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Enzymatic Hydrolosis of 16s Ribosomal RNA and 30s Ribosomal Subunits

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ENZYMATIC HYDROLYSIS OF 16S RIBOSOMAL RNA

AND 30S RIBOSOMAL SUBUNITS

BY

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OF

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1970

ABSTRACT

E. coli 30S ribosomal subunits and protein-free 16S RNA have been mildly hydrolyzed with pancreatic ribonuclease and the RNA fragments analyzed by polyacrylamide gel electrophoresis. The protein-free RNA gives nine discrete fragments and the 30S subunits give six discrete fragments. A comparison of electrophoretic mobilities, indicates that at least three fragments from 16S RNA are distinct from the fragments from 30S. The kinetics of the hydrolysis reaction is pseudo first-order for the protein-free 16S RNA and pseudo second-order for the 30S ribosomes. The rate of hydrolysis of the protein-free 16S RNA is much faster than that of the 30S subunit. These data suggest that in the protein-free 16S RNA there are certain regions exposed to the ribonuclease which are not exposed in the ribosome, and that this is due to either some shielding by the specific proteins or to a different conformation of the RNA in the ribosome.

ACKNOWLEDGMENTS

I am grateful to Dr. Karl A. Hartman, advisor and chairman of the thesis committee, for his constant guidance and support throughout this research. I am also indebted to Dr. Eugene M. Schachter for his invaluable experience with the polyacrylamide gel technique and his enlightening discussions on this work. Thanks are due also to Dr. Paul S. Cohen for his suggestions and corrections during the preparation of the manuscript.

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TO   MARY

who would rather not
look at "those moving
slimy gels."

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I. INTRODUCTION

Ribosomes are cytoplasmic organelles whose function is the
biosynthesis of proteins. In the case of the bacterium Escherichia
coli, they are composed of 63 percent ribosomal ribonucleic acid
(rRNA*) and 37 percent of specific ribosomal protein. The physical
and functional unit, the 70S ribosome, is formed from the association
of a 50S and a 30S subunit. The 50S subunit is, in turn, made up
of two rRNA components (23S and 5S RNA) plus some 36 specific
proteins, while the 30S subunit consists of a 16S RNA component and
some 20 specific proteins. Efforts to elucidate the spatial arrange-
ment of the protein molecules and the conformation (secondary and
tertiary structures) of the RNA in each subunit have not yet provided
a model of the ribosome.

Previous work suggests that protein-free 23S and 16S RNA in
aqueous solution have a secondary and a tertiary structure, and that
the secondary structure consists of helical "hairpin-like" regions
where the bases are paired in a Watson-Crick fashion (1). A fundamental
question in studying the structure of the ribosome is whether the
conformation of the protein-free RNA is the same as in the ribosome.
This problem has been approached with methods such as cation binding,

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*Abbreviations employed here are: rRNA is ribosomal ribonucleic
acid; 70S is a sedimentation coefficient (S is the svedberg unit,
10^{-13} sec.); TMV is Tobacco Mosaic Virus; RNase is a ribonucleolytic
enzyme; pRNase is RNase A from bovine pancreas.

X-ray diffraction, hypochromicity effect, optical rotatory dispersion
(ORD), and enzymatic hydrolysis.

By applying Beer-Lambert's law to the infrared spectra of the
individual nucleotides and paired polynucleotides, it has been indicated
that yeast rRNA at 30°C contains about 60 percent of its bases partici-
pating in double helical regions (2).

No detectable difference has been found in the ability to bind
Ca^{++} and Mg^{++} between protein-free RNA and ribosomes from E. coli
as measured by equilibrium dialysis (3). The authors have concluded
that Mg^{++} binding only occurs at the phosphate groups, and that all of
these groups are available as binding sites for small cations. This
conclusion has lead the authors to suggest that the organization of
the ribosome must be such that the phosphate groups are not directly
involved in the protein-RNA interaction.

Powder patterns from X-ray diffraction studies on isolated rRNA
and ribosomes from rat liver, yeast and E. coli are similar and have
been taken as an indication that the conformation of the rRNA may be
similar in all cases (4,5).

Protein-free rRNA as well as ribosomes from the various
sources investigated have been reported to exhibit equally low
extinction coefficients at 2600 A (property known as hypochromism).
The fact that the hypochromicities of the isolated rRNA and ribosomes
change identically as the RNA is being denatured by heat or by
hydroloysis has been interpreted by the authors as evidence that the
secondary structure of rRNA does not change significantly after

removal of the proteins (6,7,8).

It has also been reported that RNA in rabbit reticulocyte ribosomes give an ORD spectrum similar to that of the rRNA dissociated from the ribosomal proteins. This has lead the authors to suggest that the conformation of rRNA in the ribosome is similar to that in the protein-free state (9,10).

Pancreatic ribonuclease (pRNase) has been used as a probe to investigate the internal organization of ribosomes. It has been established that portions of the rRNA are exposed at the surface of the ribosome instead of being entirely coated by the protein (11) since the 70S ribosome is hydrolyzable by ribonuclease but to a lesser extent than the isolated rRNA. Using gel electrophoresis, it has been shown that specific cleavage is obtained from unfractionated yeast protein-free rRNA by the action of T_1 and pancreatic ribonucleases (12). Likewise, rRNA extracted from purified subunits of rabbit reticulocyte ribosomes and treated with pRNase has been reported to hydrolyze initially into a number of discrete fragments resolvable by gel electrophoresis (13). The authors have suggested that discrete fragments are produced because certain sites in the RNA are preferentially attacked as a result of a specific folding of the polymer chain into a tertiary structure (12, 13).

From the data obtained in previous work, it appears that no conclusive answer can yet be given to the question of whether the conformation of rRNA changes upon removal of the proteins. The error of the ion binding measurements is too large to clearly show that no

phosphate bonds are directly involved in protein-RNA interactions. The quality of the X-ray diffraction data is not sufficiently high to even distinguish between heated-and-cooled TMV RNA and a ribosome (4), and therefore the data cannot be used to detect changes in the conformation of the rRNA. The level of hypochromicity is correlated to the mole fraction of paired bases in the structure and is not sensitive to the positions of the short base paired regions. The ORD curve for different conformation states of a given polymer is only a function of the average helical content and will not discriminate between two different secondary structures with the same percent of helical content. Therefore these spectroscopic methods, if used together, merely analyze the total helical content of rRNA.

The present work is a comparative study of the pRNase mediated hydrolysis of purified 30S subunits and of protein-free 16S RNA from E. coli. The factors underlying this enzymatic approach are first, the presence of sites of rRNA which are specifically hydrolyzed by pRNase, and second, the existence of dissimilar rates of hydrolysis depending on individual steric conditions of the substrate.

pRNase at low concentrations is known to exclusively hydrolyze distal to the phosphate group attached to 3' carbon of the pyrimidine nucleosides in RNA. These residues are assumed to be distributed along the polymer chain as dictated by the primary structure.

