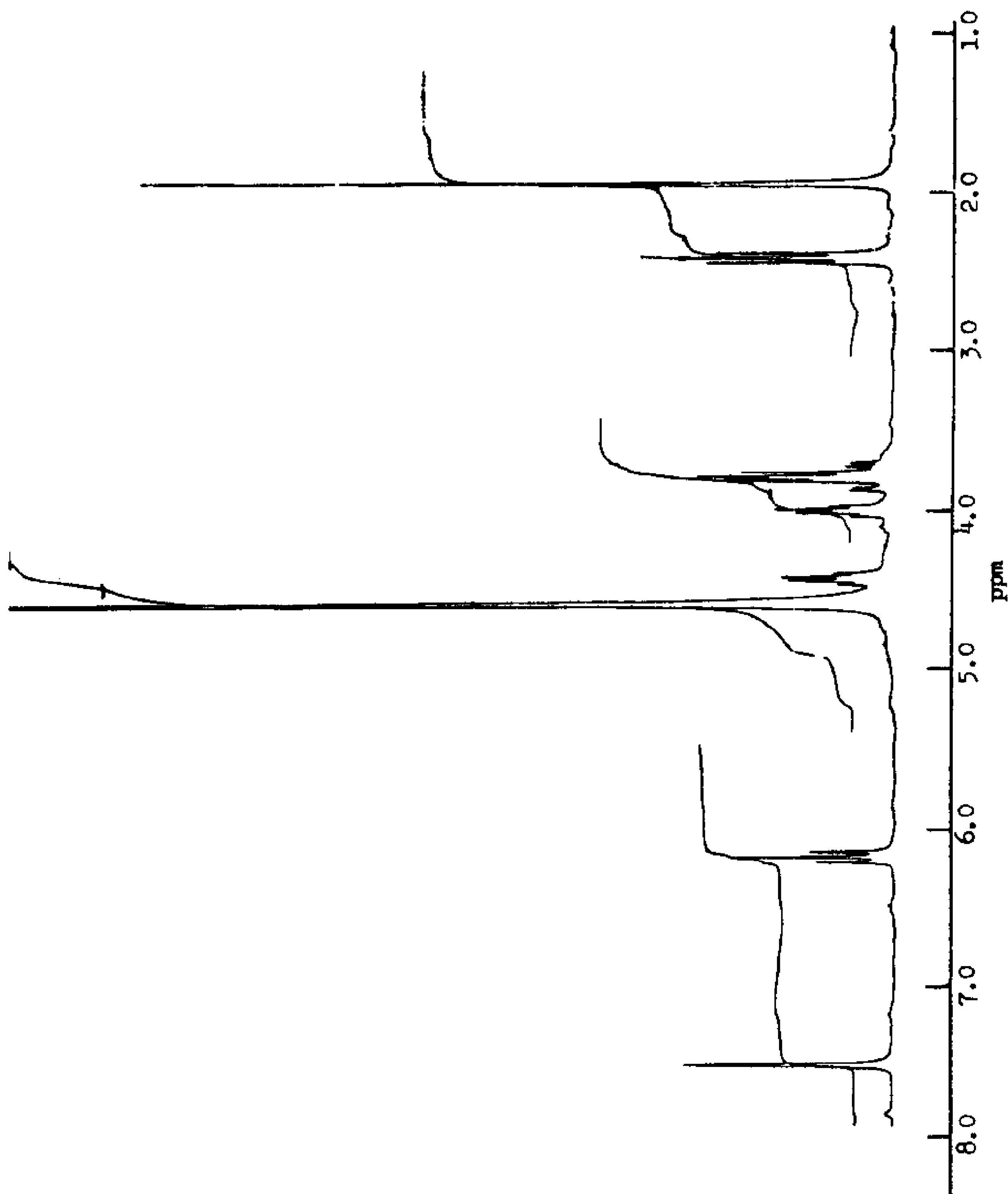


Figure 18.

TABLE 5

THYMIDINE ^{1}H-NMR

H	Shift (ppm)	Multiplicity	Integral
CH_3	1.91	singlet	3
H-2'	2.40	doublet-doublets	2
H-5(S)'	3.80	doublet-doublets	1
H-5(R)'	3.35	doublet-doublets	1
H-4'	4.03	doublet-doublets	1
H-3'	4.49	doublet-doublets	1
H-1'	6.28	triplet	1
H-6	7.65	singlet	1

Figures 19-20. Thymidine ^{13}C-NMR

The ^{1}H-coupled and ^{1}H-decoupled ^{13}C-NMR spectra at 50.3 MHz of a 4.0 mg/mL D_2O solution of thymidine were determined. The reference was Me_4Si. The ^{1}H-coupled ^{13}C-NMR spectrum is presented in Figure 19. The sample was scanned 120,150 times. The ^{1}H-decoupled ^{13}C-NMR spectrum is presented in Figure 20. The sample was scanned 75,000 times.

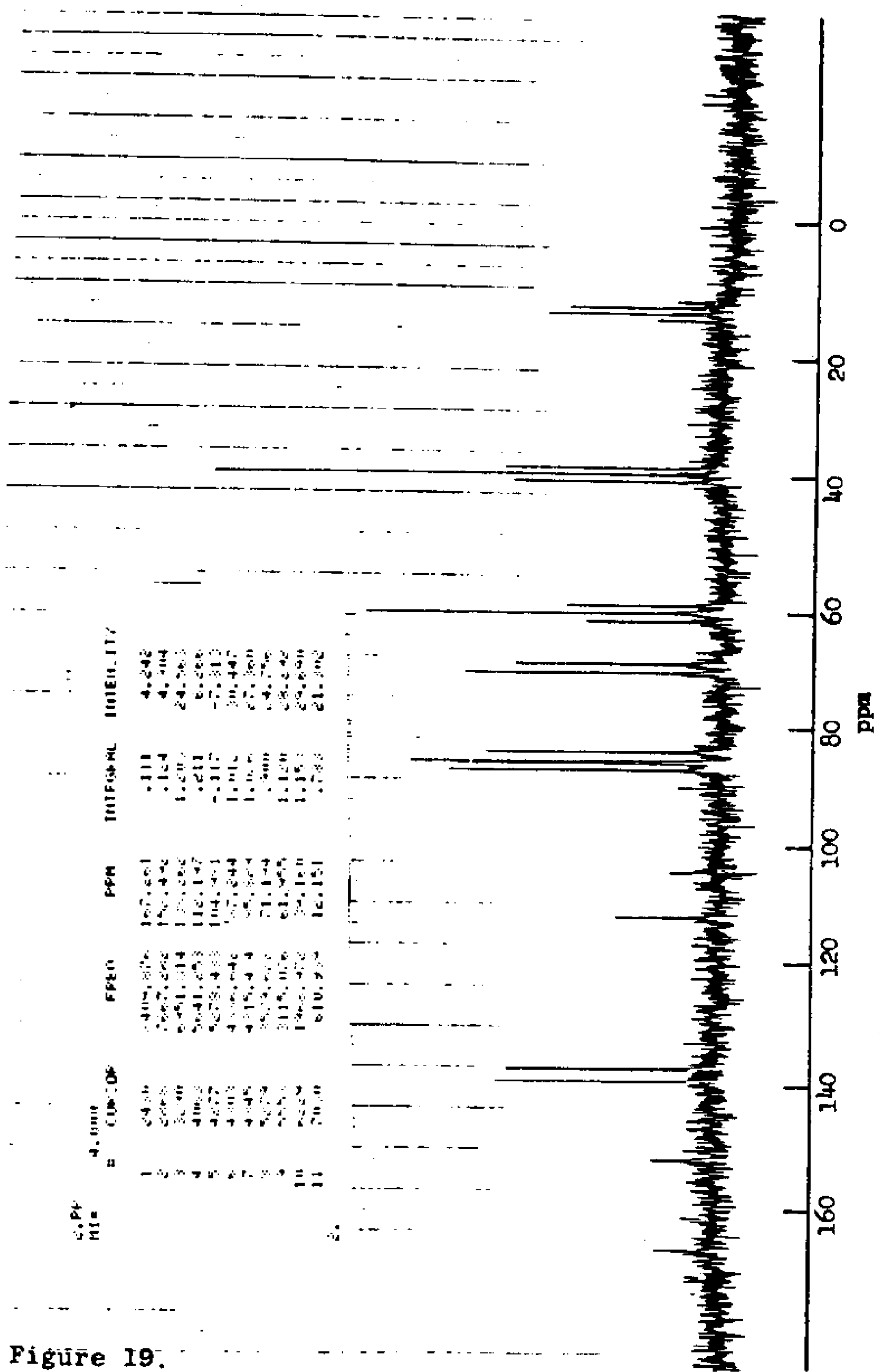


Figure 19.

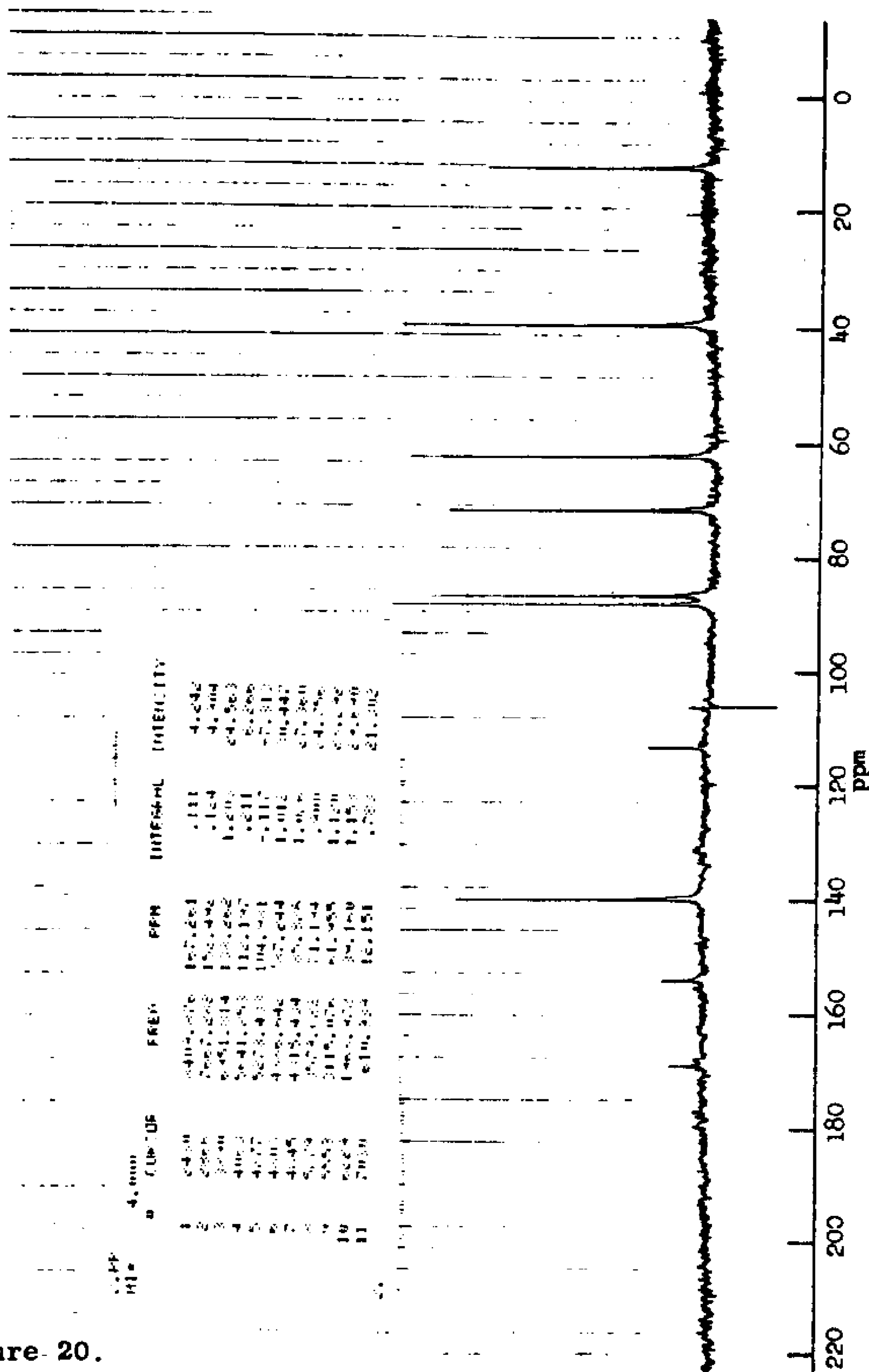


Figure 20.

TABLE 6

THYMIDINE ^{13}C-NMR

C	Shift (ppm)	C-H
CH3	12.151	3
C-2'	39.160	2
C-5'	61.955	2
C-3'	71.194	1
C-4'	85.828	1
C-1'	87.244	1
C-5	112.197	0
C-6	138.262	1
C-2	152.492	0
C-4	167.261	0

Figure 21. Thymidine Fragmentation Pattern

The major fragmentation pattern for the Thymidine Electron Impact Mass Spectrum is Illustrated.

