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BIOLOGICAL MACROMOLECULAR SEPARATIONS TECHNOLOGY PROJECT

PROGRESS REPORT FOR THE PERIOD

JANUARY 1, 1971, THROUGH MARCH 31, 1971

Compiled by:

A. D. Kelmers

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CHEMICAL TECHNOLOGY DIVISION

BIOLOGICAL MACROMOLECULAR SEPARATIONS TECHNOLOGY PROJECT\*  
PROGRESS REPORT FOR THE PERIOD  
JANUARY 1, 1971, THROUGH MARCH 31, 1971

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MAY 1971

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SUMMARY

Improvements have been made in the flowsheet for the recovery of crude calf liver tRNA. An increase of approximately 50% in the recovery of tRNA from a given weight of liver was achieved by elimination of the isopropanol precipitation and the Sephadex G-100 gel chromatography steps. The isolation of serine and phenylalanine tRNAs from crude calf liver tRNA has been accomplished by BD-cellulose chromatography. These tRNAs were upgraded five- to eightfold by this operation. A simplified method for the recovery of calf liver aminoacyl-tRNA synthetases has been developed. Active stable synthetases were obtained when the processing was completed in 6 to 8 hr.

Small columns (0.63 cm in diameter by 33 cm long) have been adapted for the rapid separation of RNAs using the RPC-5 reversed-phase packing. These columns have been used for separating both tRNAs and rRNAs. The chromatographic resolution is comparable to that obtained with larger columns (1 cm in diameter by 240 cm long). The small columns offer the advantage of speed (chromatograms can be completed in 30 to 60 min) and require only small quantities of sample. Chromatographic peaks have been obtained with as little as  $60 \times 10^{-6}$   $A_{260}$  unit of [ $^{14}C$ ]phenylalanyl-tRNA or with 0.1  $A_{260}$  unit of ribosomal RNA.

Physical properties of the Plaskon (polychlorotrifluoroethylene) support used in the RPC-5 and RPC-6 systems have been measured. As compared with the Chromosorb supports, Plaskon has a higher surface area, a much finer particle size (median, 10  $\mu$ ) within a narrow size distribution, and a highly textured surface.

The distribution of purified samples of E. coli tRNAs was continued. A total of 878 mg was shipped during this report period.

## 1. INTRODUCTION

This is the third in a series of quarterly reports describing progress in the Biological Macromolecular Separations Technology Project. These reports were initiated as a means of providing rapid communication of new advances. They are, therefore, summary in style and briefly describe the status of the various problems under investigation. Preceding reports in this series were ORNL-TM-3150 and ORNL-TM-3280.

A change in format will be noted in the current report. Since the research and development activities in the Analytical Chemistry Division are decreasing, we have decided to describe both this work and the work done by the Chemical Technology Division under a new composite heading, "Experimental Activities."

## 2. EXPERIMENTAL ACTIVITIES

### 2.1 Bovine Liver tRNAs

Improvements that were made in the flowsheet shown in the previous report for the preparation of crude calf liver tRNA (Fig. 1, ORNL-TM-3280) have resulted in approximately a 50% increase in the recovery of tRNA from a given weight of liver. Five preparations of bovine liver tRNA were completed during this report period. Approximately 5 kg of liver was used in each preparation. Previously, an average of 3.4 micromoles of tRNA per kilogram of liver was obtained. A systematic evaluation of each of the processing steps showed that both the isopropanol precipitation step and the Sephadex G-100 chromatography step failed to remove any significant amount of contaminants but did cause appreciable losses of tRNA. Elimination of these two steps increased the yield to about 5 micromoles of tRNA per kilogram of liver.

Two tRNAs can be isolated from the bulk of the crude calf liver tRNA by benzoylated-DEAE-cellulose (BD-cellulose) column chromatography. Calf liver tRNA in a buffered solution of 0.4 M NaCl was sorbed on a BD-cellulose column. The solution was also 0.01 M in  $\text{NaC}_2\text{H}_3\text{O}_2$  at pH 4.5 and 0.002 M in  $\text{Na}_2\text{S}_2\text{O}_3$ . A solution 0.95 M in NaCl and containing the other constituents

(at point A in Fig. 1) was used to remove the bulk of the crude tRNA, leaving phenylalanine and serine tRNAs still bound to the column. Then a dual concentration gradient, from 0.95 to 2.00 M NaCl and from 0 to 25% ethanol plus other constituents, was started at point B to effect elution of the phenylalanine and serine tRNAs. The eluate associated with the two peaks was pooled separately, precipitated with ethanol, and tested for amino acid acceptance. Results showed that the specific activities of the phenylalanine and serine tRNAs had increased from 23 and 40 pmoles per  $A_{260}$  unit, respectively, to 178 and 179 pmoles per  $A_{260}$  unit. Further purification of the phenylalanine and serine tRNAs and separation of the iso-accepting serine tRNA can be achieved by reversed-phase chromatography.

A modified procedure was devised for the preparation of calf liver aminoacyl-RNA synthetase. In previous preparations, several synthetases (particularly those for glutamic acid, isoleucine, methionine, serine, and valine tRNAs) were quite labile and, even when stored in liquid  $N_2$ , remained active for less than a week. Time proved to be the critical element in the preparation scheme; that is, these synthetases were stable only if the entire preparation was completed in 6 to 8 hr. The new flowsheet is shown in Fig. 2. The composition of the buffer solutions used are as follows: Buffer A, 0.05 M Tris-HCl (pH 7.5), 0.01 M  $MgCl_2$ , 0.01 M  $\beta$ -mercaptoethanol, 0.1 M KCl, 0.001 M EDTA, 0.25 M sucrose, 15% glycerol; Buffer B, 0.01 M potassium phosphate at pH 7.5, 0.02 M  $\beta$ -mercaptoethanol, 0.001 M EDTA, 0.004 M  $MgCl_2$ , 15% glycerol; Buffer C, 0.01 M potassium phosphate at pH 6.5, 0.25 M KCl, 0.001 M glutathione, 0.005 M  $MgCl_2$ , 0.001 M EDTA, 0.0001 M ATP, 50% glycerol.