It is widely accepted that pRNase principally attacks the single regions of RNA. In addition to this selectivity, the access of pRNase to sites in single stranded regions can be hindered by a tertiary structure of the RNA and the specific or associated proteins

in the ribosome. Therefore the method used here is a probe for single stranded regions of rRNA with pyrimidine residues which are not shielded by protein or by the conformation of the RNA.

For these reasons, this technique would allow me to correlate changes in the rate constants of individual sites with changes in their conformation, as opposed to the techniques that have been used to study the changes in conformation by measuring average structural properties.

Experiments have been designed using mild enzymatic hydrolysis to answer the following questions:

1) Does pRNase hydrolyze protein-free 16S RNA and the RNA in 30S ribosomes into sets of discrete and specific fragments, and if so, what are the molecular weights of such fragments?

2) What is the order of the reaction kinetics for the rRNA in either state?

3) Is it possible to give a simple mechanism for the early stages of the reactions?

4) Are there any differences between the fragments or the rate constants for the rRNA in either state?

5) Is it possible to detect any conformational differences for the rRNA in either state in this in vitro system?

II. EXPERIMENTAL

1. Growth of the Bacteria

E. coli D10 (RNase I⁻) strain, which were kept and propagated in slant tubes with nutrient agar, were used to make inoculant seed cultures in sterile 1.3% tryptone medium containing 0.7% NaCl and 0.2% dextrose. The seed cultures were incubated at 37°C in a gyratory shaker to a final density of 400-500 Klett ($8-10 \times 10^8$ cells per milliliter). Large scale exponential cultures were obtained by inoculating 12-liter volumes of the same medium with the seed cultures to a density of 30 Klett, and then incubating in fermentor at 37°C for two or three hours until cells reached a density of 200 Klett (2×10^8 cells/ml). The carboys were then cooled in ice and the cells collected in a Sharples centrifuge.

2. Preparation of the Ribosomes

All operations involved in the preparation of the 70S ribosomes and its subunits were carried out at 4°C. Pellets of cells were ground with an equal weight of alumina using a mortar and pestle. When the mass was smooth and sticky buffer (0.01 M Mg(Acetate)$_2$, 0.005 M Tris-Cl (pH 7.4, 0.08 M NH$_4$Cl) was added. The bacterial DNA was then hydrolyzed by making the slurry 5 μg/ml in DNase. The resulting slurry was centrifuged at 22,000g (14,000 rpm in a Sorval preparative centrifuge) for 20 minutes to separate alumina and whole cells from homogenate. After two more low speed

centrifugations, the 70S ribosomes were pelleted at 2000,000g (39,000 rpm in angle-40 rotor of ultracentrifuge Model L) for 150 minutes. The ribosome pellets were resuspended in 0.001 M Mg(Acetate)$_2$, 0.03 M KCl and 0.005 M Tris-Cl (pH 7.4).

The 30S subunits were separated from the 50S subunits by zonal centrifugation using a sucrose gradient of the form developed by Eikenberry (14), and approximated by means of a constant volume exponential gradient (Eikenberry, personal communication). The detailed procedure of the approximated exponential gradient adapted for the Beckman Ti-14 rotor is as follows. A Mariotte flask with 900 ml of 50% (w/w) sucrose (Fisher Scientific Co.) feeds a mixing chamber initially containing 295 ml of 7% (w/w) sucrose, and whose volume remains constant throughout the loading of the gradient. The gradient is set in the rotor at 3,000 rpm by pumping into it the output of the mixing chamber. When the rotor has been filled, some 300-500 mg of ribosomes previously placed in a linear sucrose gradient (1 - 7% sucrose in 25 ml volume) are pumped on top of the exponential gradient. An additional volume of 240 ml of buffer is pumped on top of the sample, 40 ml of which are afterward discarded by pumping a 40 ml cushion of the heavy sucrose onto the bottom of the gradient. After 4 hours of centrifugation at 45,000 rpm the 50S and 30S particles reached good separation. The speed was then reset at 3,000 rpm, and the gradient unloaded by injecting 600 ml of 50% sucrose and collected in 10 ml fractions. The absorbance of the fractions was measured at 3000 A.

The resulting concentration profile facilitates the pooling of the fractions containing 30S and 50S respectively. Subunits thus obtained were dialyzed and concentrated in a single operation using ultrafiltration in an Amicon cell with a UM-20E membrane. Upon several additions of a buffer containing 0.001 M Mg(Acetate)$_2$, 0.005 M Tris-Cl (pH 7.4), the concentration of K$^+$ was lowered by a factor of 500, the sucrose was lowered to less than 0.1% and the concentration of the 30S ribosomes was adjusted to an absorbance (2600 A) of thirty. Then the solution was aliquoted, frozen in liquid nitrogen, and stored at -15°C, ready to be used for enzymatic hydrolysis.

3. Preparation of 16S RNA

A 30S ribosome solution of concentration not greater than 2 mg/ml was deprotenized with phenol at room temperature according to the following procedure. One volume of water-saturated phenol (24°C) was shaken for 10 minutes with one volume of the ribosome suspension, and the aqueous phase separated after centrifugation. The ionic strength of the aqueous phase was then increased with 1/10 volume of 20% Na Acetate and the RNA precipitated at -15°C with 3 volumes of ethanol. After the white precipitate was sedimented at 22,000g for 20 minutes, the pellet was redissolved in about one volume of 0.005 M Tris-Cl (pH 7.4), and the RNA was again precipitated with ethanol after the addition of 20% Na Acetate . The precipitation step was done three times. This RNA, free of phenol, was dissolved in

0.005 M tris to an absorbance (2600 A) of 30 and stored at -15°C in small aliquots.

By extracting unfractionated RNA with phenol as a function of the concentration of the RNA, it was possible to show that the ratio of 16S to 23S was abnormally low for input concentrations greater than 1.5 mg/ml. Best recoveries of 16S RNA were obtained working at 1 mg/ml or less, and using one volume of phenol at room temperature.

4.   The Ribonuclease A

Ribonuclease A (bovine pancreas) from Worthington Co. was dissolved in 250 Micro grams per milliliter in 0.005 M Tris-Cl (pH 7.4, double distilled water used). Stock solutions from 250 to 25 μg/ml, made at room temperature in pyrex glass and stored at 4°C in pyrex vials, gave constant activity over a period of months. Dilutions of low concentrations of enzyme were not stored because they were found to lose activity in a matter of hours.

5.   The Hydrolysis

Hydrolysis of 30S ribosomes and 16S RNA was performed at enzyme-to-substrate molar ratios of 0.01 to 0.001, and at 0°C. The ionic conditions chosen, 0.005 M Mg(Acetate)$_2$, 0.005 M Tris (pH 7.2), were those found to give a maximum rate of RNA hydrolysis (15). A typical hydrolysis kinetics point was obtained by mixing in a 6 x 50 mm Kimax culture tube the following volumes: 20 microliters (λ) of aliquoted RNA, 10λ of 0.005 M Tris buffer, 10λ of 0.025 M Mg(Acetate)$_2$ (in Tris buffer), and 10λ of 0.01 mg/ml of RNase A. At a given time

between 0 and 60 minutes of incubation, the reaction was stopped by adding 10λ of 2% sodium dodecylsulfate (SDS;Matheson, Coleman & Bell Co.) and the tube stood at room temperature afterward. The density of the reacted sample was increased with a few grains of sucrose.