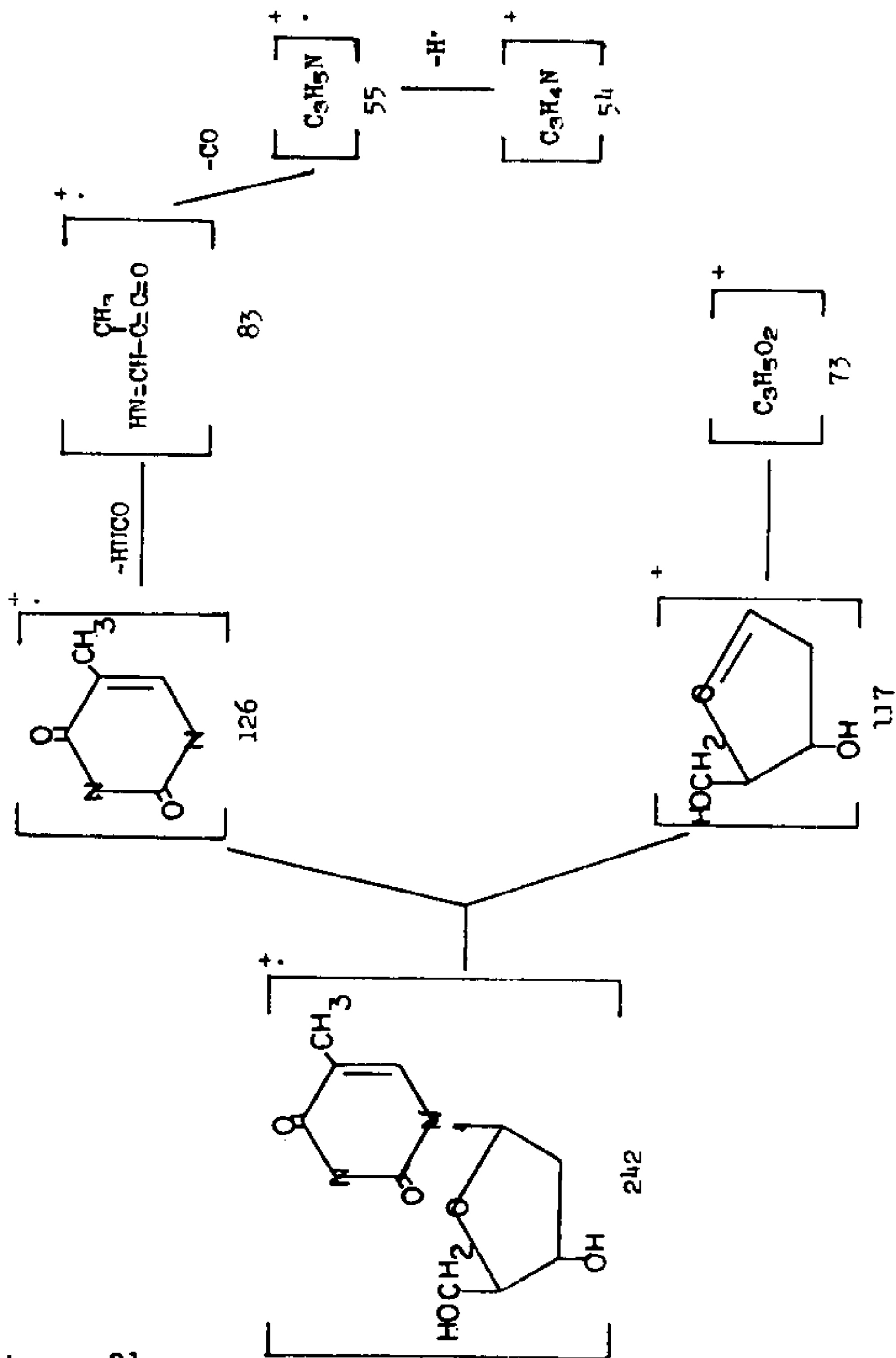


Figure 21.

TABLE 7

THYMIDINE MASS SPECTRAL DATA

m/z	Relative Intensity	m/z	Relative Intensity
40	11.0	85	3.5
41	19.5	86	4.5
42	9.5	87	2.5
43	20.5	90	2.5
44	30.0	91	5.0
45	45.0	96	2.5
47	2.5	97	6.0
51	3.5	98	10.0
52	6.5	99	21.5
53	7.5	108	2.0
54	18.5	109	2.5
55	30.0	110	13.5
56	5.0	116	2.5
57	10.5	117	100.0
60	3.5	120	3.0
67	4.0	126	90.0
68	5.5	128	3.0
69	14.0	132	4.5
70	5.0	138	9.0
71	15.0	139	2.0
73	45.0	147	2.0
74	8.5	149	2.0
75	3.5	150	3.5
80	6.0	152	2.0
81	6.0	153	6.5
82	6.0	154	3.7
83	10.0	161	3.5
84	3.5	242	10.0

3.5 Discussion

The antineoplastic activity of thymidine has been established in a large number of laboratories.[30-50] It has recently entered clinical trials as a chemotherapeutic agent.[30-32] Thymidine has shown activity in clinical trials with patients with leukemia and lymphoma.[30] Activity versus T-cell leukemia and myelogenous leukemia cells has been demonstrated. Patients with B-cell lymphoma have not shown a significant response. The toxicity expressed was primarily related to the central nervous system, gastrointestinal tract, and bone marrow. Overall, the toxic effects were tolerable and non-life threatening.[30] Studies have also been initiated on patients with a variety of hematologic malignancies and solid tumors.[31-32]

Lee has demonstrated the regression of human tumor xenografts established in nude mice after treatment with thymidine.[33] The growth of human melanoma, teratocarcinoma, lung carcinoma and breast carcinoma has been inhibited by continuous infusion by thymidine in mice. Complete regression of 60% of the melanomas and teratocarcinomas occurred. In 67% of the cases of lung carcinoma, complete regression occurred. In all cases a significant reduction of growth rate was observed. In a separate study, human melanoma and

colon carcinoma have exhibited reduced growth and regression in nude mice.[34] The *in vivo* antitumor effects have been confirmed in other human tumor xenograft models.[35-36]

Harris,[37] Zielke,[38] Eidinoff,[39] have demonstrated the antineoplastic effect of thymidine on human acute lymphoblastic leukemia *in vitro*. Harris has shown the inhibition of T-lymphoma cells *in vitro*.[37] Interestingly, the growth inhibition of the SK-17 cell line, a human lymphoma derived from B-lymphocytes is markedly less.[40] Human colonic cancer cell growth is inhibited *in vitro* as demonstrated by Tsuboi.[41] Lazarus proved the *in vitro* sensitivity of cultured human leukemia lymphocytes.[42]

Apple described growth inhibition of murine tumors by thymidine.[43] Lowe proved the effectiveness against the mouse L1210 lymphocytic-leukemia cell line *in vitro* and *in vivo*.[44] Reiter[45] and Stadecker[46] demonstrated the inhibition of EL4 thymoma tumors in mice *in vitro* and *in vivo*. The growth of mouse neoplastic mesenchymal cells are inhibited, as shown by Lee.[34]

The antineoplastic activity of thymidine is due to the inhibition of DNA synthesis when cells are exposed to excess amounts. The excess thymidine is converted to thymidine

triphosphate by thymidine kinase. Thymidine triphosphate exerts end-product feedback inhibition of ribonucleotide reductase. This causes depletion of the deoxycytidylate triphosphate pool used in DNA synthesis by inhibiting the conversion of cytidylate triphosphate to deoxycytidylate triphosphate by ribonucleotide reductase. This is t metabolic mechanism considered responsible for the inhibition of growth of normal and neoplastic cells.[33-34] The increased sensitivity of the neoplastic cells is due to the fact that when exposed to excess thymidine the neoplastic cells continue to replicate, whereas the normal cells go into a period of growth arrest.[42-47-48-49] The cytotoxic effects of thymidine are expressed during the repl: cation and the neoplastic cells die. The inhibition can be blocked by adding deoxycytidylate, which removes the metabolic block.[50] It is not known why the neoplastic cells do not enter into a period of growth arrest.

CHAPTER 4. ISOLATION AND CHARACTERIZATION OF THE HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE

4.1 Introduction

Isolation attempts by Prescott[21] and Horton[8] have indicated the presence of a relatively high molecular weight antineoplastic agent as previously described. Prescott obtained an active fraction consisting of carbohydrate, protein, and unidentified material. The active principle was non-dialyzable, heat stable to 37°C at an unspecified pH, and had a molecular weight of 8000-10000. The antineoplastic agent lost activity when heated to 100°C. Prescott's attempted isolation of Paolin 1 and Paolin 2 from oysters and abalone respectively, yielded an active fraction with similar properties.[18-20] However, the antibiotic and antiviral activities were stable when the fraction was heated to 95°C for 45 min at an unspecified pH. The possibility exists that the activities are expressed by the same agent. The presence of nucleic acids could not be demonstrated in any active fraction. These studies suggest that the high molecular weight antineoplastic principle may be a protein or polysaccharide that can express the activity itself or act as

a carrier for a low molecular weight, active principle.

4.2 Methods

4.2.1 Protein Quantitation

The Bio-Rad Protein Assay was used to determine protein content.[51] The assay is based on the shift of absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 from 465 to 595 nm when binding to protein occurs.[52] A series of standard solutions of bovine gamma globulin of 0.2 mg/mL to 1.4 mg/mL were prepared. Into a dry test tube, 0.1 mL of standard or sample was pipetted. The blank consisted of 0.1 mL sample buffer. To the test tube, 5.0 mL of diluted dye reagent was added and the solution vortexed for several seconds. After 30 min, the OD_{595} was measured versus reagent blank. The OD_{595} was plotted versus standard concentration and the concentration of the sample determined from the resulting graph.

4.2.2 SDS Polyacrylamide Disc-Gel Electrophoresis

SDS Polyacrylamide disc-gel electrophoresis was performed according to the procedure of Fairbanks.[53-54] A homemade disc-gel electrophoresis apparatus patterned after that of Rodgers[55] with a Gelman model 38201 power supply was employed.

Bio-Phore 7.5% and 12% Gels were used. The sample was dissolved in a buffer of 0.0205M Tris, 0.0205M acetic acid, 0.04M 1,4-dithiothreitol, 01% (w/v) SDS, pH 6.6 and heated at 100°C for 10 min. To 25-80 μg of protein in a volume of 5-25 μL were added one-half the volume of Bio-Phore neutral and basic tracking solution. The resulting solution was vortexed for several seconds and layered on the gel by a syringe. The solution was carefully covered by the buffer used in both the upper and lower chambers: 0.205M Tris, 0.205M acetic acid, 0.04M 1,4-dithiothreitol, 0.1% (w/v) SDS, pH 6.6. The sample was run into the gel at 4 mA/tube. The remainder of the run was carried out at 7 mA/tube for a period of 6-7 h. The gels were removed from the tube by shattering the glass. Gels were prepared for protein staining by fixing for 1 h in an aqueous solution containing 40% (v/v) isopropyl alcohol and 10% (v/v) acetic acid in water. The gel was stained overnight with 0.05% (w/v) Coomassie Brilliant Blue 4-250 in 7% (v/v) aqueous acetic acid. Alternatively, the gel was prepared for glycoprotein staining by fixing for 1 h in 12.5% (w/v) TCA, reacting for 1 h with 1% (w/v) periodic acid in 3% (v/v) acetic acid, quenching for 0.5 h in 5% (w/v) potassium metabisulfite, and staining for 4 h in 0.5% (w/v) Alcian Blue in 3% (v/v) acetic acid in

water. All gels were destained overnight with an aqueous solution of 10% (v/v) acetic acid: 10% (v/v) isopropyl alcohol.

For molecular weight determinations, the gels were calibrated with Bio-Rad Low Molecular Weight Electrophoresis Standards. A standard curve of relative mobility versus logarithm of the molecular weight was constructed and the molecular weight of the sample determined from the graph.

4.2.3 Molecular Weight Determination by Gel-Permeation Chromatography

A 1.6 X 70 cm column of Sephacryl S-200 Superfine was calibrated by globular protein standards from the Pharmacia Gel Filtration Calibration Kit. The buffer employed was 0.05M Tris pH 7.4. Detection was by UV 254 nm. The void volume was determined by use of Blue Dextran 2000. The calibration curve was prepared by measuring the elution volumes of the protein standards, calculating the corresponding K_{av} values, and plotting the K_{av} values versus the logarithm of their molecular weight. The molecular weight of the sample of interest was determined from the calibration curve once its K_{av} value was calculated from the measured elution volume. The K_{av} is calculated in the following manner:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Where: V_e = elution volume for the sample

V_o = void volume

V_t = total bed volume

4.2.4 Protein Amino Acid Analysis

Samples were prepared for protein amino acid analysis in the following manner:

A 1 mg sample of lyophilized protein was placed into a 16 X 125 mm heavy-walled Pyrex culture tube that had been adapted for a vacuum connection and was necked at the middle to flame seal. The protein was suspended in 2.0 mL of 6N HCl. The sample was frozen in an isopropyl alcohol-Dry Ice bath and connected to the vacuum line. The system was evacuated with an oil pump to 60 microns and the sample was withdrawn from the bath and allowed to thaw. This removed traces of dissolved air from the solution. The tube was then flame sealed under vacuum. The hydrolysis was conducted in an oven at 115°C for 20 and 70 h. The hydrozylate was quantitatively transferred to a round-bottomed flask and evaporated under diminished pressure at 45°C on a rotovap. The residue was dissolved in 0.20N sodium citrate buffer pH 2.20, containing

0.1% phenol and 0.2% thiodiglycol. The sample was filtered to remove any particulate matter and injected for analysis.

4.3 Instruments and Equipment

The following instruments and equipment were used.