By using the improved flowsheet, we obtained a stable crude aminoacyl-RNA synthetase preparation that was active for all the tRNAs except phenylalanine. The phenylalanine synthetase is known to be easily eluted from DEAE-cellulose at low salt concentrations and may have been discarded in the Buffer B column effluent. The crude synthetase mixture prepared by this new flowsheet has been found to be stable for at least four weeks when stored in liquid  $N_2$ ; evaluation of the storage life is continuing.

## 2.2 Small Columns for Rapid RNA Separations

### 2.2.1 tRNA Separations

Two new reversed-phase chromatographic systems, designated RPC-5 and RPC-6, were described in the previous progress report (ORNL-TM-3280). These systems employ Plaskon (polychlorotrifluoroethylene) as the inert support for the quaternary ammonium salt and yield superior chromatographic resolution of tRNAs, as compared with the previous RPC systems (which utilize diatomaceous earth). Satisfactory small columns could not be constructed with the diatomaceous earth-supported systems, and relatively large (1 cm in diameter by 240 cm long) columns and 2- to 3-liter elution volumes were required for the resolution of small quantities of tracer-labeled tRNAs. With the new RPC-5 system, we have been able to construct small (0.63 cm in diameter by 33 cm long) columns, which employ only 100-ml elution volumes. Thus a chromatographic run can be completed in 30 to 60 min, as compared with the 1 to 2 days previously required. The experimental equipment used is shown in Fig. 3.

The separation of the five leucyl-tRNAs present in E. coli is shown in Fig. 4. The minor peaks are shown in an expanded scale in the insert in the figure. The  $\text{tRNA}_{2}^{\text{Leu}}$  was clearly defined even though the peak tube had a radioactivity level equal to only 12 counts of  $^3\text{H}$  per minute. The separation of isoaccepting tRNAs is comparable to that obtainable on a 1-cm-diam by 240-cm-long column with RPC-5. Under the selected conditions, the run with the small column required only 30 min. The recovery of [ $^3\text{H}$ ]leucyl-tRNA was 84%.

The separation of several aminoacyl-tRNAs from calf liver is shown in Fig. 5. In general, the tRNAs from calf liver contained larger numbers of isoacceptors than those from E. coli. Recoveries of the calf liver tRNAs averaged almost 100%.

A series of experiments was carried out with E. coli [ $^{14}\text{C}$ ]phenylalanyl-tRNA to determine the minimum amount of tRNA that could be readily resolved. Carrier crude tRNA was added in these experiments. Figure 6 is a chromatogram of [ $^{14}\text{C}$ ]phenylalanyl-tRNA having a radioactivity level of 44 counts/min. In order to overcome problems with counting statistics, it was necessary to

count the fractions for 100 min. After correcting for a background of 48.7 counts/min, a total of 24.8 counts/min (or 56% of the initial activity) was detected in the chromatographic peak. This chromatographic peak represents  $3.5 \times 10^{-9}$  g or  $60 \times 10^{-6}$   $A_{260}$  unit of phenylalanyl-tRNA.

### 2.2.2 rRNA Separations

Small reversed-phase chromatographic columns (Fig. 3), particularly RPC-5, have also been utilized for the rapid separation of 5S, 16S, and 23S ribosomal RNAs from E. coli. Good resolution of 16S and 23S rRNAs was obtained with only 0.1  $A_{260}$  unit in about 30 min (Fig. 7). The 30-ml gradient was generated with two conical chambers (Buchler Instruments). The column effluent was monitored with a Laboratory Data Control UV Monitor, Model 1205, recording full-scale absorbance of 0.02.

Mixtures of 5S, 16S, and 23S rRNA were separated in about 90 min (Fig. 8). The separation could probably be achieved in half this time by doubling the flow rate. As shown previously (ORNL-TM-3280), tRNAs are also eluted in the region of the 5S rRNA and between the 5S and 16S rRNAs under these conditions.

E. coli ribosomes have also been chromatographed using the RPC-5 system. In this case, the eluent was 0.001 M in EDTA and contained no  $MgCl_2$ . Peaks for both 16S and 23S rRNAs were obtained, although in different relative amounts than for rRNA preparations. When sodium dodecyl sulfate was added to the eluent to increase the RNA recovery, the extractant was apparently washed off the column.

As compared with the other analytical separation techniques of ultracentrifugation, electrophoresis, and MAK (methylated albumin on kieselguhr) chromatography, this RPC system gives more effective resolution and is faster and more reproducible while using only small amounts of material, which can be recovered.

The RPC-5 technique is also being evaluated for the separation of mammalian rRNAs. Samples of rRNAs from calf liver and rat liver have been prepared. Preliminary chromatographic results indicate that mammalian rRNAs are more difficult to separate than bacterial rRNAs, as reported by other investigators using MAK columns.

### 2.3 Chromatographic Technology

Physical properties of the Plaskon support used in the RPC-5 and RPC-6 systems have been determined in order to more fully understand the dramatic improvement in chromatographic resolution and concomitant reduction in size of the chromatographic column. The sizes of 200 particles of separated powder (after treatment with chloroform and agitation to break up clumps) were measured using a Zeiss particle size analyzer. The size distribution covered a narrow range, 3.5-20  $\mu$ ; the median particle size was 10  $\mu$ . The surface area was 3.82 m<sup>2</sup>/g, as measured by B.E.T. nitrogen absorption, and the porosity was 9.9%. An electron micrograph of a replicate of a particle (see Fig. 9) shows a highly textured surface.

The superior performance of Plaskon, in comparison with Chromosorb W (the support used in previous RPC systems), can readily be understood as a result of these physical properties. The surface area, which is nearly four times that for Chromosorb W, and the 10- $\mu$  size allow many times as much surface available for chromatographic resolution in a given column volume. The narrow particle size distribution and the hard, noncompressible, and nonfriable nature of the Plaskon particles permit aqueous flow through the columns even at high pressures of 300 to 500 psi. The polar hydrophobic surface of the Plaskon holds the quaternary ammonium chloride extractant, permitting little or no loss to the aqueous solution. All these favorable properties result in a superior support for use in reversed-phase chromatography.