Zero-time controls with and without RNase showed that hydrolysis did not continue in the presence of 0.4% SDS even at room temperature for several hours. To test whether the adsorption of RNase to the glass was significant, controls were also run in polyethylene tubes, and the results showed no significant difference in activity.

6. The Polyacrylamide Gels

Acrylamide, N,N'-methylene bisacrylamide, and N,N,N',N' – tetramethylethylenediamine (T.M.E.D.) were purchased from Eastman Chemicals. The gels were polymerized and kept in E buffer (0.001 M EDTA, 0.02 M Na(Acetate), 0.04 M Tris-Cl pH 7.2).

I found that the method of Bishop (16) for the preparation of polyacrylamide gels for the analysis of RNA can be modified for the sake of simplicity. His method calls for the recrystallization of the acrylamide and the N,N' -methylenebisacrylamide, and for presoaking the gels for several days. Gels of recrystallized materials were made and presoaked for about ten days. Gels of un-recrystallized materials were also made and allowed to presoak for 8 and 24 hours. Then the three kinds of gels were scanned versus distance along gel in the ultraviolet between 2600-2900A, before and after passing a 5-mAmp current through them in the electrophoresis buffer (prerun). No

significant difference was found in the background level of all gels after 20 minutes of prerun. All of the scans had a smooth, low and flat background, except for the gels with long presoaking which probably exhibited some light scattering from dust.

For a typical batch of 48 gels of 4.4% acrylamide, the polymerization reaction was started in a 250 ml flask in the following manner: 42.5 ml of 15% (w/v) acrylamide, 30 ml of 0.725% (w/v) of bisacrylamide, 7.3 ml of 20xE buffer, and 64 ml of H_2O were first mixed and degassed for 30 seconds. Then 60λ of T.M.E.D. solution was added followed by the addition of 1.2 ml of freshly made 10% (w/v) aqueous solution of ammonium persulfate. Immediately after, 3 ml aliquots were placed in vertical plexiglass tubes of 1/4" internal diameter. The solution was polymerized for 30 minutes, and the gels were then extruded and soaked in E buffer for a minimum of eight hours.

7. Electrophoresis of the RNA

The gels to be used in an electrophoretic run were cut to 10.5 cm in length, leaving the ends flat and straight. Before loading the sample, a 5 mAmp/gel current was passed through the gel for 30 minutes (pre-run) in E-SDS (0.2% SDS) buffer. The total reacted volume (little over 60) was loaded onto a gel, and then electrophoresed with a current of 5 mAmp/gel for 3 or 3.5 hours using a Canalco disc-electrophoresis instrument.

8. Reading the Gels

The gels were scanned for absorbance at 2600 A in a DU-Gilford spectrophotometer with mechanical gel scanner and recorder. Quantitative

analysis of the products of the reaction was done by both cutting the
tracing of the recorder and weighing the areas, and by direct measure-
ment of the heights of the bands. To supplement the results from the
UV-scan, gels in 0.2% SDS were first washed in distilled water in a
test tube for 24 hours (with one change of water) and then stained with
one-tenth volume of 0.2% methylene blue in 0.4 M acetate buffer
(pH 4.7). The staining could be stopped anywhere between 4 and 8 hours
by placing the gel in a test tube with distilled water, with no need
of further washing.

III. RESULTS

Protein-free 16S rRNA was mildly hydrolyzed with 0.02 μg/ml of pRNase, and the products of the reaction were analyzed in a poly-acrylamide gel to give the pattern shown in figure 1. Nine product bands even at the earliest kinetic points were observable by either staining the gel with methylene blue or by scanning the gel at 2600 A. These nine bands correspond to nine discrete RNA fragments whose molecular weights have been calculated from their individual mobil-ities (16) and reported in table 1.

In order to follow a brief history of the bands of figure 1, I have arbitrarily divided the course of mild hydrolysis into two stages according to the remaining amount of input RNA namely, when intact 16S RNA remaining is 25% or more, and when it is less than 25%. Toward the middle of the first stage most of the new bands were observed to attain maximum height and best resolution. In the second stage, band 16A lost considerable height, the general background was raised, and the other bands lost their original shape. Bands 16A and 16G remained particularly prominent until the onset of the second stage (figure 1b, 1c).

30S ribosomal subunits, in ionic conditions identical to those used for the protein-free rRNA, were midly hydrolyzed with 0.4 μg/ml of pRNase. The products of the reaction were deproteinized with SDS, and then analyzed in a polyacrylamide gel to give the pattern shown in figure 2. Six small bands were observable by either staining the gel

with methylene blue or by scanning the gel at 2600A. Bands 30A and 30B appeared almost fused into one broad and flat band, but were well resolved bands in the stained gels. Notice the 30C and 30D are the highest bands among the products. The molecular weights of the RNA fragments range between 0.5 and 0.02 times that of the 16S, and their values are given in table 2.

By comparing the gel patterns of the RNA fragments from protein-free rRNA and 30S subunits, two major observations were made. a) Upon reaction with the ribonuclease, the protein-free RNA generated more fragments than the RNA in the ribosome. b) The bands that exhibited greater absorbance did not correspond to the larger molecular weight, but rather to some intermediate molecular weight fragments.

The kinetic study of the hydrolysis of RNA was done by measuring the height of the 16S band of the gel pattern as a function of time since the height of the band is directly proportional to the concentration of unhydrolyzed 16S RNA. In order to learn the order of the kinetics of the reactions, the data from both protein-free 16S RNA and 30S ribosomes were treated as a zeroth-order, first-order, and second-order reaction with respect to the substrate. The rate of decomposition of a reactant that follows zeroth-order kinetics obeys the rate equation $dC/dt = -k_o$, whose integrated form,

$$C_o - C_t = k_o t \qquad \text{Equation 1.}$$

represents a straight line of slope k_o. In turn, the rate of decomposition of a reactant that follows first-order kinetics obeys the rate equation $dC/dt = -k_1 C$, whose integrated form,

$$\ln C_o/C_t = k_1 t \qquad \text{Equation 2}$$

thus plotting $\ln C_o/C_t$ versus t gives a straight line of slope k_1.

If the decomposition of a reactant follows second-order kinetics, then

the rate equation is $dC/dt = k_2 C^2$, whose integrated form,

$$1/C_t - 1/C_o = k_2 t \qquad \text{Equation 3}$$

thus plotting $(\frac{1}{C_t} - \frac{1}{C_o})$ versus t gives a straight line of slope k_2.

Pseudo first-order and pseudo second-order rate constants (k_1', k_2')

which are proportional to the enzyme concentration, are commonly used

for enzyme-catalyzed reactions.