Beckman Automatic Amino Acid Analyzer Model 119

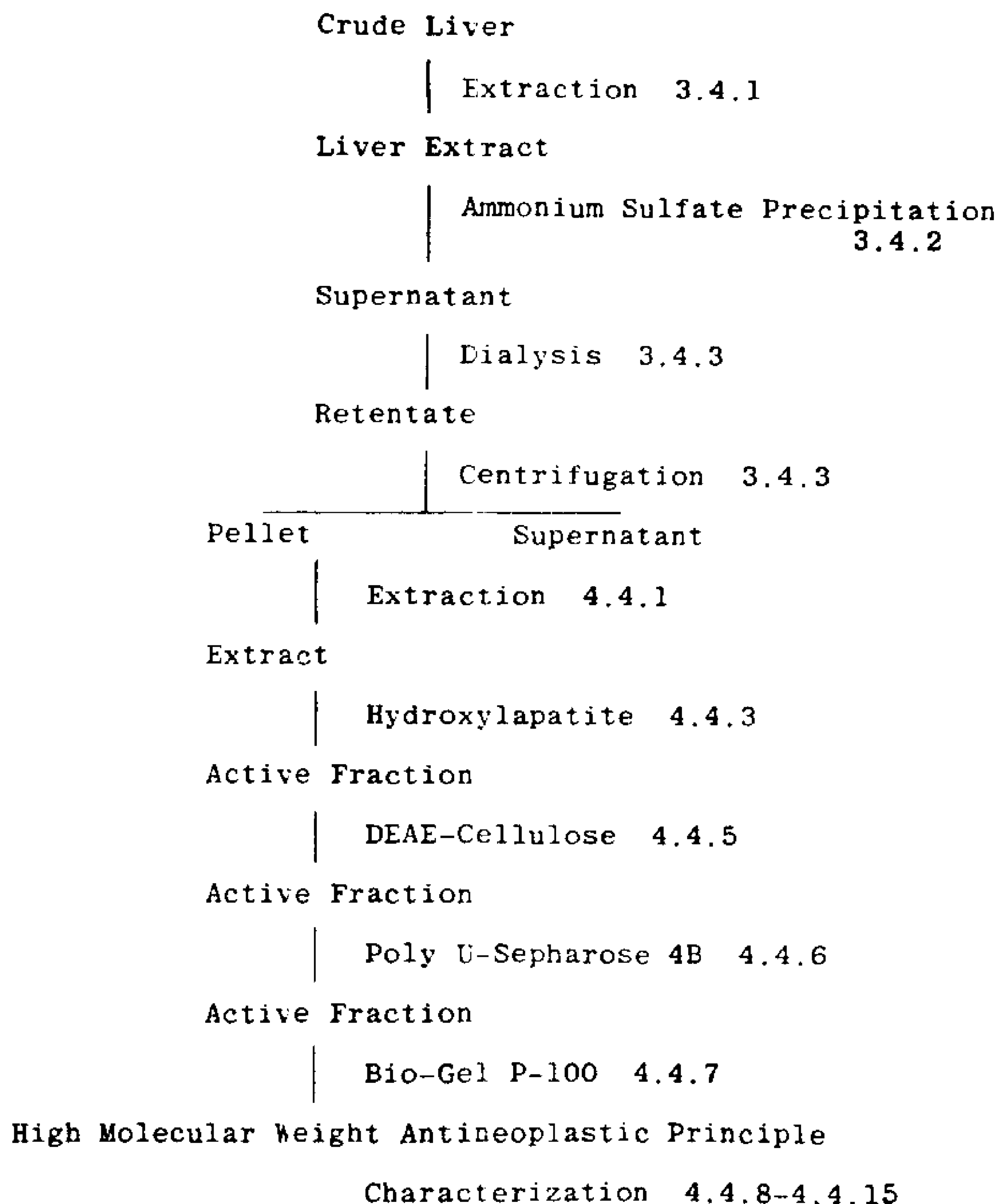
UV Cary Spectrophotmeter

UV Detector Pharmacia UV

Centrifuge Servall Refrigerated-Automatic
Rotor SS-34

Figure 22. Flow Chart of the Isolation Scheme for the Major High Molecular Weight Antineoplastic Principle

The isolation scheme for the high molecular weight antineoplastic principle is summarized. The section of the text for each individual fractionation and characterization step is indicated next to that step.


Crude Liver
Extraction 3.4.1
Liver Extract
Ammonium Sulfate Precipitation 3.4.2
Supernatant
Dialysis 3.4.3
Retentate
Centrifugation 3.4.3
Pellet
Supernatant
Extraction 4.4.1
Extract
Hydroxylapatite 4.4.3
Active Fraction
DEAE-Cellulose 4.4.5
Active Fraction
Poly U-Sepharose 4B 4.4.6
Active Fraction
Bio-Gel P-100 4.4.7
High Molecular Weight Antineoplastic Principle
Characterization 4.4.8-4.4.15

4.4 Isolation and Characterization of the High Molecular Weight Antineoplastic Principle

The isolation scheme is summarized in Figure 22.

4.4.1 Extraction of the High Molecular Weight Principle

The extraction of the activity from the retentate pellet was investigated in several systems to optimize recovery of the activity. The following general procedure was used:

In a 100-mL beaker, 100 mg of lyophilized retentate pellet was suspended in 40 mL of the specified aqueous solution at 4°C in 0.01M sodium phosphate buffer pH 6.8 or 0.05M Tris buffer pH 7.4. The suspension was stirred at 4°C for 8 h. Aliquots were taken at the specified times. The aliquots and the final suspension were centrifuged at 4°C, 5000 RPM, for 30 min. The supernatant and the final pellet were dialyzed versus distilled water for 24 h at 4°C, lyophilized, and assayed. The control was extraction with 0.01M sodium phosphate buffer pH 6.8 at 4°C. The results are presented in Table 8. PBS is Dulbecco's formula phosphate buffered saline. For extraction at pH 2.0, an aqueous HCl solution was prepared by dropwise addition of HCl to the desired pH.

Phosphate buffered saline, salts, sonication, and chaotropic ions appear to have little effect in solubilizing the activity. Repeated extraction with control and extraction with 0.2% SDS were effective in solubilizing the activity. Solubilization of the active principle after extraction from the retentate pellet occurred completely at pH 6.8 or pH 7.5 at subsequent purification steps in the buffer systems previously described. The crude extract is stable at pH 2.0 in aqueous HCl. A relatively large percent of the activity is solubilized under these conditions. Solubilization can be decreased by addition of 0.01M UMP to the buffer before extraction. For preparative work, repeated extraction with 0.01M sodium phosphate buffer pH 6.8 at 4°C was used in a slightly revised procedure. In a 600-mL beaker, 1.5 g of the retentate pellet was suspended in 200 mL of 0.01M sodium phosphate buffer pH 6.8 at 4°C. The suspension was stirred at 4°C for 6 h and centrifuged as before. The resulting pellet was reextracted with 100 mL of 0.01M sodium phosphate buffer pH 6.8 at 4°C for 6 h and centrifuged as previously described. The supernatants were combined and dialyzed for 24 h at 4°C versus distilled water, lyophilized, and stored at -20°C or used directly in the subsequent fractionation step on hydroxylapatite. The extraction yielded 130 mg of product with ID_{50} 0.20 mg corresponding to 620 units of total activity.

TABLE 8

EXTRACTION OF THE HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE[a]

Solution	pH	% Inh. (0.20 mg/mL) 1h	2h	4h	8h	Pellet	% wt Supernatant
PBS	7.4	75	50	-	35	100	15
15% NaCl	6.8	80	50	30	10	65	10
15% NaCl Sonicated	6.8	30	50	40	55	55	10
$NaClO_4$ 0.10M	7.4	30	20	-	20	90	10
SDS 0.2%	6.8	50	70	-	60	10	25
HCl	2.0	100	100	-	100	35	10
HCl 0.01M UMP	2.0	100	70	-	70	100	10
Control	6.8	70	70	-	80	70	10

[a]Experimental conditions are described in the text.

4.4.2 Fractional Precipitation of the Retentate Pellet Extract

After extraction of the retentate pellet, it was hoped that fractional precipitation by ammonium sulfate would serve as a means of further purification. Into a centrifuge tube, 100 mg of lyophilized retentate pellet was added and solubilized by addition of 30 mL of 0.05M sodium phosphate buffer pH 6.8 at $4^{o}C$. Solid ammonium sulfate was added to the solution to yield the desired concentration. The solution was stirred at $4^{o}C$ for 20 min and centrifuged at 5000 RPM for 30 min at $4^{o}C$. The supernatant fluid was transferred to another centrifuge tube and the pellet was dialyzed for 12 h versus distilled water at $4^{o}C$, lyophilized, and assayed. The precipitation was repeated at the next ammonium sulfate concentration in the same manner. The final supernatant fluid was dialyzed for 24 h versus distilled water at $4^{o}C$, lyophilized, and assayed. The results are presented in Table 9. The activity was precipitated over a wide range of ammonium sulfate concentrations. It was concluded that fractional precipitation by ammonium sulfate was not satisfactory as a means of purification.

TABLE 9

AMMONIUM SULFATE FRACTIONAL PRECIPITATION OF THE RETENTATE PELLET EXTRACT[a]

% Ammonium Sulfate	% Inh.(0.10 mg/mL)	% Recovery
0	45	-
10	0	15
40	70	25
50	50	15
60	20	15
70	60	30
Final Supernatant	0	5
15	0	20
35	30	5
45	35	10
55	55	25
65	45	25
85	45	15
Final Supernatant	0	1

[a]Experimental conditions are described in the text.

4.4.3 Hydroxylapatite Adsorption and Column Chromatography

The adsorption of the high molecular weight principle onto hydroxylapatite was investigated in the following manner. Into a centrifuge tube, 100 mg of retentate pellet extract was added and solubilized by addition of 50 mL of 0.05M sodium phosphate buffer pH 6.8 at 4°C. Solid hydroxylapatite, Bio-Gel HTP, was added to the desired weight and the centrifuge tube gently agitated by hand for 10 minutes at 4°C. The suspension was centrifuged at 4000 RPM for 5 minutes at 4°C. An aliquot of the supernatant fluid was taken and dialyzed for 12 hours versus distilled water at 4°C, lyophilized, and assayed. The procedure was repeated to the final weight of hydroxylapatite added. After the final addition of hydroxylapatite and subsequent centrifugation, the supernatant fluid was dialyzed as before, lyophilized, and assayed. The hydroxylapatite pellet was extracted with 50 mL of 1.0M potassium phosphate buffer pH 6.8 at 4°C as described before. The supernatant fluid was dialyzed for 24 hours versus distilled water at 4°C, lyophilized, and assayed. The recovery of total activity was 95%. The results are presented in Table 10. The activity can be adsorbed completely onto the hydroxylapatite and eluted quantitatively. The active principle is stable under the conditions employed. The

experiment was repeated at 18°C with similar results. It was concluded that hydroxylapatite column chromatography could be employed for preparative work. Hydroxylapatite column chromatography was performed in the following manner:

A 1.6-cm diameter column was packed to a height of 10 cm with Bio-Gel HT on a 0.5 cm bed of Sephadex G-25. The Sephadex G-25 bed improved the flow rate, as the fine hydroxylapatite particles tend to tightly pack at the end of the column if not supported. Before being used, the column was washed with 1.0M potassium phosphate buffer pH 6.8 and equilibrated with 0.01M potassium phosphate buffer pH 6.8. To 130 mg of lyophilized extract, 20 mL of the equilibration buffer was added. The solution was loaded onto the hydroxylapatite column and eluted stepwise with potassium phosphate buffer pH 6.8 from 0.01M to 1.0M. Each fraction was dialyzed 24 hours at 4°C versus distilled water, lyophilized, and assayed. The results are presented in Table 11. For routine preparative work, the extract supernatant fluid was loaded directly onto the hydroxylapatite column and eluted stepwise with 0.01M-0.05M-1.0M potassium phosphate buffer pH 6.8. All hydroxylapatite column chromatography was performed at 15°C. The activity was eluted from the hydroxylapatite column at 0.05M buffer concentration. For 130 mg of extract, the yield was 30 mg in the 0.05M eluant fraction with ID_{50} 0.11 mg corresponding to 318 units of total activity. Recovery was 51% of the activity applied.

No synergistic relationship between all the combinations of the eluant fractions could be established. This indicated that no loss of a reversible cofactor occurred during hydroxylapatite fractionation.

Since extraction with 0.2% SDS was effective, SDS hydroxylapatite column chromatography was investigated as a purification method. To extract 1.5 g of the retentate pellet, 50 mL of 0.2% SDS 0.01M potassium phosphate buffer pH 6.8 at 4°C was used. The extraction was performed as previously described. The extract supernatant fluid was loaded directly onto the hydroxylapatite column and eluted stepwise at 4°C with 0.01M-0.25M potassium phosphate buffer pH 6.8 at 15°C. Each fraction was collected, dialyzed for 36 h at 4°C versus distilled water, lyophilized, and assayed. The results are presented in Table 12. The 0.20 M fraction contained the majority of the activity but activity was present in all fractions. SDS exhibited an ID_{50} 0.04 mg in the assay system. Apparently, the tightly bound SDS can not be easily removed. No attempts to remove the SDS were employed when SDS was shown to be significantly active. It was concluded that SDS hydroxylapatite fractionation was not appropriate because of the intrinsic criticism of adding activity to the isolation matrix.

TABLE 10

HYDROXYLAPATITE ADSORPTION OF THE RETENTATE PELLET EXTRACT[a]

Fraction	Hydroxylapatite(g)	Supernatant %Inh (0.25 mg/mL)
Retentate	0.00	85
Pellet Extract	0.20	65
	0.50	40
	1.00	30
	1.50	25
	2.00	10
	2.50	0
Potassium Phosphate Eluant 1.0M		80

[a]Experimental conditions are described in the text.

TABLE 11

HYDROXYLAPATITE COLUMN CHROMATOGRAPHY OF THE HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE[a]

Eluant Molarity	% Inh (0.20 mg/mL)	%wt Recovery
0.01	10	50
0.05	90	35
0.10	5	5
0.20	10	5
1.00	15	5

[a]Experimental conditions are described in the text

TABLE 12

SDS HYDROXYLAPATITE COLUMN CHROMATOGRAPHY OF THE HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE[a]

Eluant Molarity	%Inh (0.01 mg/mL)	%wt Recovery
0.01	35	5
0.04	15	10
0.07	20	10
0.10	15	15
0.15	20	25
0.20	100	30
0.22	15	5
0.25	20	5
1.00	20	5

[a]Experimental conditions are described in tbe text.

With SDS in the eluant buffer, the activity is bound more tightly to the hydroxylapatite. Hydroxylapatite exhibits an affinity for negatively charged molecules of relatively high molecular weight.[56] An increase in the affinity of the active principle toward the hydroxylapatite suggests that SDS and the active principle bind. This result indicates that the active principle may be a protein or a protein-cofactor complex.