### 3. SHIPMENTS OF PRODUCTS

During this report period, the distribution of samples of purified tRNAs was continued in accordance with recommendations of the NIGMS review committee. Forty-three samples, containing a total of 878 mg of purified tRNAs, were shipped. Samples of all five purified tRNAs (arginine, glutamic acid, formylmethionine, phenylalanine, and valine) were distributed. The supply of the arginine, phenylalanine, and valine tRNAs has now been exhausted and will not be replenished since all large-scale flowsheet

development work has been terminated. The distribution of purified glutamic acid and formylmethionine tRNAs will be continued since we still have a total of 11.4 g of these two tRNAs on hand.

4. TABLES AND FIGURES

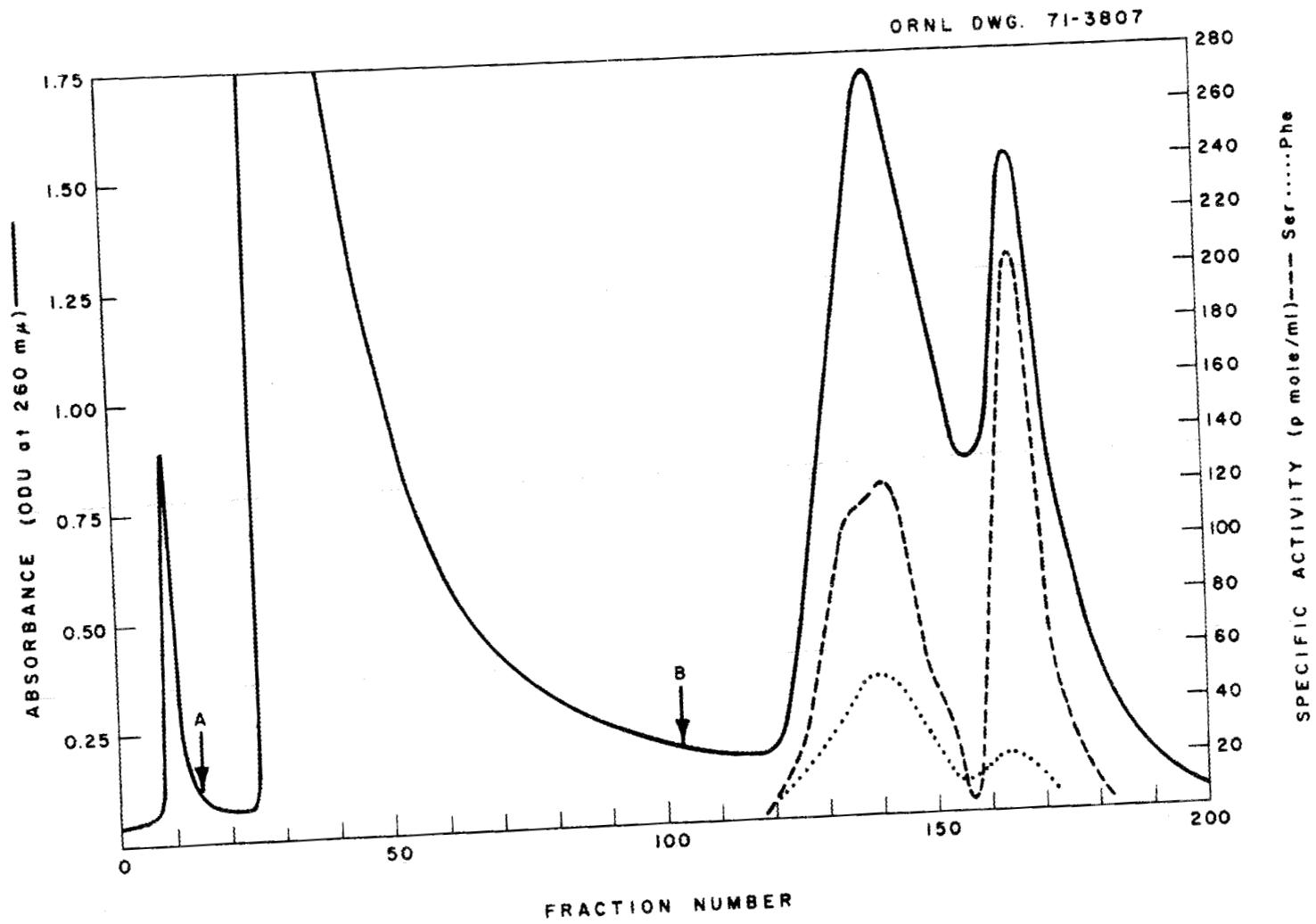


Fig. 1. Chromatogram of Calf Liver tRNA on a BD-Cellulose Column.