The kinetic data for the hydrolysis of protein-free 16S RNA

best fit equation 2 for the first stage of the reaction (figure 3b).

For an enzyme concentration close to 0.01 μg/ml, the pseudo first-order

rate constant (k_1') was estimated from the slope of figure 3b to be

equal to 2.6×10^{-2} min^{-1}, and the corresponding half-life for the

protein-free 16S RNA was approximately 25 minutes. When the same

set of data was treated according to the zeroth-order and second-order

rate equations, curved lines were observed to fit the points (figure 3a).

It was observed that the order of the reaction could change if the

extent of hydrolysis passed the first stage.

The kinetics for the hydrolysis of 30S ribosomes with 0.4 and

0.8 μg/ml pRNase was found to fit a straight line according to

equation 3 (figure 4c). Starting with an input of 0.5 mg/ml of the

substrate, the pseudo second-order rate constants were 8.6×10^{-2}

and 17.2×10^{-2} ml/mg·min, and the half-life times were 24 and 12

minutes for 0.4 and 0.8 μg/ml of pRNase respectively. Attempts to

fit these data to equations 1 and 2 for zeroth-order and first-order kinetics respectively, gave the curves shown in figures 4a, b.

When 30S ribosomes and 16S RNA were treated at RNase concentrations which largely hydrolyzed 16S RNA, little or no hydrolysis of the 30S ribosomes was observed (figure 3b). It was therefore necessary to employ for the ribosomes RNase in concentrations 20 to 40 times that needed for the protein-free RNA.
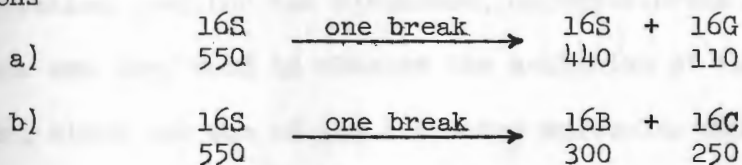
IV. DISCUSSION

The observation that mild hydrolysis of protein-free 16S rRNA results in a number of discrete fragments suggests the RNA has a tertiary structure. The appearance of discrete fragments of rRNA upon mild hydrolysis has been already reported for yeast rRNA and fractionated E. coli rRNA (12), rabbit reticulocyte 19S rRNA (13), and for 23S rRNA from E. coli (17). Evidence is given that a tertiary structure assumed by the RNA is selectively exposing the few sites that are primarily hydrolyzed while there exist many other sites which remain temporarily "guarded" by the tertiary structure of the RNA. The seven larger fragments (16A - 16G), produced during the first stage of mild hydrolysis of the protein-free RNA, are equally hydrolyzable into smaller fragments during the second stage of the reaction (figure 1d). It has been reported (13) that during the course of mild hydrolysis of rRNA, the fragment pattern changes as the large fragments are hydrolyzed into smaller fragments which, in turn, originate new bands. Furthermore, small fragments can be produced by the action of pRNase (about 2S) of rRNA which are known to contain both helical and single stranded regions (19). Therefore, the presence of many hidden sites strongly suggests that there is a tertiary structure in the protein-free 16S RNA determining, over the dictates of the primary and secondary structures, which sites are most likely to be hydrolyzed.

By examination of the molecular weights in table 1, it is possible to suggest a mechanism for the hydrolysis of protein-free 16S rRNA. It is likely that fragment 16A (M.W. 440,000) proceeds from the 16S

by a one-break reaction since that 16A has a very high molecular weight, and the enzyme-to-substrate molar ratio in this hydrolysis is very low (about 1/1000). Making also the consideration that the absorbance of RNA fragments of molecular weight less than 100,000 is quite low, it is then reasonable to expect from the reaction an RNA component with a molecular weight in the neighborhood of 110,000. Fragment 16G (M.W. 130,000) fits best the desired condition and, with its molecular weight reassigned as 110,000, it could be used as a marker together with 16S to correct the weights of the intermediate fragments. Taking the corrected molecular weights of table 1, I propose that the hydrolysis in question could be initiated by, at least, two simple parallel reactions

a) $\quad$ 16S $\quad$ one break $\quad$ 16S + 16G
$\qquad$ 550 $\qquad\qquad\qquad$ 440 $\quad$ 110

b) $\quad$ 16S $\quad$ one break $\quad$ 16B + 16C .
$\qquad$ 550 $\qquad\qquad\qquad$ 300 $\quad$ 250

The RNA fragments shown in figure 2 suggest that the 30S sub-unit has a definite conformation. Two features of figure 2 deserve to be discussed. a) The fact that bands 30C and 30D are the highest bands could be due to a major accumulation of these fragments or to a high content of single stranded regions in the fragments. b) All of these are small bands which are observed to appear simultaneously even at the earliest kinetic points thus ruling out the accumulation of some intermediates.

For making a comparison between the fragment patterns of figures 1 and 2, it should be considered that the conformation of the rRNA in the ribosome determined by the interactions present between the

rRNA and the ribosomal proteins. It is apparent from figures 1 and 2 that there are fewer sites available in the ribosome. Bands 16A, 16B and 16C, of figure 1, are clearly not present in figure 2. A narrow site on the RNA might be strategically exposed after the removal of the proteins so that fragments 16A and 16G could be produced. It is possible that if three out of the nine fragments in figure 1 do not coincide with any of those in figure 2 at least 3 and perhaps 6, or even all 9, of them are characteristic of the protein-free rRNA.

Since no two RNA fragments from the hydrolysis of the subunit add up to 550,000 daltons, it is concluded that no single-break reaction could be detected in the present conditions. At the high enzyme concentration used for the ribosomes, multiple-break reactions are more probable and they tend to obscure the mechanism of the total reaction. However, since the sum of the corrected molecular weights (table 2) of fragments 30A through 30F equals 583,000 daltons, it is possible to suggest at least one mechanism for the total reaction. The mechanism would require the existence of n neighboring sites which originate n equally probable reactions on the ribosome. A model of the 30S ribosome that makes the mechanism feasible could be one having a large surface protected by protein, and a "cavity" by which the enzyme could reach unprotected regions of the RNA. This interpretation would imply that the reaction for the entire set of sites would occur by random collisions while n -1 of the sites would not react independently, as if the enzyme remained "trapped" in a cavity after the first reaction.

The hydrolysis of protein-free 16S RNA follows first-order kinetics and the hydrolysis of 30S ribosomes is found to fit a second-order rate equation when the half-lives of both substrates are comparable. There is not a clear cut differentiation between the first, second and zeroth-order plots for the rate data for 16S. This is still unexplained although the complexity of the reaction and the particular degree of saturation of the enzyme may determine this behavior. The several sites open to hydrolysis in the protein-free RNA may originate parallel reactions of diverse order which, on the average, follow first order kinetics. The order of the kinetics for 16S RNA may be a function of the extent of the reaction. On the other hand, the hydrolysis of 30S ribosomes was not done at saturation of the enzyme (figures 4a, b), and the order of the kinetics did not change with the extent of the reaction. It should be mentioned here that hydrolysis of 50S subunits and 23S RNA, under conditions identical to those for the 16S RNA, were found to follow first-order kinetics(17).