4.4.4 Gel-Permeation Column Chromatography

The extract of the retentate pellet and the 0.01M eluant fraction from the hydroxylapatite column chromatography of the extract were subjected to the gel-permeation chromatography on Sephacryl S-200 Superfine and their elution and activity profiles were compared. The gel-permeation column was calibrated with globular protein standards as previously described. The objective was to demonstrate that the active principle is retained by hydroxylapatite and to obtain an estimate of its molecular-weight range. The elution profiles of the extract and the 0.01M hydroxylapatite eluant fraction are given in Figures 22 and 23 respectively. Fractions were collected as shown and dialyzed at 4°C versus distilled water for

24 h, lyophilized, and assayed. Fractions 8-13 of the extract exhibited significant antineoplastic acitvity as defined as ID_{50} 0.10. This corresponds to a molecular weight range of 50,000-80,000 as calibrated for a globular protein. Fractions 0-7 and fractions 14-20 exhibited no significant antineoplastic activity. The 0.01M eluant fraction exhibited no significant activity as defined above. As expected, most of the relatively high molecular weight fraction adsorbed to the hydroxylapatite column. The binding of the antineoplastic activity suggests that the high molecular weight antineoplastic principle is not a polysaccharide, as most polysaccharides do not express a high affinity for hydroxylapatite. It is apparent the active principle is retained by hydroxylapatite, in agreement with the previous results.

Figure 23. Sephacryl S-200 Gel-Permeation Chromatography of the Retentate Pellet Extract

A 2.0 mL extract solution containing 40 mg of lyophilized extract in 0.05M sodium phosphate buffer pH 6.8 was loaded onto a 1.6 X 70 cm Sephacryl S-200 Superfine column previously equilibrated with the same buffer. The column was eluted at 15°C with the equilibration buffer. The flow rate was 35 mL/h. Fractions were collected every 20 min. Absorbance was measured at 254 nm at a sensitivity of 1.28X.

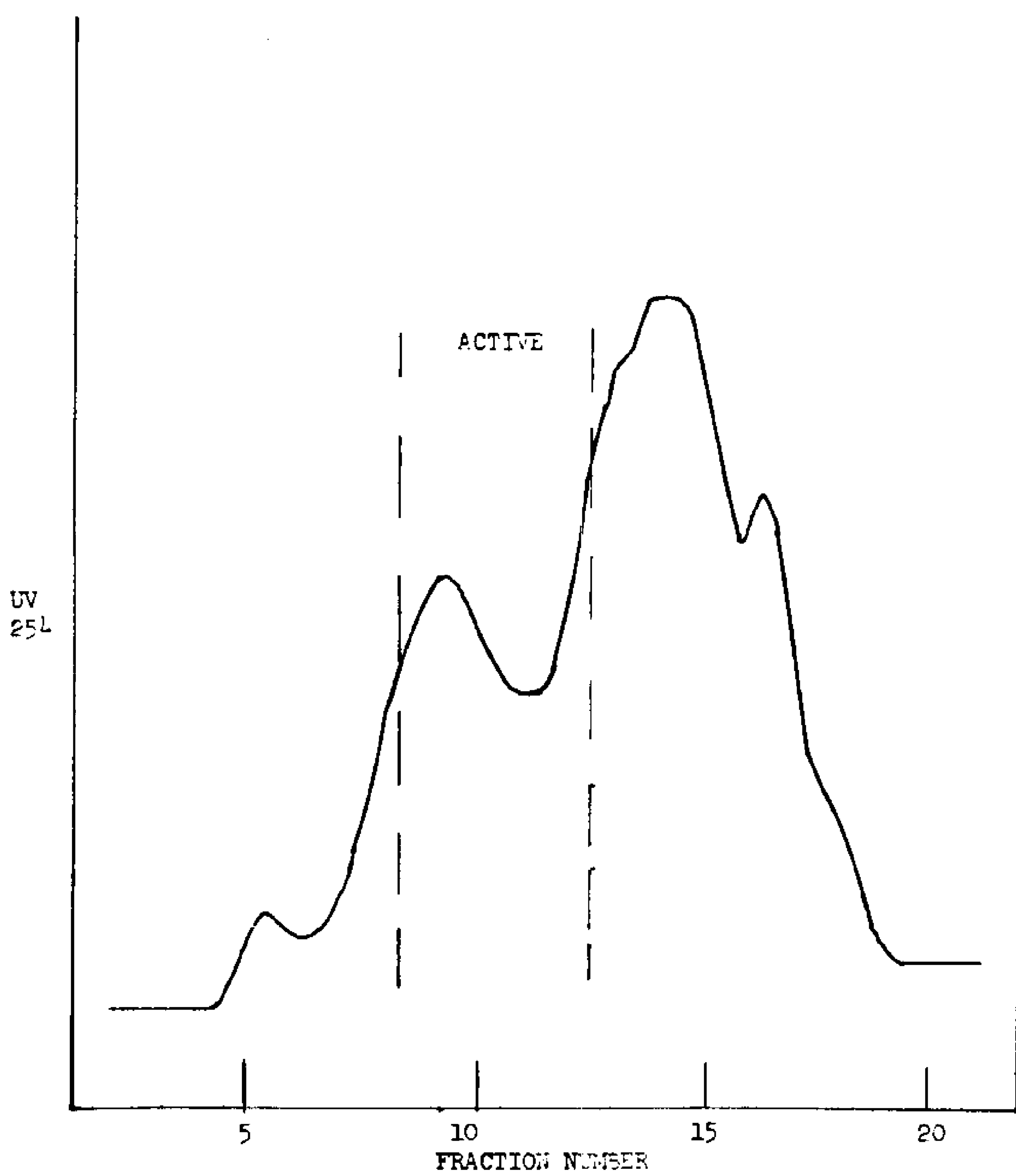


Figure 23.

Figure 24. Sephacryl S-200 Gel-Permeation Chromatography of the 0.01M Hydroxylapatite Eluant Fraction

A 2.0 mL solution containing 20 mg of lyophilized 0.01M hydroxylapatite eluant fraction in 0.05M sodium phosphate buffer pH 6.8 was loaded onto a 1.6 X 70 cm Sephacryl S-200 Superfine column previously equilibrated with the same buffer. The column was eluted at 15°C with the equilibration buffer. The flow rate was 35 mL/h. Fractions were collected every 20 min. Absorbance was measured at 254 nm at a sensitivity of 1.28X.

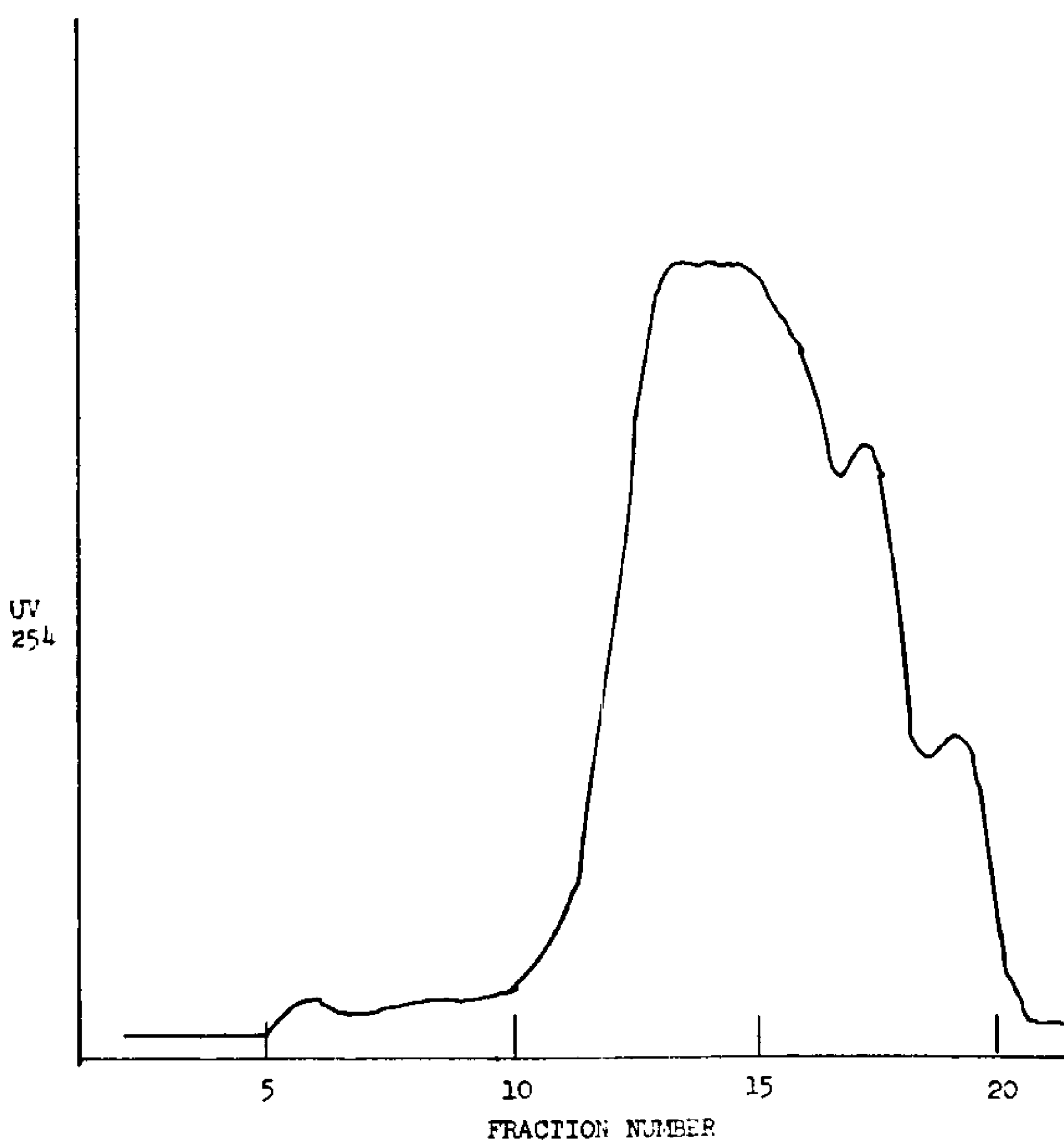


Figure 24.

4.4.5 DEAE-Cellulose Anion Exchange Chromatography

The 150 mL 0.05M hydroxylapatite eluant fraction was lyophilized, dissolved in 10 mL of distilled water, and dialyzed at 4°C for 12 hours versus distilled water and 24 hours versus 0.05M Tris buffer pH 7.4. The resulting 20-30 mL solution was loaded onto a 1.6 X 8 cm DEAE-cellulose column which had been equilibrated with 0.05M Tris buffer pH 7.4. The column was washed at 15°C with the equilibration buffer and then eluted with a 500 mL linear gradient of 0.01M-1.00M NaCl. The elution profile is shown in Figure 25. Fractions were collected as indicated, dialyzed at 4°C for 24 h versus distilled water, and assayed. For routine fractionation, the column was eluted with 1M NaCl. Fractions 1-6 were significantly active as previously defined and were pooled. The yield was 12 mg with ID_{50} 0.04 corresponding to 300 units of total activity. Recovery was 94% of the applied activity. No activity could be demonstrated binding to the column as fractions 7-21 were totally inactive. The active principle does not bind strongly, but does interact with the support matrix, as tailing is observed. The result is not due to column overload as doubling the column length does not alter the observed results. For routine fractionation, the active fraction was lyophilized and stored at -20°C or used directly in the next step.

Figure 25. DEAE-Cellulose Column Chromatography of the Active Hydroxylapatite Fraction

A 20 mL solution containing 35 mg of the 0.05M hydroxylapatite eluant fraction in 0.05M Tris buffer pH 7.4 was loaded onto a 1.6 X 8 cm column of DEAE-cellulose that had been equilibrated with the same buffer. The column was washed at 15°C with the equilibration buffer and eluted with a 500 mL linear gradient of 0.01M-1.00M NaCl. The flow rate was 20 mL/h. Fractions were collected every 25 min. Absorbance was measured at 254 nm at a sensitivity of 0.16X.

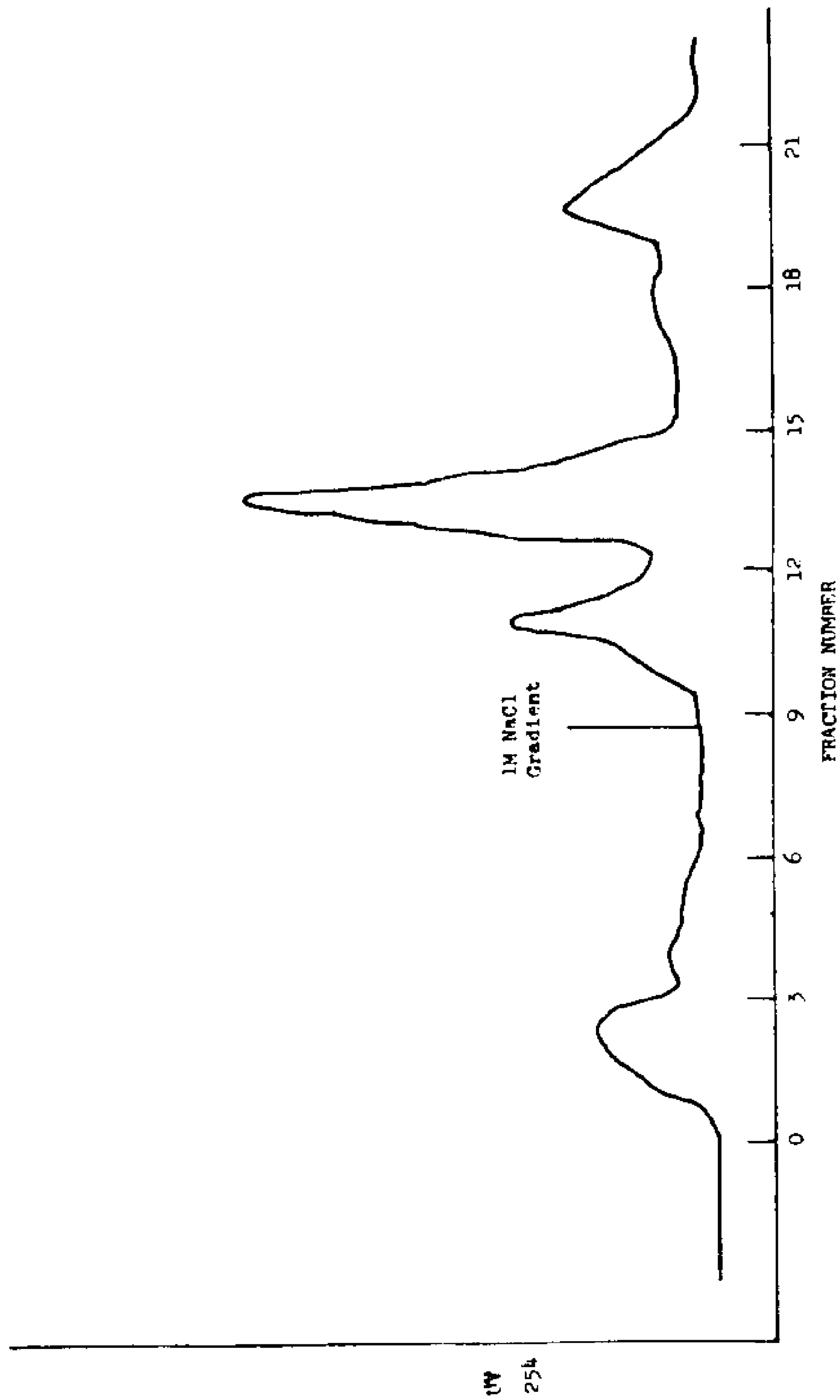


Figure 25.