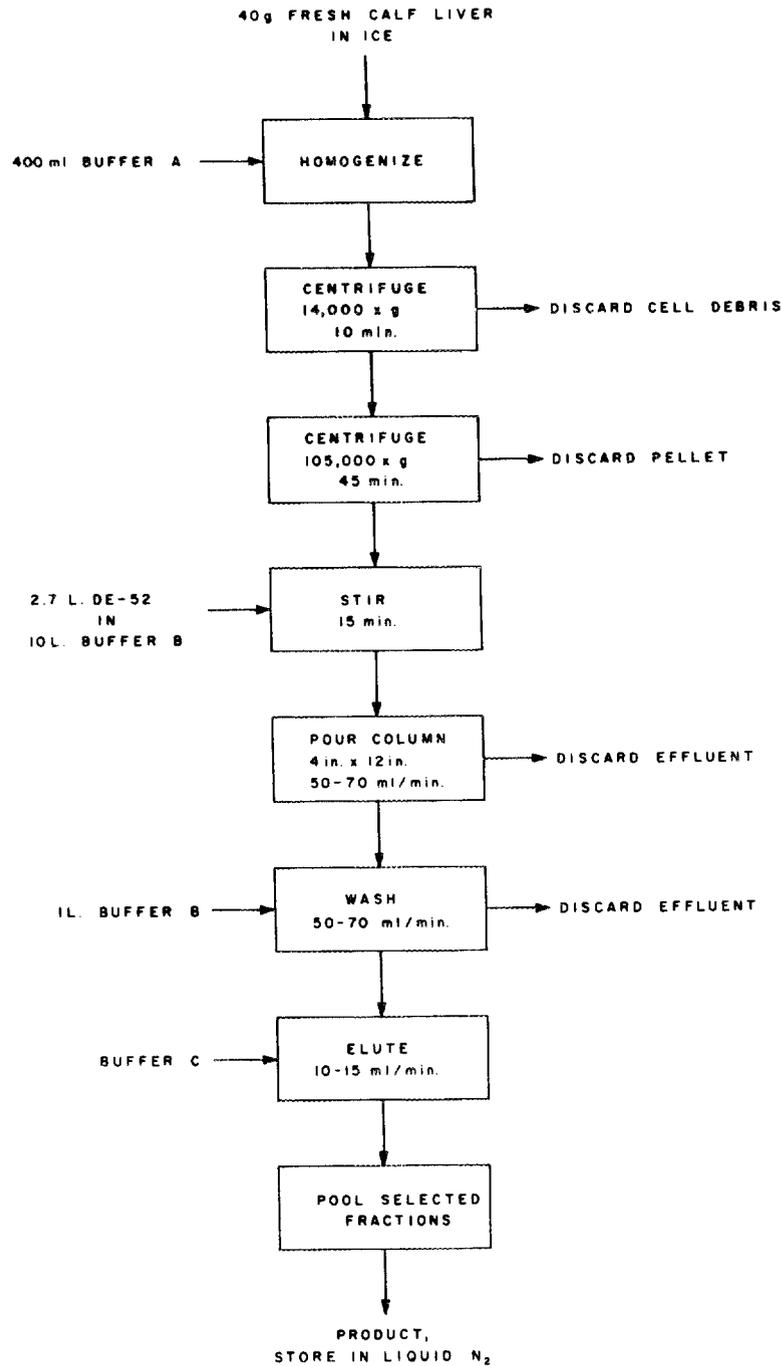


Fig. 2. Modified Flowsheet for the Preparation of Calf Liver Aminoacyl-RNA Synthetase.

ORNL DWG 71-3810

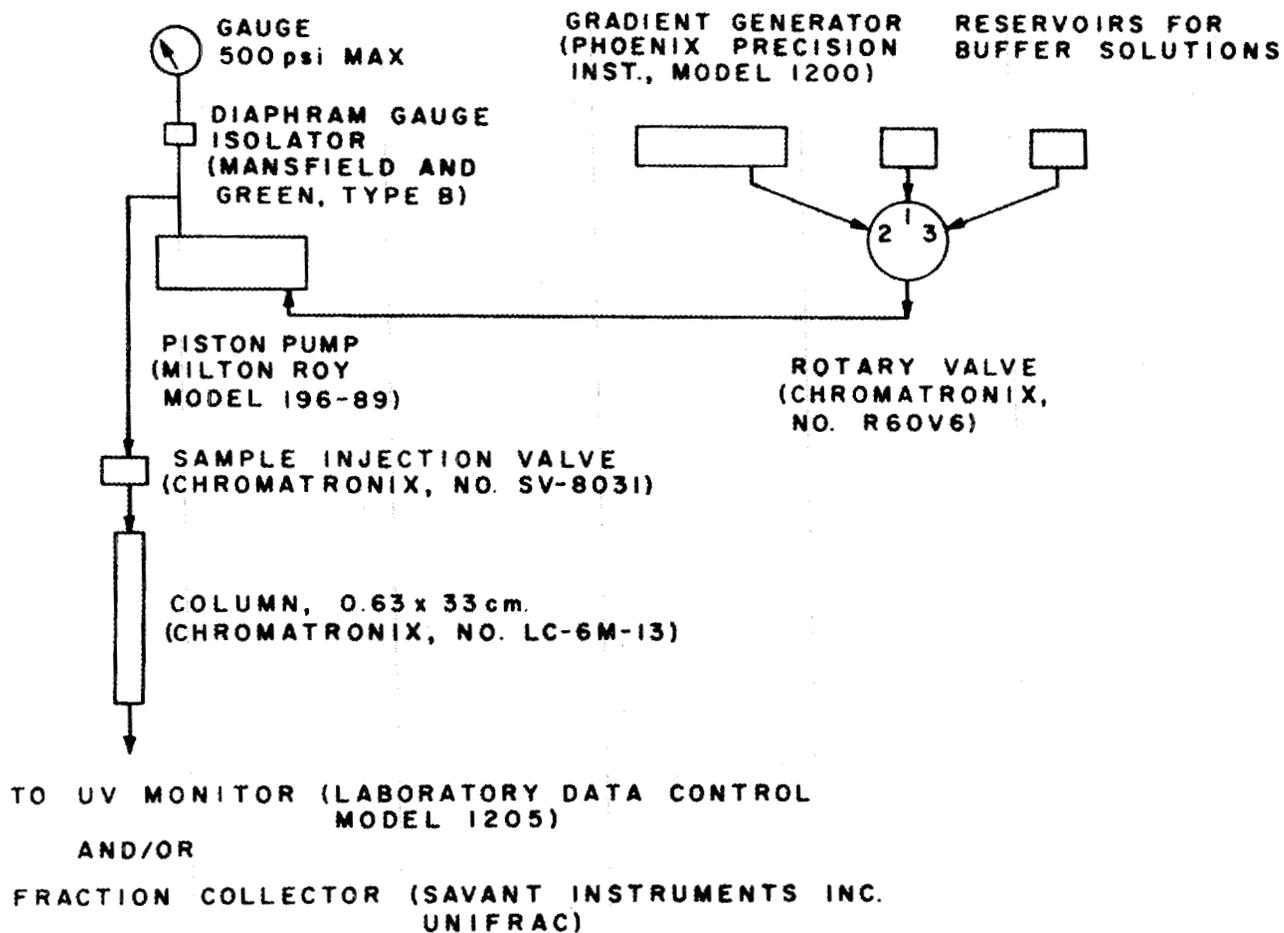


Fig. 3. Experimental Apparatus Used in Runs with Small RPC-5 Columns.