The hydrolysis rate of the protein-free 16S rRNA is substantially greater than that of the 30S ribosomes. In order to make the half-lives about 25 minutes, the subunits required a 40-fold increase of the ribonuclease. This striking difference between the 30S subunit and 16S RNA contrasts with the fact that the 50S subunit has a first-order rate constant equal to twice that of the 23S RNA (17).

The RNA in the 30S ribosomal subunit is mostly protected from the action of the ribonuclease. The significantly smaller hydrolysis rate of the ribosomes could be partly a result of shielding by the

proteins, and by an increase in the energy of activation of the reaction caused by a change in conformation due to the protein-RNA interactions.

It is not clear yet whether the conformation of 16S rRNA in the protein-free state is different from that in the ribosomal state, however, data presented here indicate that they are not necessarily the same. Two facts are underlying this statement. 1) at least, fragments 16A, 16B, and 16C do not appear in the hydrolysis of the subunits indicating that there exist regions open to hydrolysis in the protein-free state which are not available in the ribosomal state. 2) The hydrolysis rate of the 30S subunit is low enough to suspect that the RNA may undergo conformational change upon removal of the proteins.
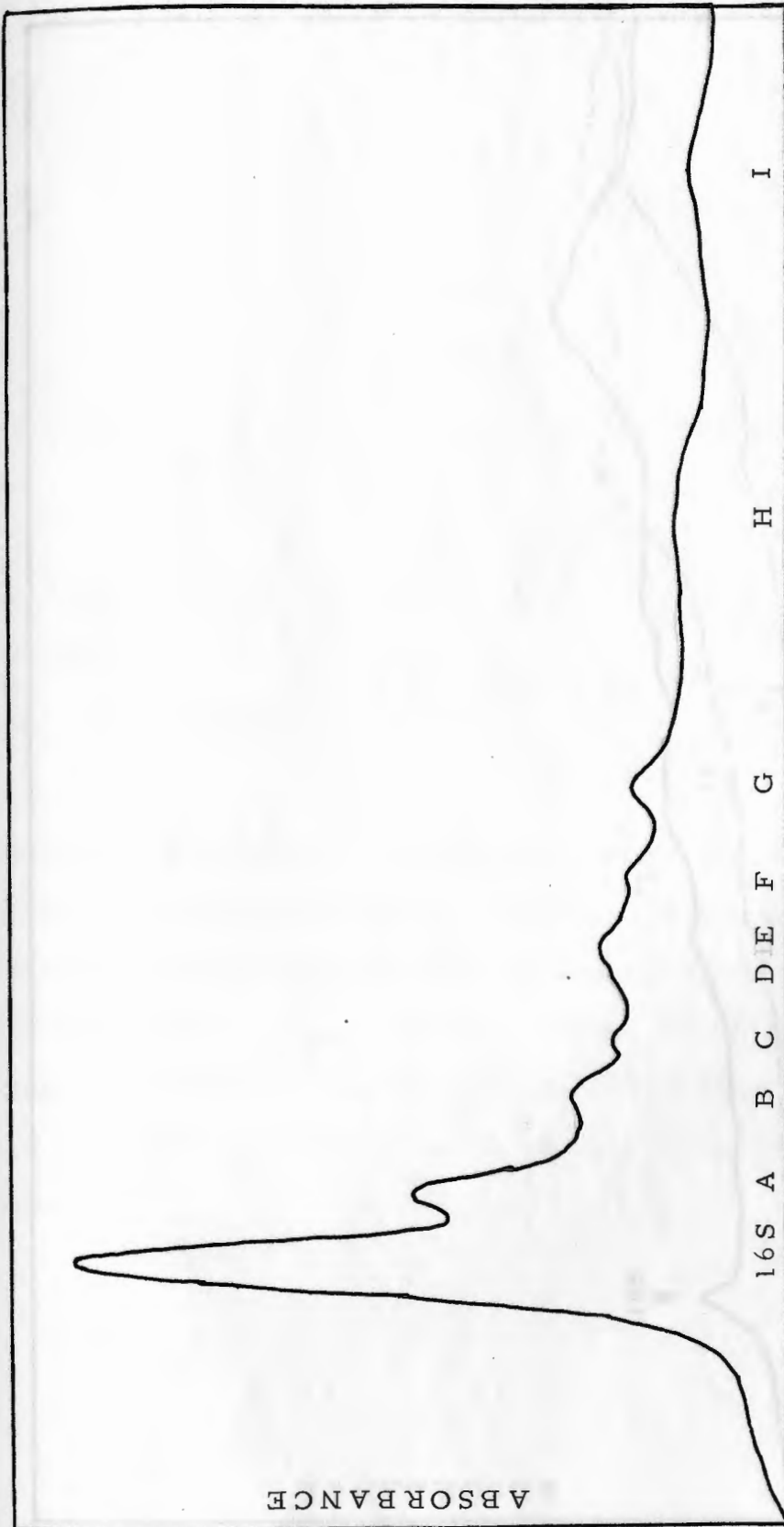
Figure 1

Mild hydrolysis of 16S rRNA

a. Input RNA in a 4.4% polyacrylamide gel. Single band of completely
    undigested protein-free 16S rRNA to which SDS was added before
    the pRNase.

b. Fragment pattern corresponding to the middle of the first stage
    of mild hydrolysis of protein-free 16S rRNA. The reaction was
    carried out with 0.02 μg/ml of pRNase.

c. Fragment pattern in the second stage of mild hydrolysis of
    protein-free 16S rRNA. Note the differences of this pattern as
    compared to that in figure 1b.