4.4.6 Affinity Chromatography

The activity spectrum of the crude extracts, evidence the activity is expressed through a high molecular weight biopolymer, possibly a protein, and the stability to acidic conditions are consistent with properties expressed by the interferons. It was thought that the active principle may express other properties that are exhibited by the interferons. Therefore, the use of affinity chromatography as a purification technique was investigated using polyuridylic acid-Sepharose 4B as the affinity matrix. Interferons exhibit a high affinity for nucleic acids.[57] The affinity matrix has also been used to purify m-RNA,[58] plant nucleic acids,[59] and reverse transcriptase.[60] The lyophilized active fraction obtained by DEAE-cellulose column chromatography was dissolved in 5 mL of 0.05M Tris buffer pH 7.4 and dialyzed at 4°C versus the same buffer for 12 hours. The fraction was then loaded onto a 1.6 X 5 cm column of polyuridylic acid-Sepharose 4B which had been equilibrated with 0.05M Tris buffer pH 7.4. The column was washed at 15°C and then eluted with a 250 mL linear gradient of 0.01M-1.0M NaCl. The elution profile is presented in Figure 26. The

fraction not adhering to the support was collected, dialyzed at 4°C versus distilled water for 24 h, and assayed. The fraction eluted by the NaCl gradient was collected and prepared for assay in the same manner, except that the dialysis was performed for 36 h. The fraction not adhering was inactive, while the fraction binding to the column expressed significant activity. The yield was 3.0 mg with ID_{50} 0.01, corresponding to 300 units of total activity. Recovery of activity was 100%.

4.4.7 Bio-Gel P-100 Gel-Permeation Chromatography of the High Molecular Weight Antineoplastic Principle

The lyophilized active fraction obtained by affinity chromatography on Poly U-Sepharose 4B was dissolved in 1.5 mL of 0.05M Tris buffer pH 7.4 and purified by Bio-Gel P-100 gel-permeation chromatography. The elution profile is shown in Figure 27. The fractions were collected, fraction 1 and 2 were dialyzed at 4°C versus distilled water for 12 h, lyophilized, and assayed. Fraction 3 was handled in a similar manner, except the dialysis was performed for 4 h. Fraction 1 was active. The yield was 1.5 mg with the ID_{50} 0.02, corresponding to 75 units of

total activity. Fractions 2 and 3 were inactive. Fraction 1 gave a positive protein assay. Its estimated molecular weight was between 70,000 to 85,000. Total recovery of applied activity was 25%.

The isolation of the high molecular weight antineoplastic principle is summarized in Table 13.

4.4.8 Synergism

The synergistic relationship between the high molecular weight antineoplastic principle and fraction 3 from Bio-Gel P-100 gel-permeation chromatography, thymidine, and all inactive fractions obtained from the isolation of the high molecular weight active principle was investigated as previously described. No synergistic relationship could be established except with fraction 3 from the Bio-Gel P-100 gel-permeation chromatography. The observed ID_{50} was 0.02, corresponding to 125 total units of activity for the Bio-Gel P-100 gel-permeation chromatography purification step. Accounting for the observed synergism, 42% of the applied activity was recovered.

Figure 26. Affinity Chromatography of the DEAE-Cellulose Active Fraction

A 7 mL solution containing 12 mg of the DEAE-cellulose active fraction in 0.05M Tris buffer pH 7.4 was loaded onto a 1.6 X 5 cm Sepharose 4B affinity column of poly-uridylic acid-Sepharose 4B that had been equilibrated with the same buffer. The column was washed at 15°C with the equilibration buffer and eluted with a 250 mL linear gradient of 0.01M-1.00M NaCl in the equilibration buffer. The flow rate was 30 mL/h. The eluant and wash fractions were collected. Absorbance was measured at 254 nm at a sensitivity of 0.8X.

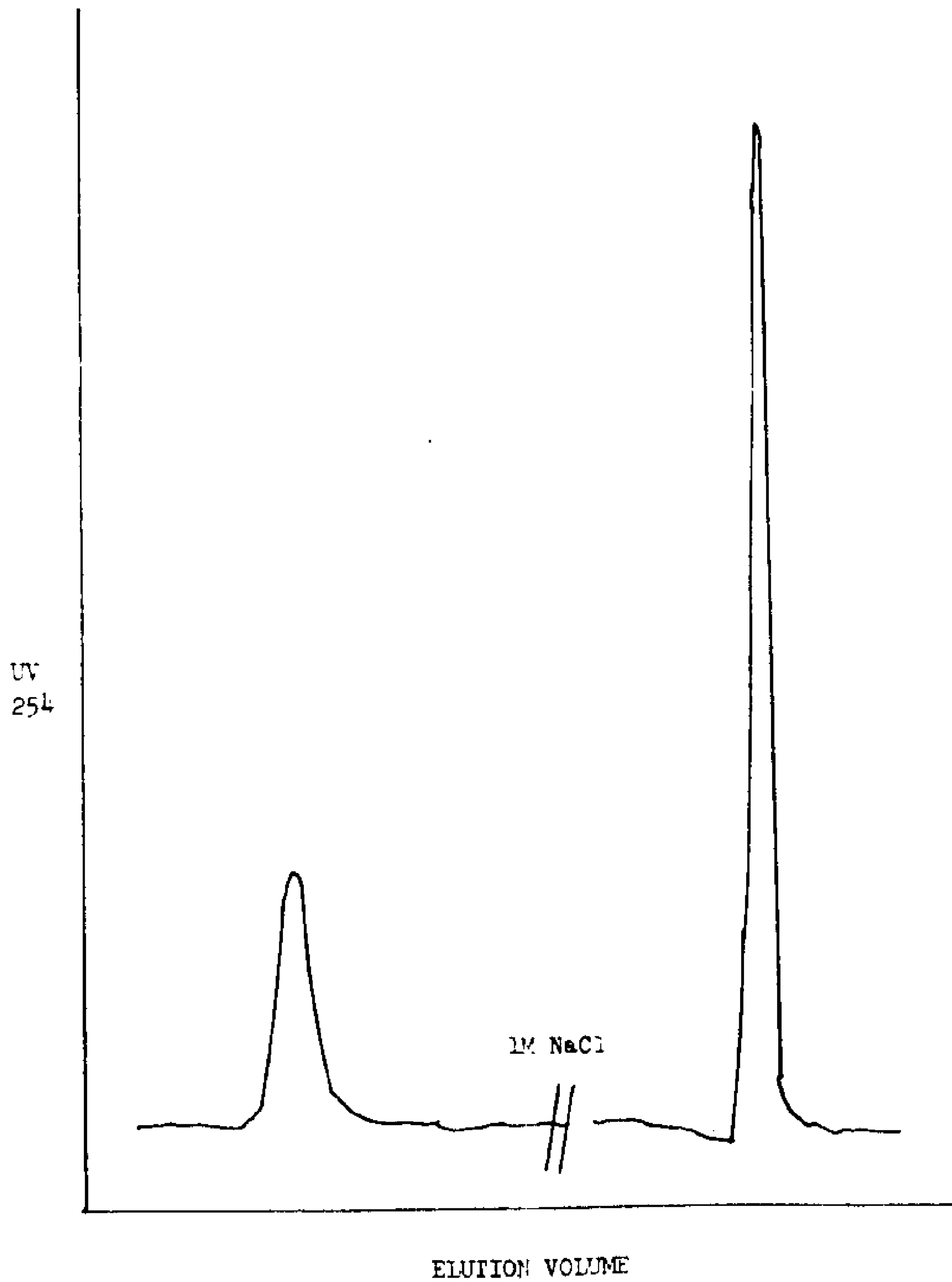


Figure 26.

Figure 27. Bio-Gel P-100 Gel-Permeation Chromatography of the High Molecular Weight Antineoplastic Principle

A 1.5 mL solution containing 3.0 mg of the affinity chromatography active fraction in 0.05M Tris buffer pH 7.4 was loaded onto a 1.6 X 40 cm column of Bio-Gel P-100 that had been washed with the same buffer. The column was eluted at 15°C with the equilibration buffer. The flow rate was 20 mL/h. Absorbance was measured at 254 nm at a sensitivity of 0.8X. Fraction 1 eluted between 15 and 25 mL and was UV active. Fraction 2 was collected between 25 and 60 mL. The UV active Fraction 3 eluted between 60 and 85 mL.

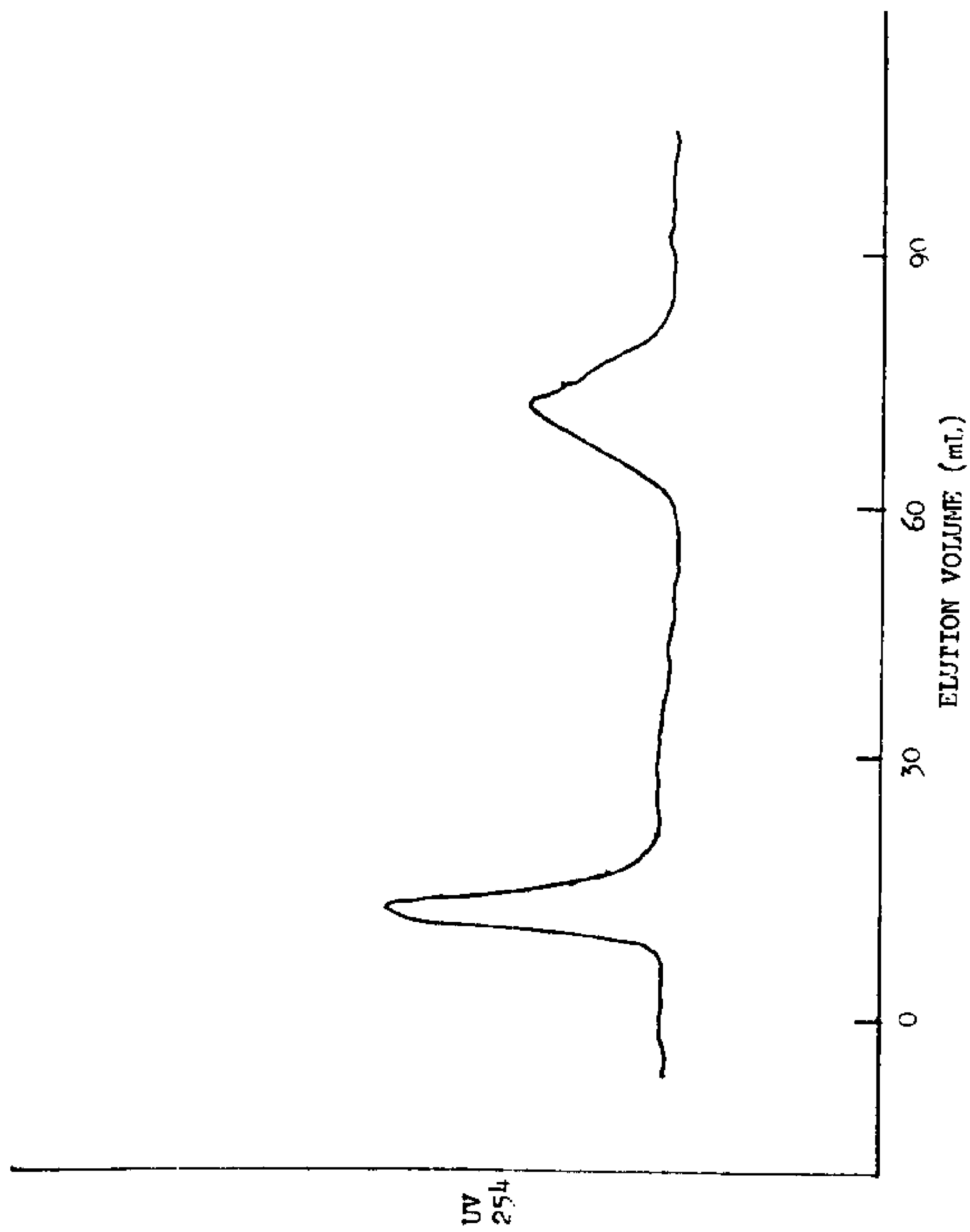


Figure 27.

TABLE 13

SUMMARY OF THE PURIFICATION OF THE

HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE

Fraction	mg[a]	Protein (mg)[b]	ID_{50}[c]	Total Activity	% Recovery
Crude Extract	130	100	0.20	630	100
Hydroxylapatite	35	31	0.11	318	51
DEAE-Cellulose	12	10	0.04	300	48
Poly U-Sepharose 4B	3	3	0.01	300	48
Bio-Gel P-100 Protein	1.5	1.5	0.02	75	12
Low Molecular Weight	1.0	0	Inactive	0	0
Protein + LMW	2.5	1.5	0.02	125	20

[a]Weight of the lyophilized, dialyzed fraction.

[b]Determined by Bio-Rad Protein Assay.