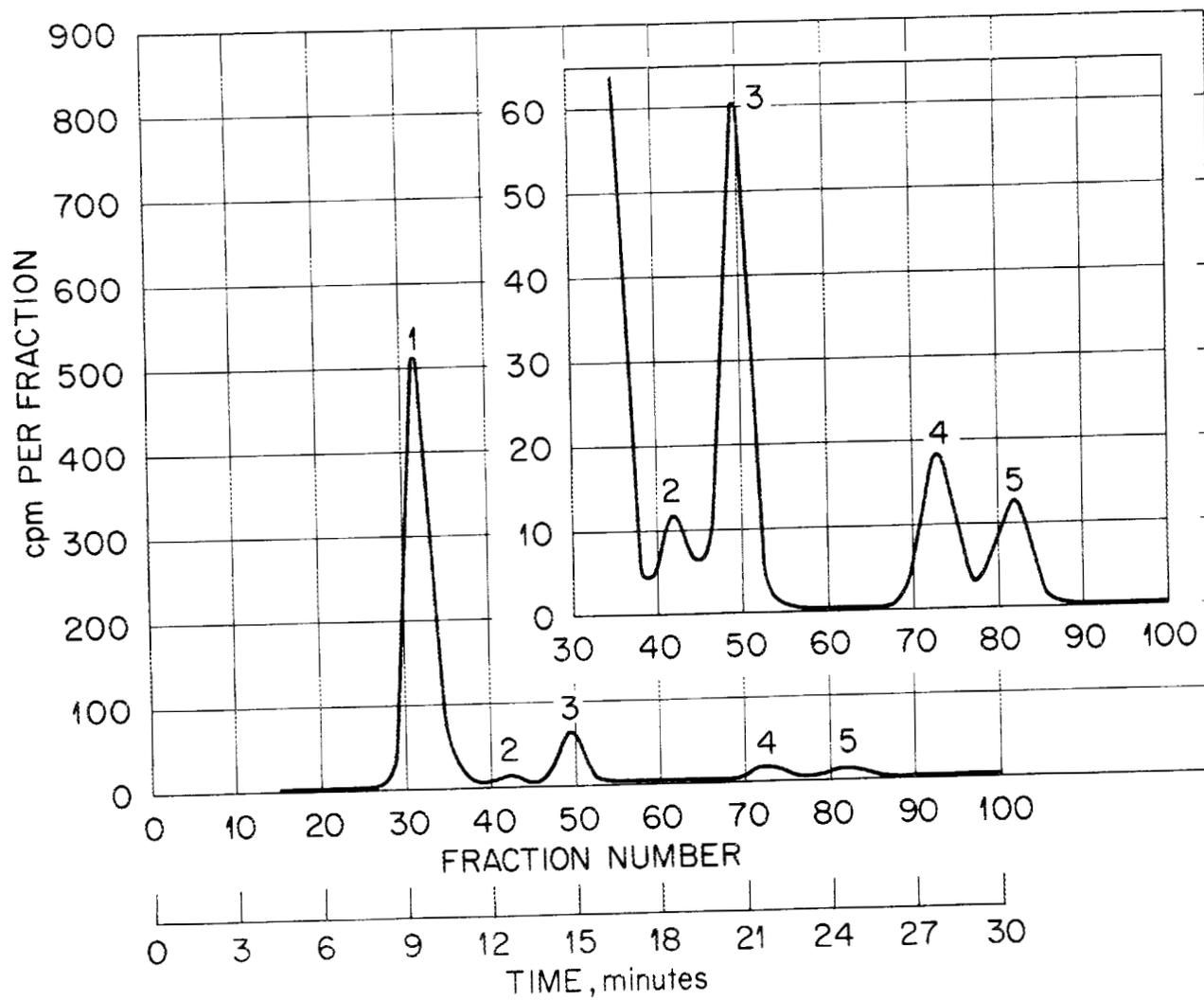


Fig. 4. Chromatogram of  $[^3\text{H}]$ Leucyl-tRNAs from *E. coli*.