d. Fragment pattern following the second stage of mild hydrolysis of
    protein-free 16S rRNA. The larger molecular weight fragments
    further disappear by slower reactions suggesting the presence
    of more hydrolyzable sites.
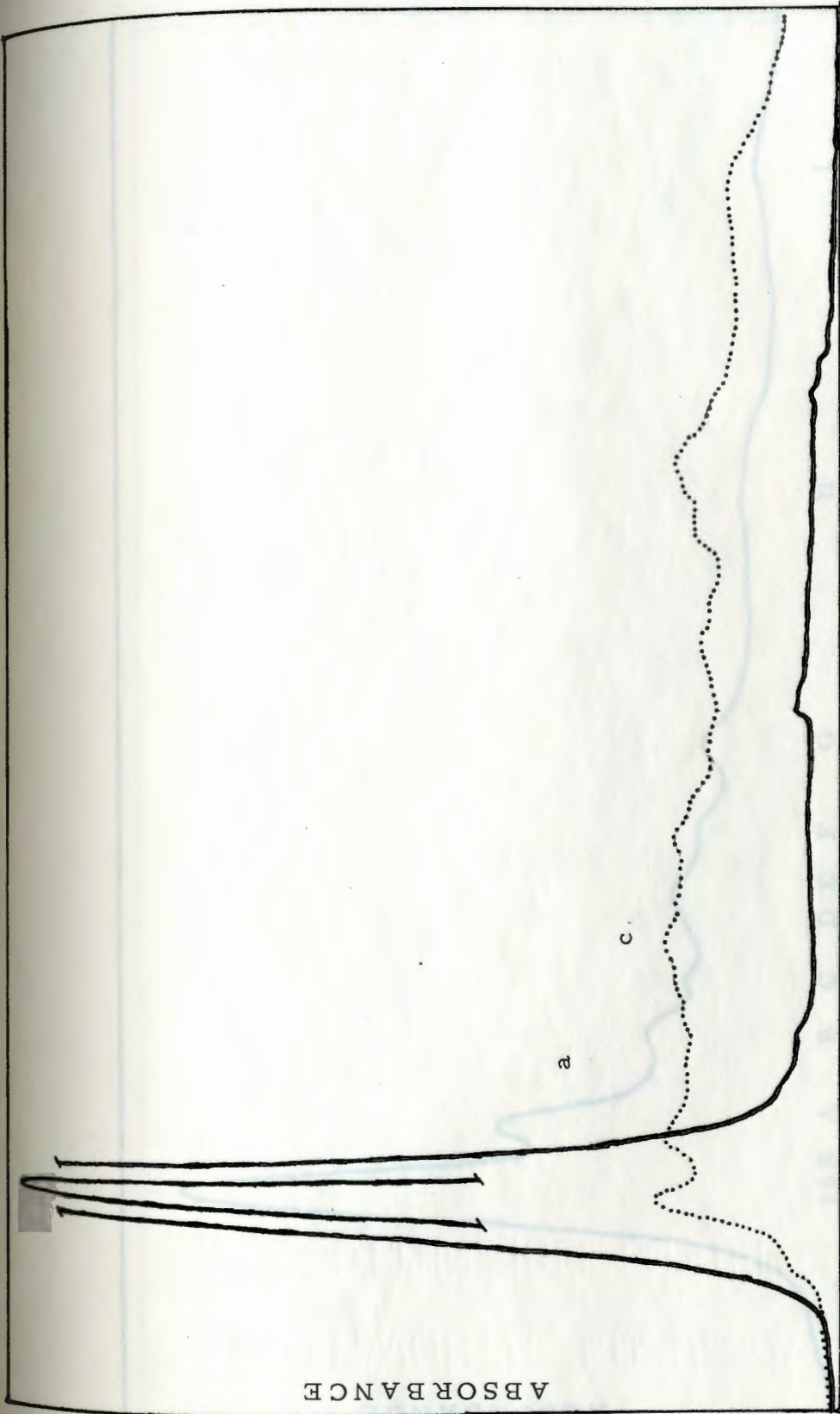
DISTANCE

Figure 1a, c

DISTANCE

Figure 1b
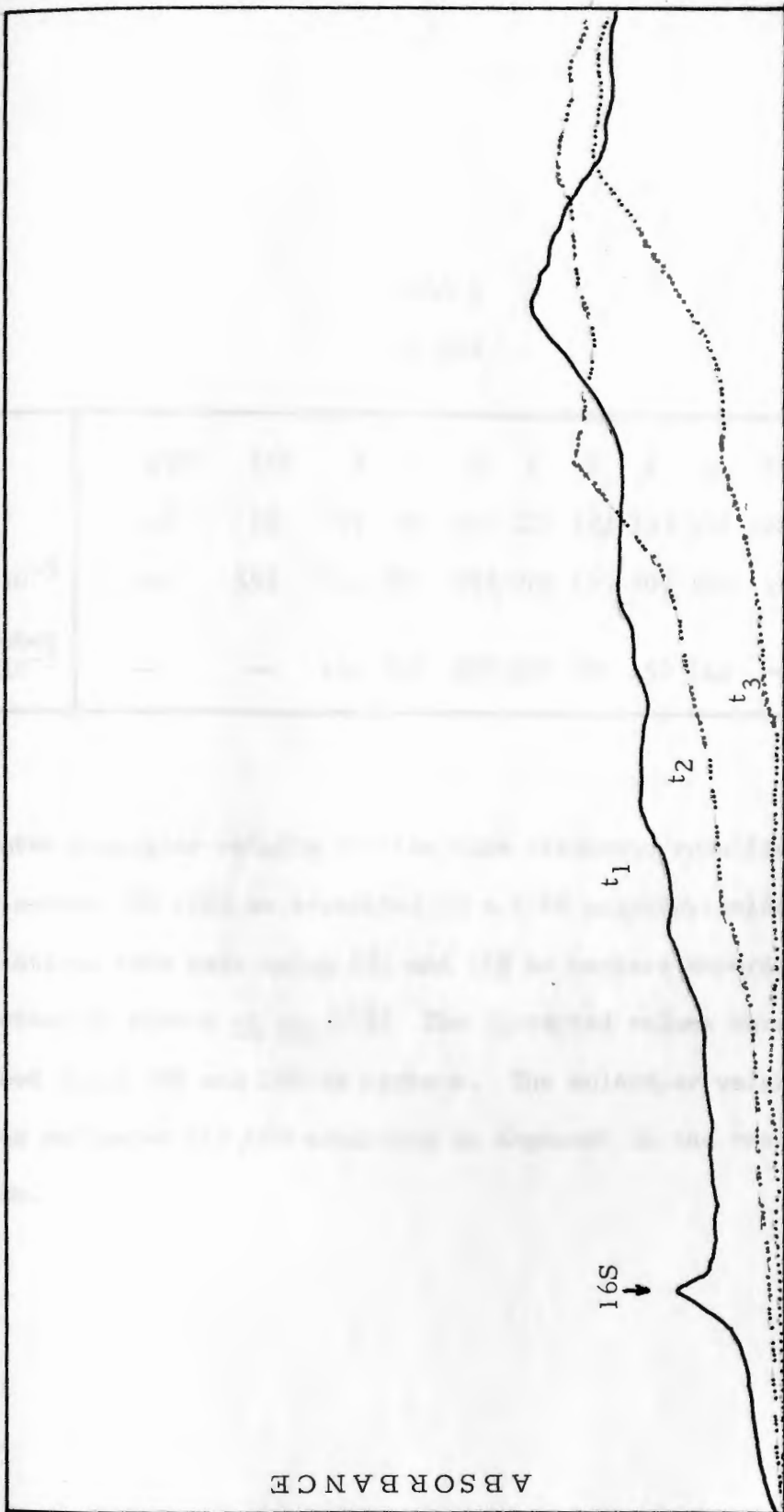
ABSORBANCE

DISTANCE

16S

t_1

t_2

t_3

Figure 1d

TABLE 1

16S RNA

Band	23S	16S	A	B	C	D	E	F	G	H	I
mm	12	66	77	95	101	114	122	133	148	192	251
M.W.x10^{-3}	1,100	550	450	325	288	225	195	160	120	51	18
corrected M.W.x10^{-3}	—	—	440	300	250	210	160	150	110	—	—