[c]Determined by L1210 Lymphocytic Leukemia Tissue Culture Assay

4.4.9 pH Stability

The stability of the active principle to acidic conditions was investigated to ascertain that the stability exhibited by the extract was still present and no stabilizing cofactor had been lost during the purification.

Two 5 mL solutions containing 4 mg of the lyophilized DEAE-cellulose active fraction were adjusted to pH 2.0 by dropwise addition of HCl at 4°C. One solution was allowed to warm to room temperature while the other was kept at 4°C. Two control solutions of 4 mg of the DEAE-cellulose active fraction in 0.05M sodium phosphate buffer pH 6.8 were used, one at each temperature. Aliquots were taken at the specified time intervals. The aliquots and the final solution were neutralized by addition of the control buffer, dialyzed at 4°C versus distilled water for 24 h, and assayed. The control solutions were handled in the same manner, but the neutralization step was omitted. The results are presented in Table 14. The active principle is stable at a pH 2.0 at 4°C or 23°C for a minimum of 3 h. The stability is consistent with that exhibited by the extract. If present, no stabilizing cofactor toward acidic conditions has been lost during the purification. The stability at pH 2.0 is similar to that expressed by a class of proteins expressing antineoplastic activity, the interferons.[61]

TABLE 14

pH STABILITY OF THE HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE[a]

Fraction	pH	Time (h)	Temp C	%Inh(0.04mg/mL)
DEAE-Cellulose Active Fraction	6.8	1.0	4	55
		2.0	4	55
		3.0	4	50
	6.8	1.0	23	55
		2.0	23	40
		3.0	23	50
	2.0	1.0	4	50
		2.0	4	50
		3.0	4	45
	2.0	1.0	23	45
		2.0	23	40
		3.0	23	50

[a]Experimental conditions are described in the text.

4.4.10 Temperature Stability of the High Molecular Weight Antineoplastic Principle

The heat stability of the high molecular weight antineoplastic principle was investigated in the following manner: To a 2 dram vial, 1.0 mg of the lyophilized active fraction from DEAE-cellulose column chromatography or Bio-Gel P-100 gel-permeation chromatography was added and solubilized by 2.0 mL of 0.05M Tris buffer pH 6.8. The vial was heated in a heating block to the desired temperature. Aliquots were taken at 5 h. Aliquots and the final solution were dialyzed at 4°C versus distilled water for 12 h, lyophilized, and assayed. Controls were maintained at 4°C for the same time interval and prepared for assay in the same manner. The results are presented in Table 15.

The high molecular weight antineoplastic principle was stable to 40°C for 10 h. Activity was lost gradually at 50°C. The observed results are in agreement with the temperature stability reported by Prescott.[21] The active fraction from Bio-Gel P-100 gel-permeation chromatography exhibited similar stability as the DEAE-cellulose active fraction. This indicated that no cofactor that conferred heat stability to the high molecular weight principle was lost during the gel-permeation chromatography.

TABLE 15

TEMPERATURE STABILITY OF THE

HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE[a]

Fraction	Temp. (C)	% Inh. (0.10 mg/mL)		% Inh. (0.01 mg/mL	
		5h	10h	5h	10h
DEAE-Cellulose	4	100	100	45	40
Active Fraction	20	100	100	45	50
	30	100	100	40	45
	40	100	100	45	40
	50	100	100	35	20
Bio-Gel P-100	4	100	100	40	45
Active Fraction	40	100	100	45	45
	50	100	100	30	20

[a]Experimental conditions are described in the text.

4.4.11 SDS Polyacrylamide-Gel Electrophoresis of the High Molecular Weight Antineoplastic Principle

The high molecular weight antineoplastic principle obtained by Bio-Gel P-100 gel-permeation chromatography was analyzed by SDS polyacrylamide-gel electrophoresis as previously described to determine its molecular weight and purity. The results are shown in Figures 28 and 29. The molecular weight was 76,000 daltons relating to protein standards. One protein band was observed, suggesting that the protein has been purified to homogeneity.

4.4.12 Molecular Weight Determination of the High Molecular Weight Antineoplastic Principle by Gel-Permeation Chromatography

The molecular weight of the high molecular weight antineoplastic protein was determined by gel-permeation chromatography on Sephacryl S-200 Superfine as previously described. The result is shown in Figure 30. The elution volume of the antineoplastic protein was 80.0 mL, corresponding to a K_{av} 0.28. From the calibration curve for the globular protein standards, the molecular weight was determined to be 72,000 daltons. This molecular weight determined by gel-permeation chromatography agrees with the molecular weight of 76,000 daltons determined by SDS polyacrylamide gel-electrophoresis.

Figures 28 and 29. SDS Polyacrylamide Gel-Electrophoresis of the High Molecular Weight Antineoplastic Principle

SDS polyacrylamide gel electrophoresis was performed as previously described. Figure 28 is the 12% gel onto which 50 μg of the protein from Bio-Gel P-100 fraction 1 has been loaded.

Figure 29 is the molecular weight determination for the protein on the calibration curve constructed for a series of protein standards on the 12% gel.

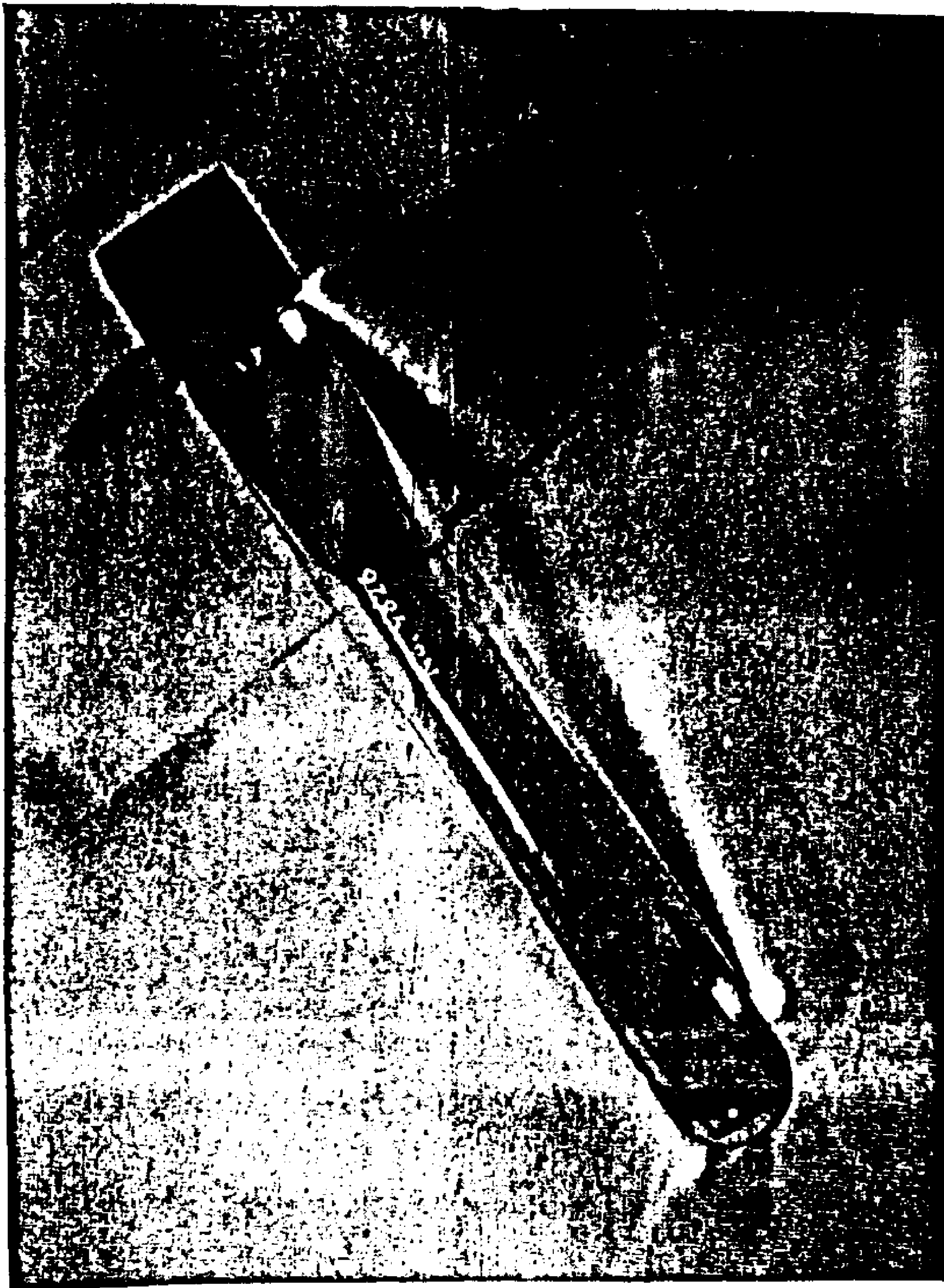


Figure 28.

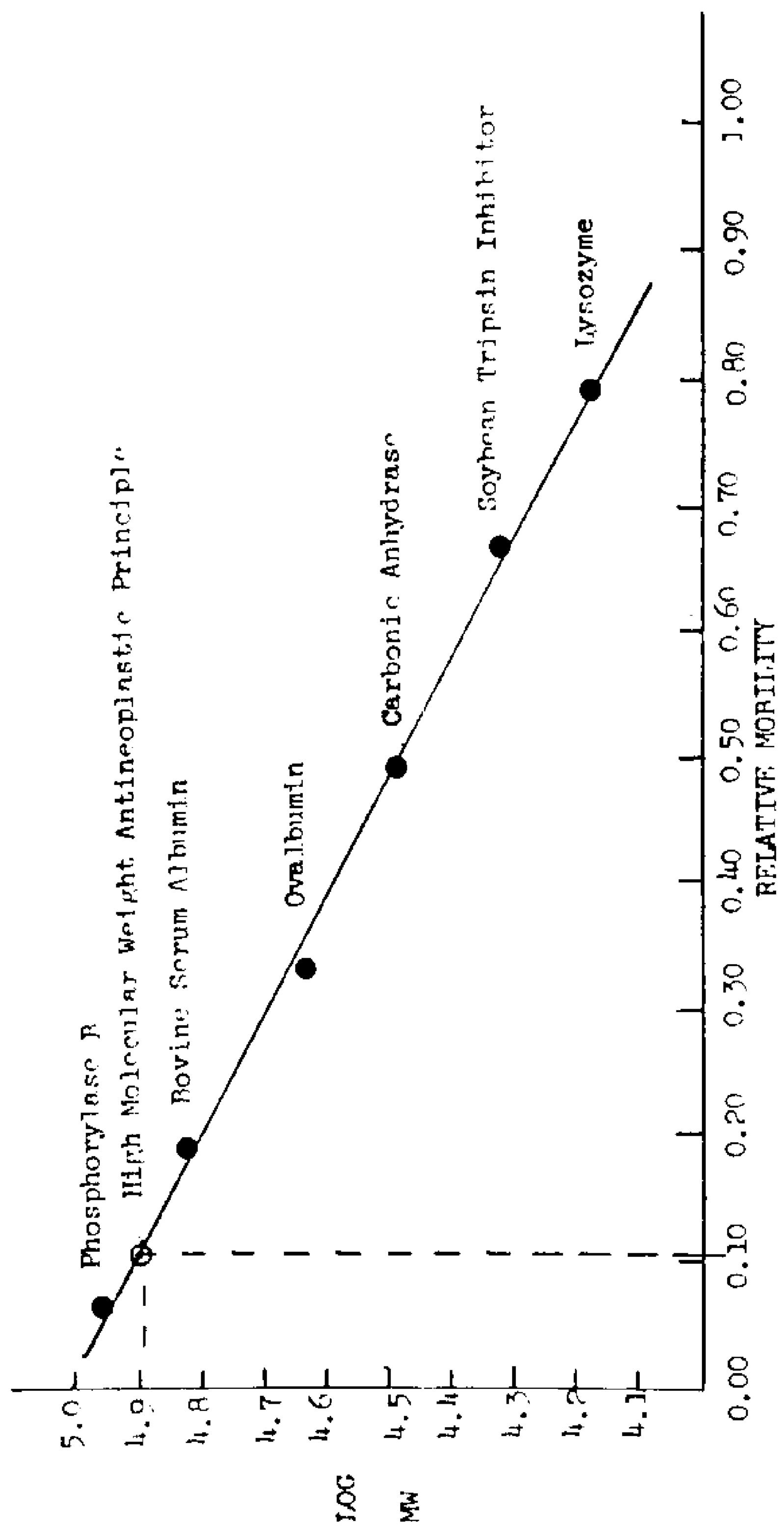


Figure 29.

Figure 30. Estimation of the Molecular Weight of the High Molecular Weight Antineoplastic Principle by Sephacryl S-200 Gel-Permeation Chromatography

The molecular weight of the high molecular weight antineoplastic principle was determined from the calibration curve of globular protein standards chromatographed on a 1.6 X 70 cm column of Sephacryl S-200 Superfine. The K_{av} of the reference proteins was plotted versus the logarithm of their molecular weight.