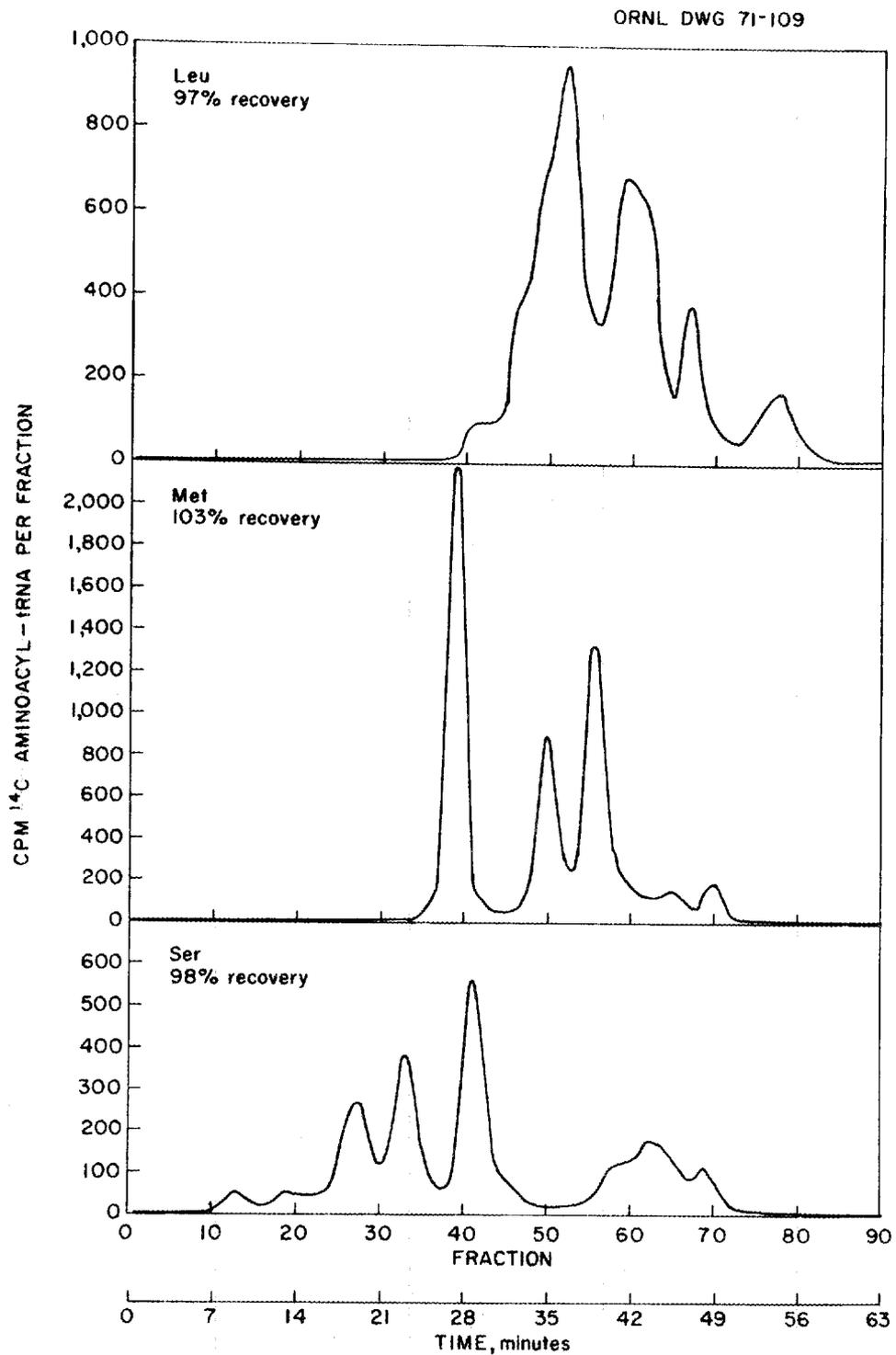


Fig. 5. Chromatograms of [<sup>14</sup>C]Aminoacyl-tRNAs from Calf Liver.

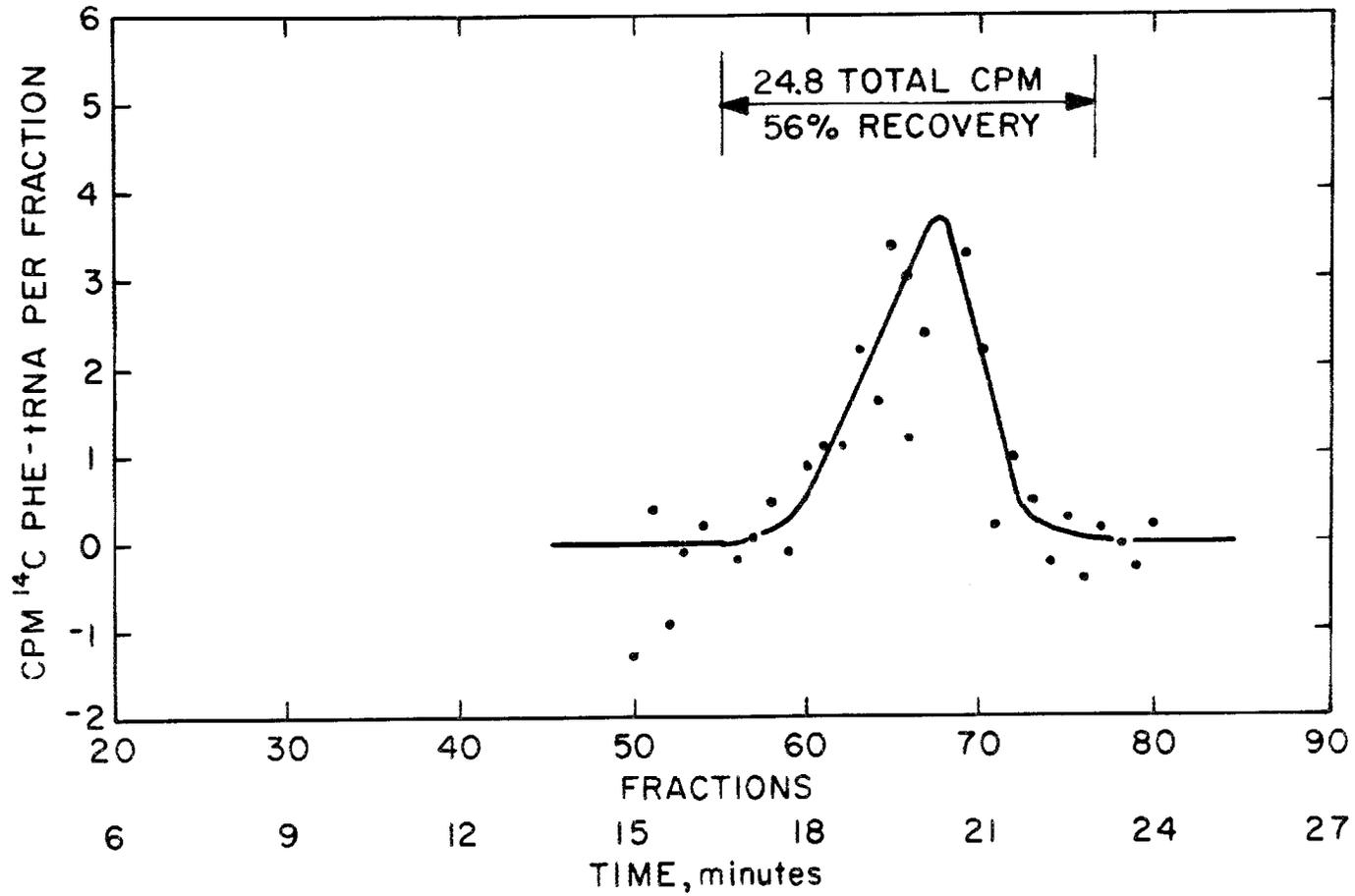


Fig. 6. Chromatogram of Minimum Quantity of [ $^{14}\text{C}$ ]Phenylalanyl-tRNA from E. coli.