Estimated molecular weights for the nine fragments resulting from
protein-free 16S rRNA as separated in a 4.4% polyacrylamide gel.
Calculations were made using 23S and 16S as markers according to
the method of Bishop et al. (16) The corrected values were
obtained using 16S and 16G as markers. The molecular weight of
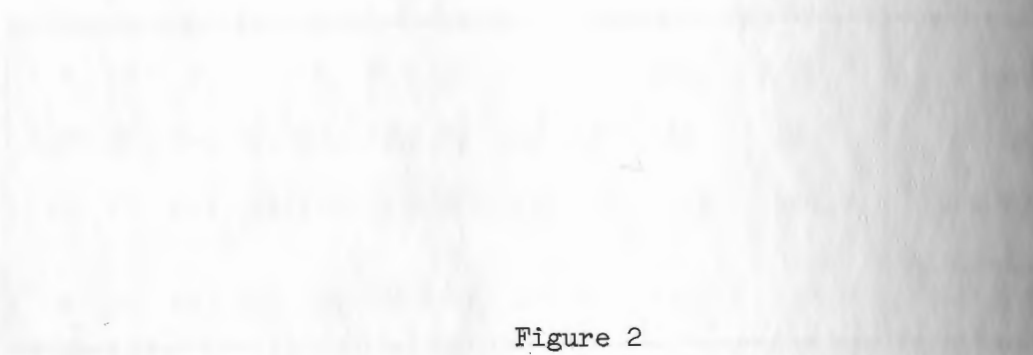16G was estimated 110,000 according to argument in the results
section.

Figure 2

Fragment pattern from the hydrolysis of 30S
ribosomal subunits as analyzed in a 4% poly-
acrylamide gel. The product bands display
maximum height attained during mild hydrolysis.
The reaction was carried out with 0.4-0.8
μg/ml of RNase. The RNA from input ribosomes
gave a single band undistinguishable from that
in figure 1a.

DISTANCE

Figure 2

ABSORBANCE

16S 30A 30B 30C 30D 30E 30F
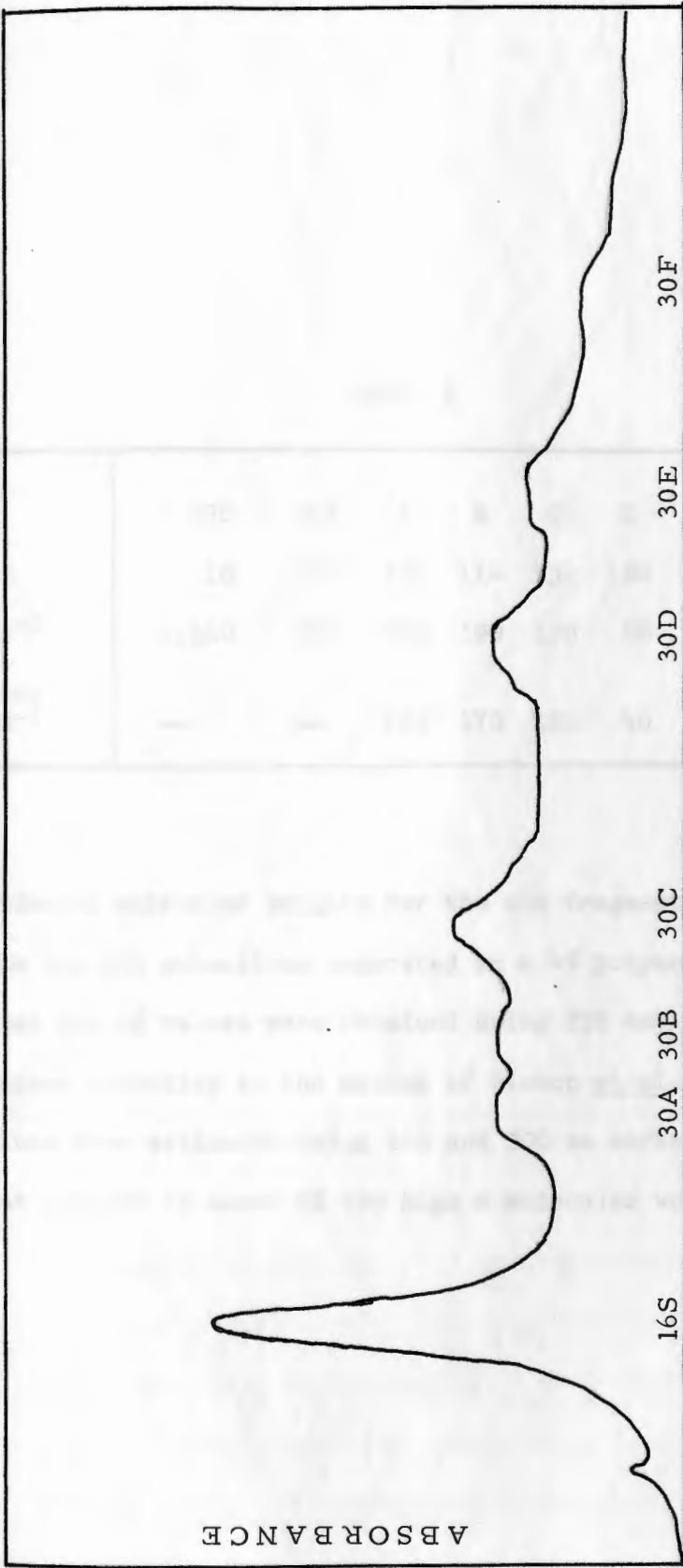
TABLE 2

Band	23S	16S	A	B	C	D	E	F
mm	10	60	101	114	132	180	209	240
M.W.x10^{-3}	1,100	550	242	190	130	50	28	15
corrected M.W.x10^{-3}	—	—	220	170	120	40	22	11