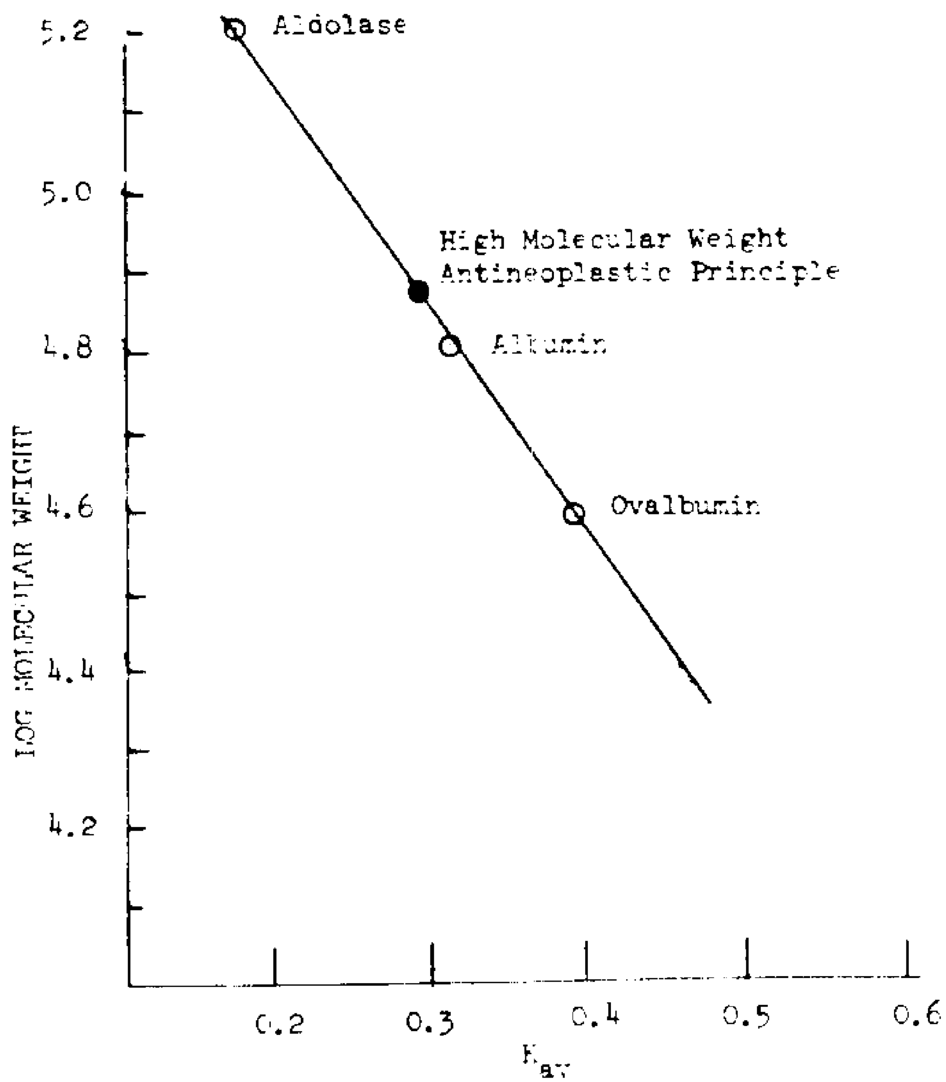


Figure 30.

4.4.13 Amino Acid Analysis of the Antineoplastic Protein

The antineoplastic protein was prepared for amino acid analysis as previously described.[62] The hydrolysis was conducted for 20 and 70 h to provide an estimate of the loss of Ser, Tyr, and Thr. Less than 5% destruction of these amino acids after 20 h hydrolysis occurred. The results are presented in Table 16. The relative ratios of the individual amino acids were based on the amount of the stable amino acids Phe and Arg which were present in the same amount. Based on the number of residues, Gly was the most abundant amino acid. The protein contained 15% Gly and 14% Ser. Over 19% of the amino acids were Ser and Thr. The large amount of NH_3 produced suggests that Asn and Gln are present in a relatively high amount. The calculated molecular weight from the amino acid composition, excluding Trp, was 69,700 daltons.

4.4.14 Sephadex G-10 Gel-Permeation Chromatography of Bio-Gel P-100 Fraction 3

The Bio-Gel fraction 3, which expressed synergism with the high molecular weight antineoplastic principle, was chromatographed by Sephadex G-10 gel-permeation chromatography. The elution profile is shown in Figure 31.

TABLE 16

AMINO ACID ANALYSIS OF THE

HIGH MOLECULAR WEIGHT ANTINEOPLASTIC PRINCIPLE[a]

Amino Acid	nM/0.1mL[b]	Relative Molar Quantity	Number Residues M.W. 76000
Asp-Asn	22.89	60.6	61
Thr	13.32	35.2	35
Ser	32.04	84.9	85
Glu-Glm	29.31	77.6	78
Gly	35.85	95.0	95
Ala	20.34	53.9	54
Cys	1.50	4.0	4
Val	12.54	33.3	33
Met	3.60	9.9	10
Leu	13.71	36.4	36
Ileu	8.58	22.8	23
Tyr	1.98	5.3	5
Phe	8.40	17.0	17
His	17.26	45.7	46
Lys	8.40	30.2	30
Arg	5.85	17.0	17
Pro	10.28	20.8	21
Trp	-	-	-

[a]Experimental conditions are described in the text.

[b]Corrected for loss during hydrolysis.

Figure 31. Sephadex G-10 Gel-Permeation Chromatography of Bio-Gel P-100 Fraction 3.

A 1.0 mL solution containing 4.0 mg of Bio-Gel P-100 fraction 3 dissolved in water was loaded onto a 1.6 X 40 cm column of Sephadex G-10. The column was eluted with distilled water at 15°C. The flow rate was 40 mL/h. Absorbance at 254 nm was measured at a sensitivity of 0.8X.

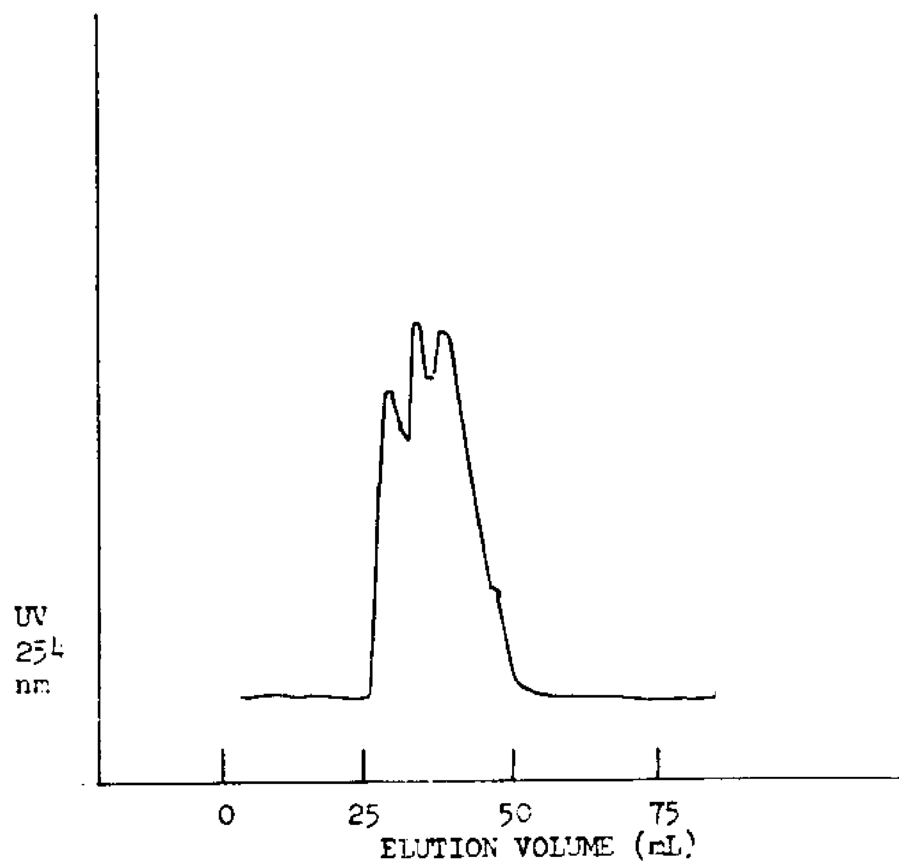


Figure 31

4.4.15 The UV Spectra of the High Molecular Weight Antineoplastic Principle and the Affinity Chromatography Active Fraction

The UV spectrum of a 1.3 X 10^{-5}M solution of the purified high molecular weight antineoplastic principle in 0.05M Tris buffer pH 7.4 was determined. The spectrum is illustrated in Figure 32. The high molecular weight antineoplastic principle exhibited an absorption maximum at 268 nm (ε = 19,800).

The UV spectrum of a 1.0 mg/mL solution of the active fraction from Poly U-Sepharose 4B in 0.05M Tris buffer pH 7.4 was determined. The spectrum is illustrated in Figure 33. The fraction exhibited an absorption maximum at 260 nm a = 45,000).

4.5.16 Discussion

The high molecular weight antineoplastic principle appears to be a protein or protein cofactor complex of relatively high molecular weight (76,000 daltons). It was isolated in 0.005% yield based on the weight of wet liver. The protein exhibited an ID_{50} 2.6 X 10^{-7}M, had an affinity for nucleic acids, and its amino acid composition exhibited a high amount of Gly and Ser (29%).

Figure 32. The UV Spectrum of the High Molecular Weight Antineoplastic Principle

The UV spectrum of a 1.3 X 10^{-5}M solution of the high molecular weight antineoplastic principle in 0.05M Tris buffer pH 7.4 was determined.

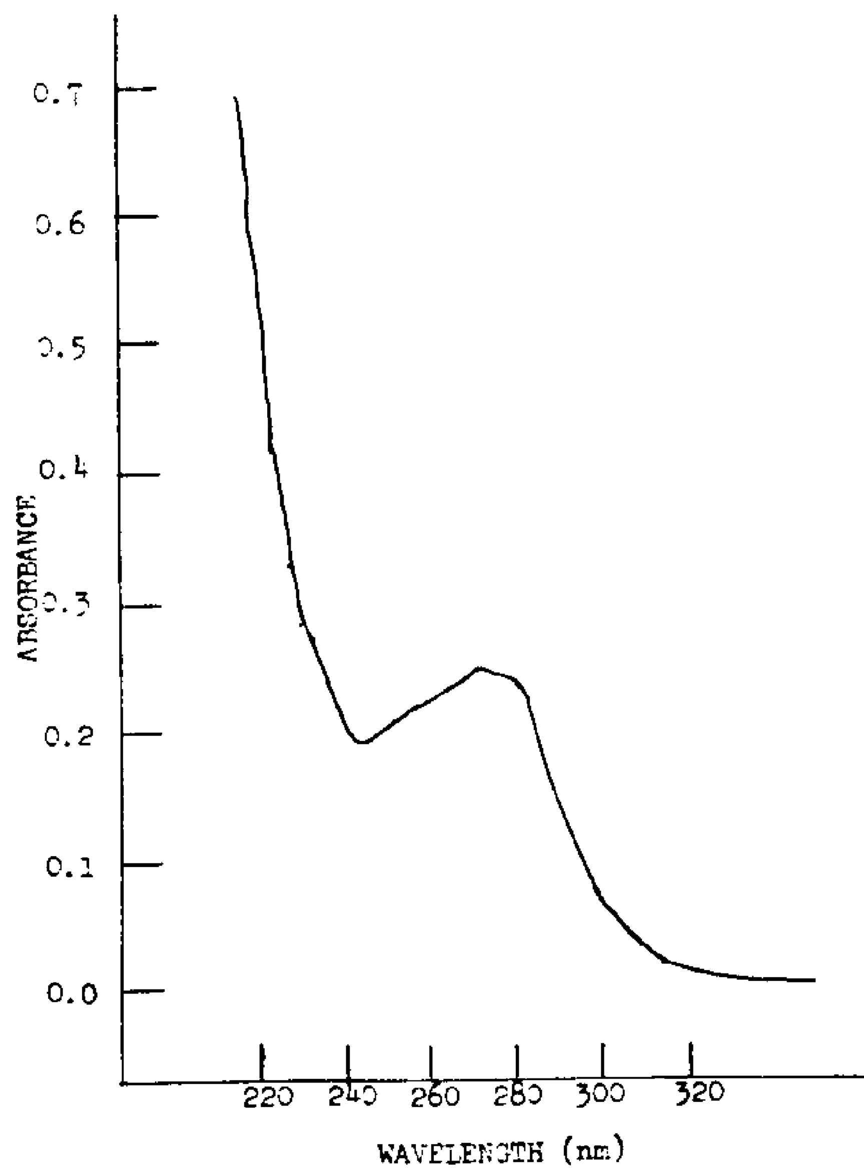


Figure 32.

Figure 33. The UV Spectrum of the Active Fraction from Poly U-Sepharose 4B Affinity Chromatography.

The UV spectrum of a 1.0 mg/mL solution of the active fraction from Poly U-Sepharose 4B affinity chromatography in 0.05M Tris buffer pH 7.4 was determined.

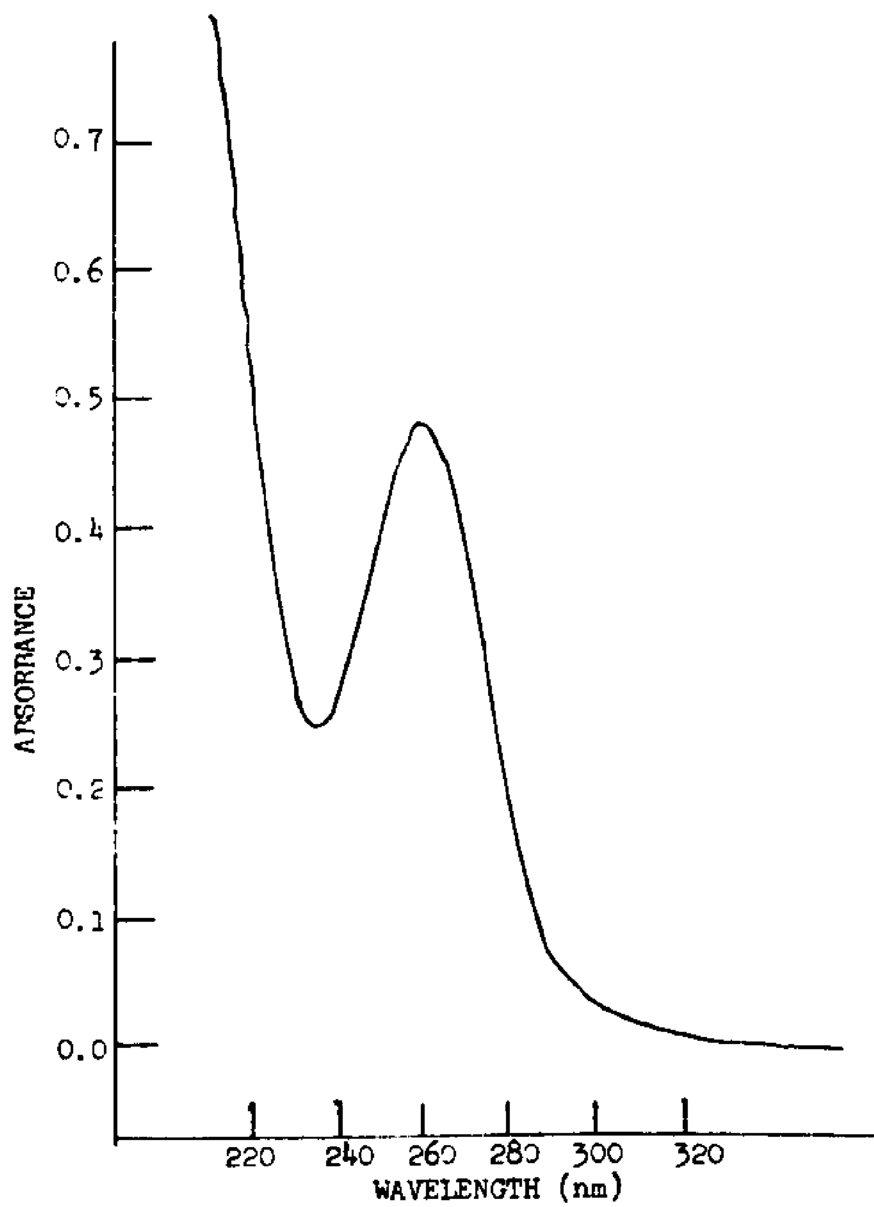


Figure 33.