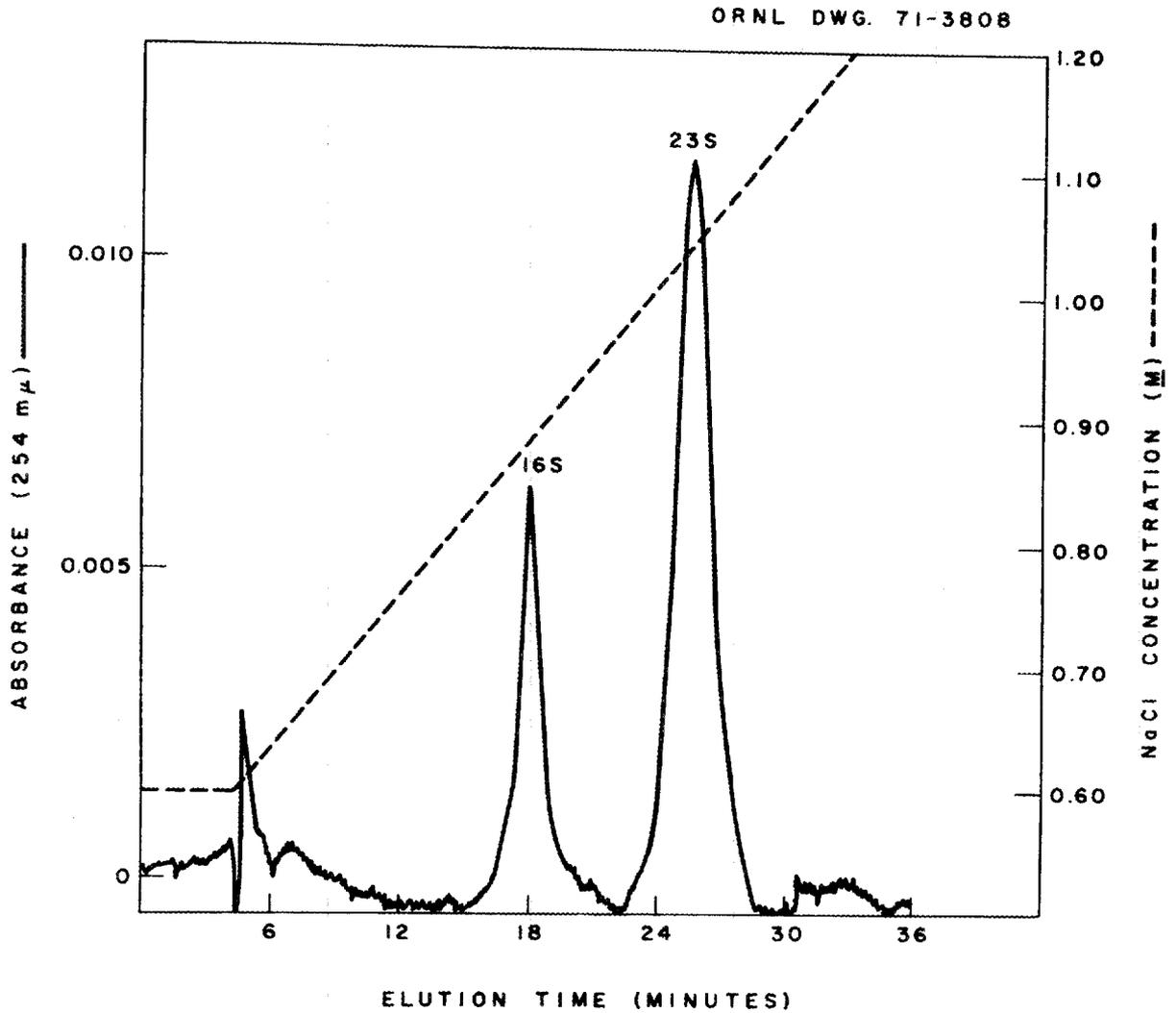


Fig. 7. Chromatogram of 0.1  $A_{260}$  Unit of E. coli rRNA. RPC-5 column, 0.63 cm diam by 30 cm long; temperature, 25°C; flow rate, 1.0 ml/min; eluent: 30 ml containing 0.01 M  $MgCl_2$ , 0.05 M Tris-HCl, pH 7.3, NaCl as shown.

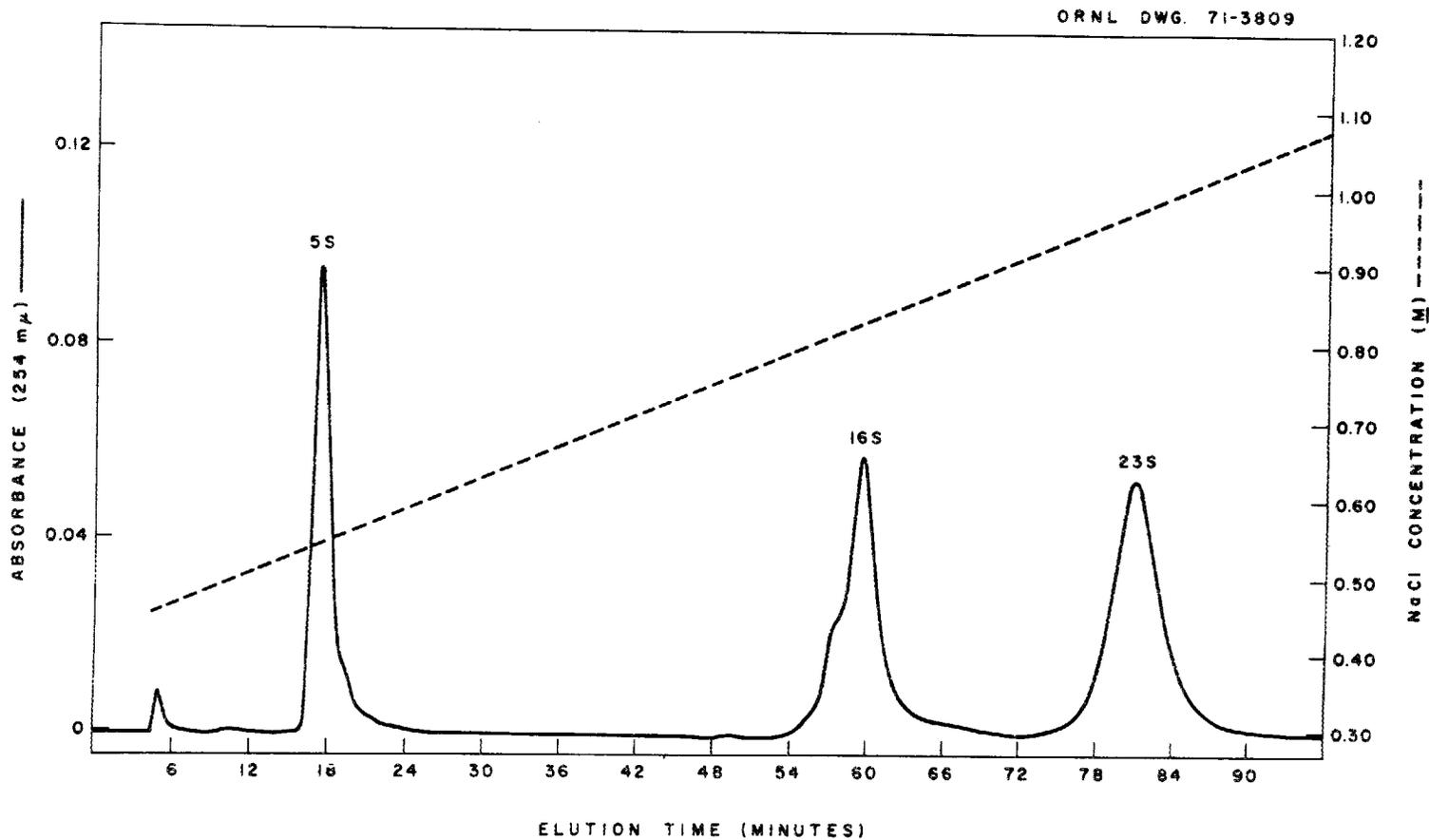


Fig. 8. Chromatogram of a Mixture of 5S, 16S, and 23S rRNA (1.2  $A_{260}$  units, total). RPC-5 column, 0.63 cm diam by 30 cm long; temperature, 25°C; flow rate, 1.0 ml/min; eluent: 100 ml containing 0.01 M  $MgCl_2$ , 0.05 M Tris-HCl, pH 7.3, NaCl as shown.

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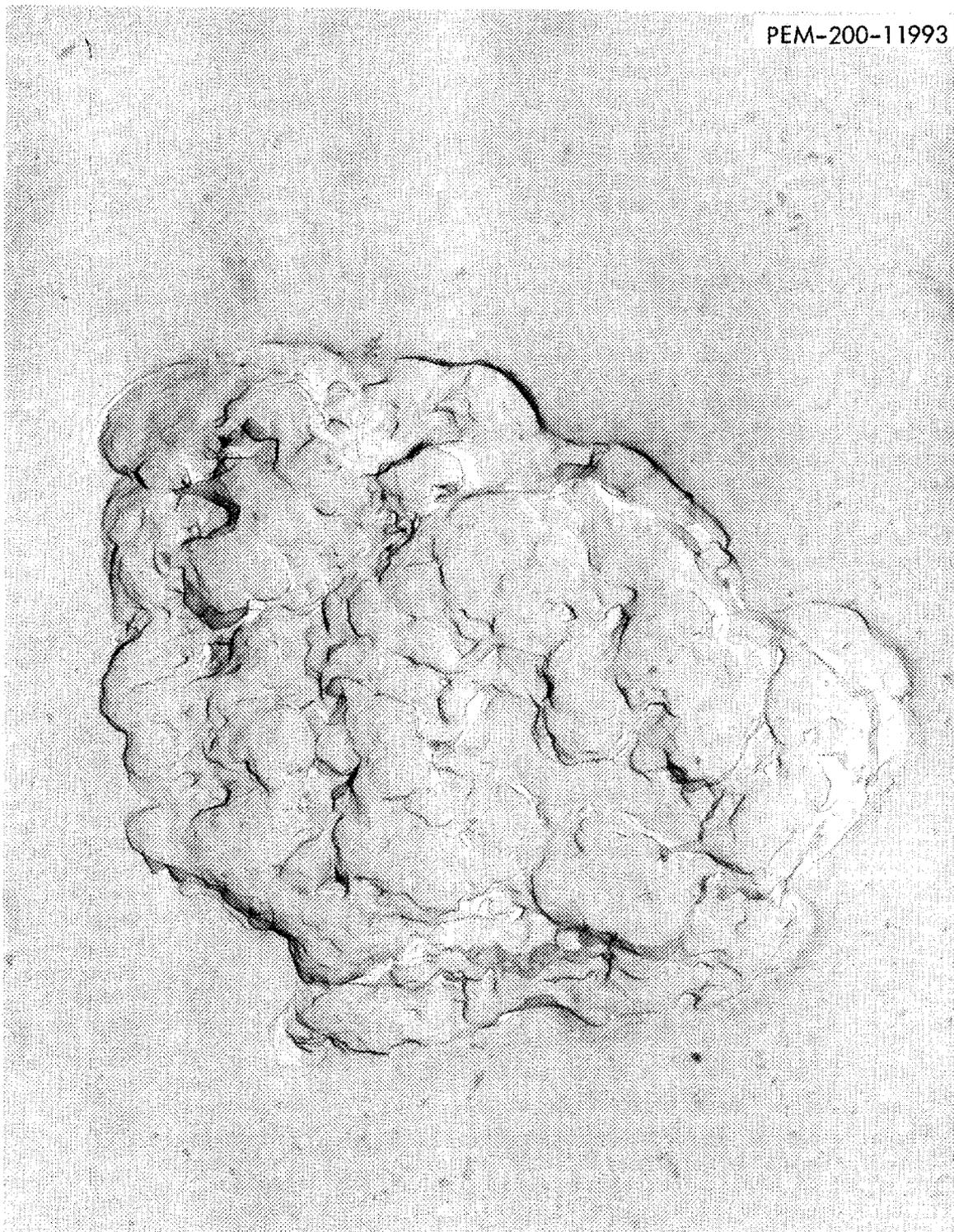


Fig. 9. Particle of Plaskon. Magnification, 10,000 X.



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