One protein band by SDS polyacrlyamide gel-electrophoresis, Sephacryl S-200 Superfine gel-permeation chromatography, Bio-Gel P-100 gel-permeation chromatography, and Poly U-Sepharose 4B affinity chromatography suggest that the protein has been purified to homogeneity.

Several types of proteins expressing antineoplastic activity have previously been isolated. Several proteins with antitumor activity have been isolated from the culture filtrates of various microorganisms of the genus *Streptomyces*.[63] The most thoroughly studied of these antitumor proteins is neocarzinostatin. Neocarzinostatin degrades DNA in order to express its antineoplastic activity. Therefore, it has an affinity toward nucleic acids, as does the high molecular weight antineoplastic principle. The affinity of neocarzinostatin for Poly U-Sepharose 4B has not been investigated. Neocarzinostatin consists of a single chain acidic protein of known primary structure of molecular weight 10,500 and a low molecular weight cofactor.[64] The protein serves only as a carrier of the low molecular weight cofactor which can degrade DNA in the absence of protein. The protein-cofactor complex exhibits significant absorption above 300 nm.[64] The UV spectrum of the high molecular weight principle shows no significant

absorbance above 300 nm, indicating that it contains no cofactor similar to that of neocarzinostatin. Also, the molecular weight of the high molecular weight antineoplastic principle (76,000 daltons) is significantly different from neocarzinostatin (10,700 daltons). All the other antitumor proteins isolated from the genus Streptomyces have molecular weights between 10,000 and 16,000 daltons. It appears unlikely that the high molecular weight antineoplastic principle is closely related to this family of proteins. The identity and function of the protein-cofactor complex have not been thoroughly investigated for the other proteins of this family.

Another class of antitumor proteins is the interferons. Many demonstrate similar pH stability, heat stability, stability in SDS, and affinity for nucleic acids as the high molecular weight antineoplastic principle.[61] The molecular weights of the interferons range between 10,000 and 40,000 daltons. The optimum antineoplastic activity approaches ID_{50} 1.0 X 10^{-11}M. It is possible that the high molecular weight antineoplastic principle is related to the interferons. In future studies, the full activity spectrum of the high molecular weight antineoplastic principle should be determined. The activity spectrum

of the crude extracts is similar to that of the interferons.[61]

Synergism is expressed between the high molecular weight antineoplastic principle and Bio-Gel P-100 fraction 3, which consists of a minimum of three components of molecular weight less than 700. The synergistic relationship was not investigated in detail. The exact nature of the observed synergism, the questions of a protein cofactor, and the identification of the Bio-Gel P-100 fraction 3 low molecular weight components should be performed.

CHAPTER 5 SUMMARY

Mercenene, the name given to the antineoplastic principles of the common quahog, <u>Mercenaria</u> <u>mercenaria</u>, was comprised of a low molecular weight antineoplastic principle and a high molecular weight antineoplastic principle. The low molecular weight antineoplastic principle was purified and identified as thymidine, a nucleoside of established chemotherapeutic value. It was isolated in 0.006% yield, based on the weight of wet liver, and exhibited an ID_{50} 1.6 X 10^{-5}M. Thymidine accounted for 24% of the total activity. The distribution of the remaining activity in the low molecular weight fraction was determined. Two additional low molecular weight antineoplastic principles of significant activity were present but their yield did not surpass the chosen criterion of 0.001% and they were not purified to homogeneity.

The high molecular weight antineoplastic principle exhibited 50% of the total activity. It was purified in a 0.005% yield and characterized. It was established that the high molecular weight antineoplastic principle was a protein or protein-cofactor complex. The ID_{50} was

2.6×10^{-7}M, calculated for a molecular weight of 76,000 daltons. The purification of the antineoplastic principles is summarized in Figure 34.

Figure 34. Summary of the Purification of the Antineoplastic Principles

The purification of the antineoplastic principles is summarized. The weight of the active fraction, ID_{50} value, and total number of activity units are given for each fractionation step. The section of the text for each fractionation step is indicated next to that step.

Crude Liver 140 g

| Extraction 3.4.1

Liver Extract 50g ID_{50} 5.6 8900U

| Ammonium Sulfate Fractionation 3.4.2

Supernatant

| Dialysis 3.4.3

Retentate

| Centrifugation 3.4.4

Supernatant 25.8g
ID_{50} 7.0 3680U

| Gel-Permeation Chromatography 3.4.4

Active Fraction 21.5g
ID_{50} 7.0 3070U

| Preparative TLC 3.4.5

Active Fractions

| HPLC 3.4.6

Thymidine 8.4 mg
ID_{50} 1.6 X 10^{-5}M
0.006% Yield 2100U

Pellet 7.5 g ID_{50} 2.0 3750U

| Extraction 4.4.1

Extract ID_{50} 0.2 3250U

| Hydroxylapatite 4.4.3

Active Fraction ID_{50} 0.11 1590U

| DEAE-Cellulose 4.4.5

Active Fraction ID_{50} 0.04 1500U

| Poly U-Sepharose 4B 4.4.6

Active Fraction ID_{50} 0.01 1500U

| Bio-Gel P-100 4.4.7

High Molecular Weight Antineoplastic Principle 7.5mg 0.005% Yield ID_{50} 2.6 X 10^{-7}M 375U

Figure 34.

BIBLIOGRAPHY

1. Nigrelli, R. F. Zoologica, 37, 89 (1952).

2. Limasset, P. Compt. Rend. Acad. Sci., 252, 3154 (1961).

3. Li, C. P., Prescott, B., Caldes, G., Martino, E. C., Proc. Soc. Exp. Biol. N.Y., 123, 460-464 (1966).

4. Li, C. P., Prescott, B., Jahnes, W. G., Martino, E. C., Trans. N. Y. Acad. Science SerII., 24, 504-509 (1962).

5. Schmeer, M. R., Science 144, 413-414 (1964).

6. Schmeer, M. R, Huala, C. V., Ann. N. Y. Acad. Sci., 118, 603-610 (1965).

7. Schmeer, M. R., Berry, G., Life Sciences, 4, 2157-2163 (1965).

8. Schmeer, M. R., Horton, D., Tanimura, A., Life Sciences, 5, 1169-1178 (1966).

9. Schmeer, M. R., Physical Chem. and Physics, 11, 415-424 (1979).

10. Li, C. P., Prescott, B., Liu, G. C., Martino, E. C., Nature (Lond.) 219, 1163-1164 (1968).

11. Lavelle, The Xth International Cancer Congress Houston, Texas., No. 1298:789.

12. Judge, J. R., Proc. Soc. Exp. Biol. Med., 123, 229-302 (1966).

13. Hegyel, A. F., Science, 146, 77-78 (1964).

14. Szent-Györgyi, A., Science, 149, 34-37 (1965).

15. Fishtein, M. D., J. Am. Osteopathic Assoc., 68, 90-92 (1969).

16. Fishtein, M. D., Sheridan, J. F., J. Am. Osteopathic Assoc., 69, 90-93 (1970).

17. Fishtein, M. D., Piscitelli, J., J. Am. Osteopathic Assoc., 70, 106-109 (1971).

18. Li, C. P., Prescott, B., Eddy, B., Caldes, G., Green, W. R., Martino, E. C., Young, A. M., Ann. N. Y. Acad. Sci., 130, 374-382 (1965).

19. Liu, O. C., Cipola, R. J., Bact. Proc., 164, 162-164 (1967).

20. Li, C. P. Prescott, B., Jahnes, W. B., Martino, E. C., Trans. N. Y. Acad. Science. SerII., 24, 504-509 (1962).

21. Prescott, B., King, M. L., Caldes, G., Li, C. P., Young, A. M., Int. J. Clin. Pharmacol., 9, 1-5 (1974).

22. Premuzic, E., Fortachr. Chem. Org. Naturstoffe, 29, 417 (1971).

23. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., Anal. Chem., 28, 350 (1956).

24. Quantitative Gas-Liquid Chromatography of Amino Acids in Proteins and Biological Substances, Gehrke, C. W., Roach, D., Zumualt, R. W., Stalling, D. L., Wall, L. L. 1-108, (1968).

25. Merck Index, 9th Edition, 9139 (1976).

26. Lowe, J. R., Grindley, G. B., Mol. Pharmacol. 12, 177-184, (1976).

27. Varian Associates High Resolution NMR Spectra Catalog, 1st Edition, 566 (1963).

28. Carbon-13 NMR Spectra, Johnson, L. F., Jankowski, W. C., 1-500 (1972).

29. Basic Principles in Nucleic Acid Chemistry, 1, 209-305, (1974).

30. Kufe, D. W., Beardsley, P., Karp, D., Parker, L., Rosowsky, Canellos, G., Frei, E., Blood, 55, 580-589 (1980).

31. Woodcock, T., Damin, L., O'Hehir, M., Hansen, H., Andreeff, M., Young, C., Proc. Am. Assoc. Cancer Res., 20., 462 (1979).

32. Chiuten, D., Wiernik, P., Zaharko, D., Edwards, L., Proc. Am. Assoc. Cancer Res., 20, 305 (1979).

33. Lee Shih-Shun, Giovanella, B. C., Stehlin, J. S., Brunn, J. C., Cancer Res., 39, 2928-2933 (1979).

34. Lee, S. S., Giovanella, B. C., Stehlin, J. S., J. Cell Physical, 92, 401-405 (1977).

35. Howell, S. B., Jenkins, R., Proc. Am. Assoc. Cancer Res., 20, 1052 (1979).

36. Lee, S. S., Giovanella, B. C., Stehlin, J. S., Brunn, J. C., Proc. Am. Assoc. Cancer Res., 20, 945 (1979).

37. Harris, A. W., Protides Biol. Fluids, 25, 601-604 (1978).

38. Zielke, R., Cancer Res., 39, 3373-3376 (1979).

39. Kim, J. H., Kim, S. H., Eidinoff, M. L., Biochem Pharmacol., 14, 1821-1829 (1965).

40 Mutgo, A. J., Fried, J., Burchenal, P., Vale, K. L., Strife, A., Woodcock, J., Young, C. W., Clarkson, B. D., Cancer Res., 40, 1543-1549 (1980).

41. Tsuboi, K. K., Kwong, L. K., Cancer Res., 38, 3745-3750 (1978).

42. Foley, G. E., Lazarus, H., Biochem. Pharmacol., 16, 659-664 (1967).

43. Apple, B., Proc. Am. Assoc. Cancer Res., 9, 3 (1968).

44. Lowe, J. K., Grindley, G. B., Mol. Pharmacol., 12, 177-184 (1976).

45. Reiter, H., Cancer Res., 39, 4856-4860 (1979).

46. Stadecker, M. J., Calderon J., Karvovsky, M. L., Unanue, E. R., J. Immunol., 119, 1738-1743 (1977).

47. Thymidine Metabolism and Cell Kinetics, Cleaver, J. E., 43-59 (1967).

48. Gentry, G. A., Morse, P. A., Potter, V. R., Cancer Res., 45, 517-524 (1965).

49. Martin, D. S., Cancer, 45, 1117-1128 (1980).

50. Deoy, C., Xeros, N., Nature (Lond.), 194, 682-683 (1962).

51. Bio-Rad Protein Assay Instruction Manual, 1-17 (1979).

52. Bradford, M., Anal. Biochem., 64, 509 (1975).

53. Bio-Rad Bulletin 1038, 1-14 (1978).

54. Fairbanks, G., Steck, T. L., Wallach, D. H., Biochem., 10, 2606 (1971).

55. Rodgers, L. J., Biochem. Biophys. Acta, 94, 324-329 (1965).

56. Giorgio, B., Kawasaki, T., Biochem. Biophysica Acta, 160, 301-310 (1968).

57. Maeyer-Guignard, J., Tovey, M. G., Gresser, I., Nature, 271, 622-625 (1978).

58. Shapiro, D. J., Schimke, R. T., J. Biol. Chem., 250, 1759-1764 (1975).

59. Grotha, R., Biochem. Physiol. Pflonzen, 170, 273-277, (1976).

60. Grandgenett, D. P., J. Virol., 20, 348-350 (1976).

61. The Interferon System, William Stewart, 1-421 (1979).

62. Moore, S., Stein, W. H., Methods in Enzymol., 6, 819-831 (1963).

63. Samy, T. S., Mahalakshmi, A., Hiroshi, M., Meienhofer, J., Biochem., 13, 1007-1014 (1974).

64. Napier, M. A., Holmquist, B., Strydom, D. J., Goldberg, I. H., Biochem. Biophys. Res. Comm., 89, 635-642 (1979).