Estimated molecular weights for the six fragments resulting
from the 30S subunit as separated in a 4% polyacrylamide gel.
Upper set of values were obtained using 23S and 16S as
markers according to the method of Bishop et al.(16) Corrected
values were estimated using 16S and 30C as markers assuming
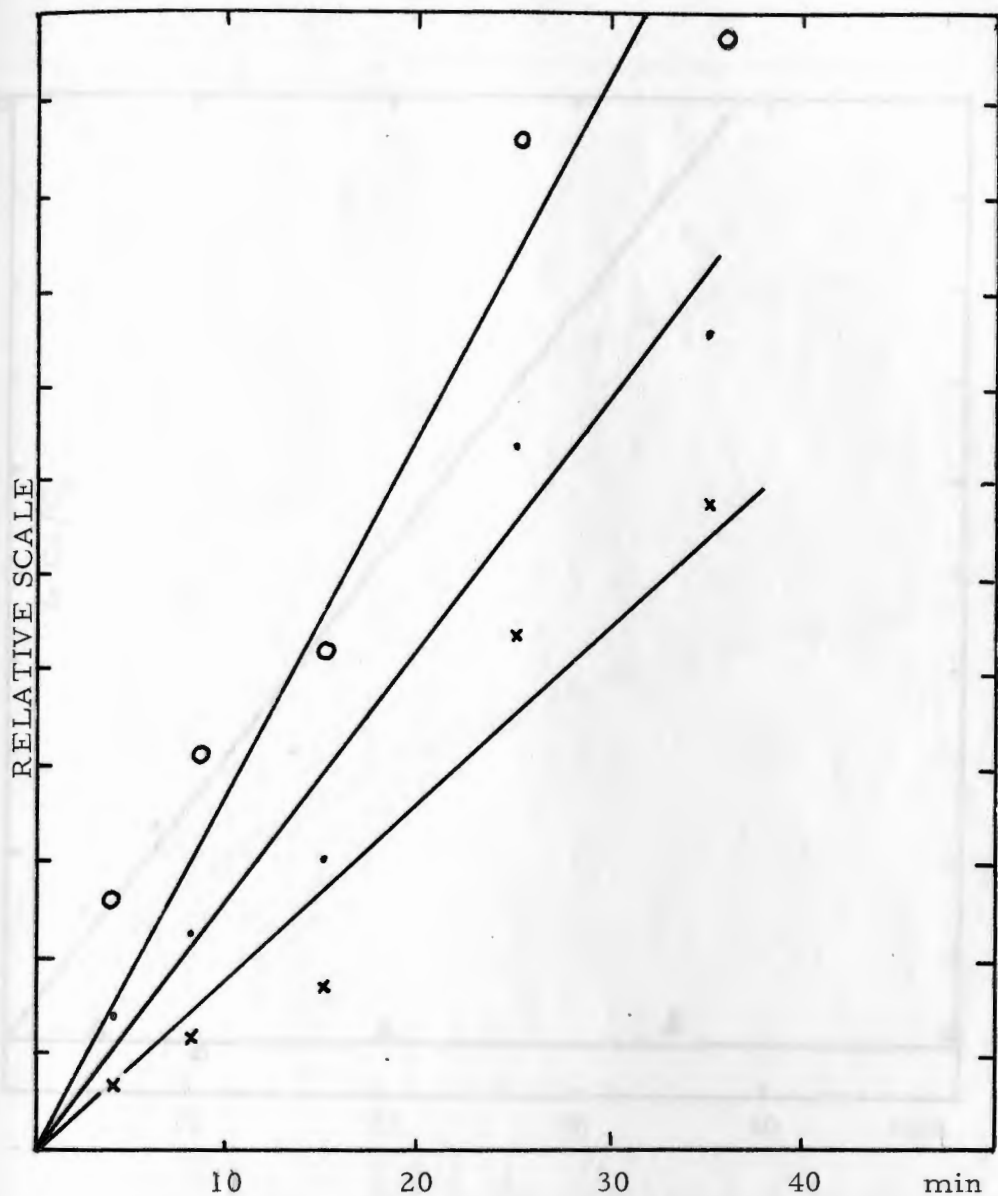that 130,000 is about 5% too high a molecular weight for 30C.

Figure 3a

Treatment of kinetic data for protein-free 16S RNA
according to the zeroth-order (o), first-order (·), and
second-order (x) rate equations. Note that lower points
acquire upward trend with zeroth-order treatment and
downward trend with second-order treatment. Trends are
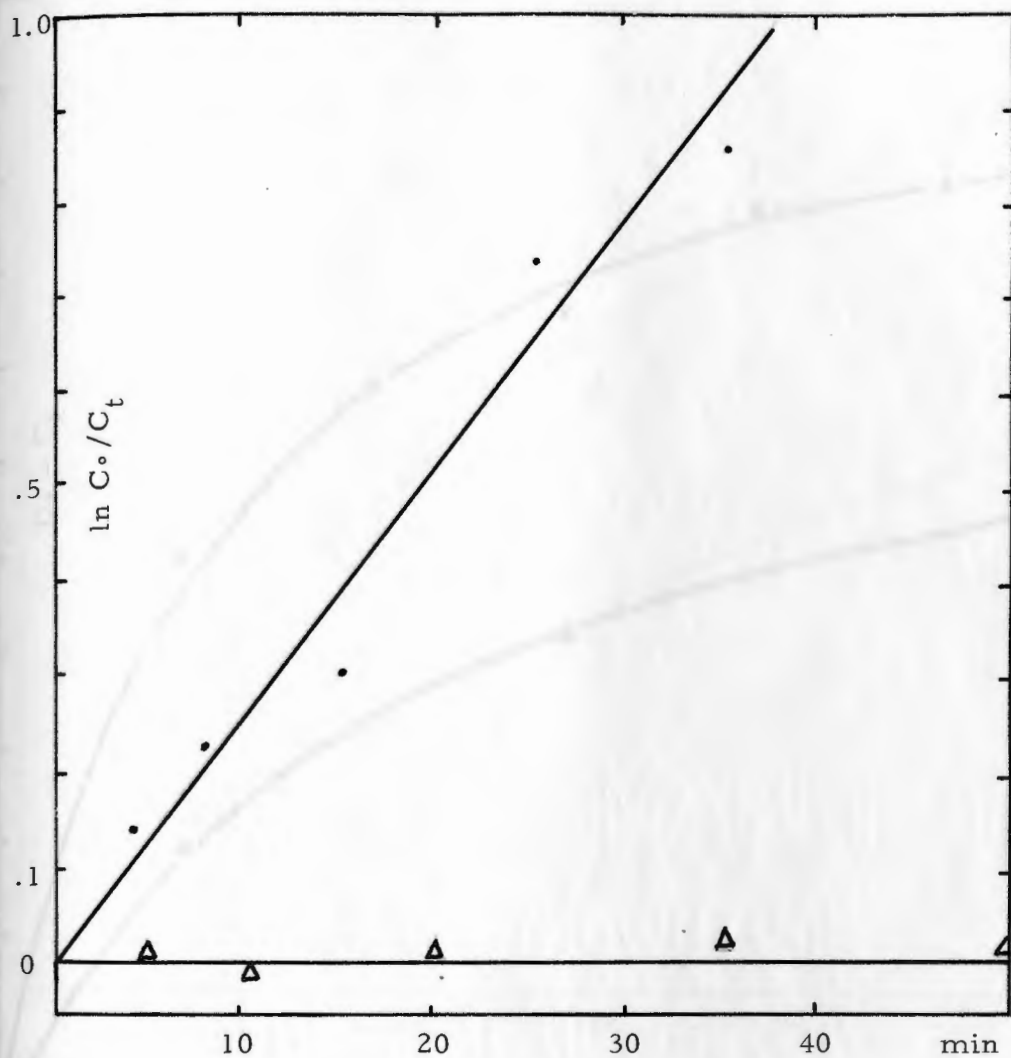opposite for upper points.

Figure 3b

Parallel hydrolysis of 16S RNA and 30S ribosomes
at about 0.01 μg/ml RNase. No appreciable hydrolysis of
the ribosomes was observed unless the enzyme concentration
was raised 20 times or more. The ionic conditions are these
given in the experimental section. (o)protein-free RNA; (Δ) 30S ribosomes.
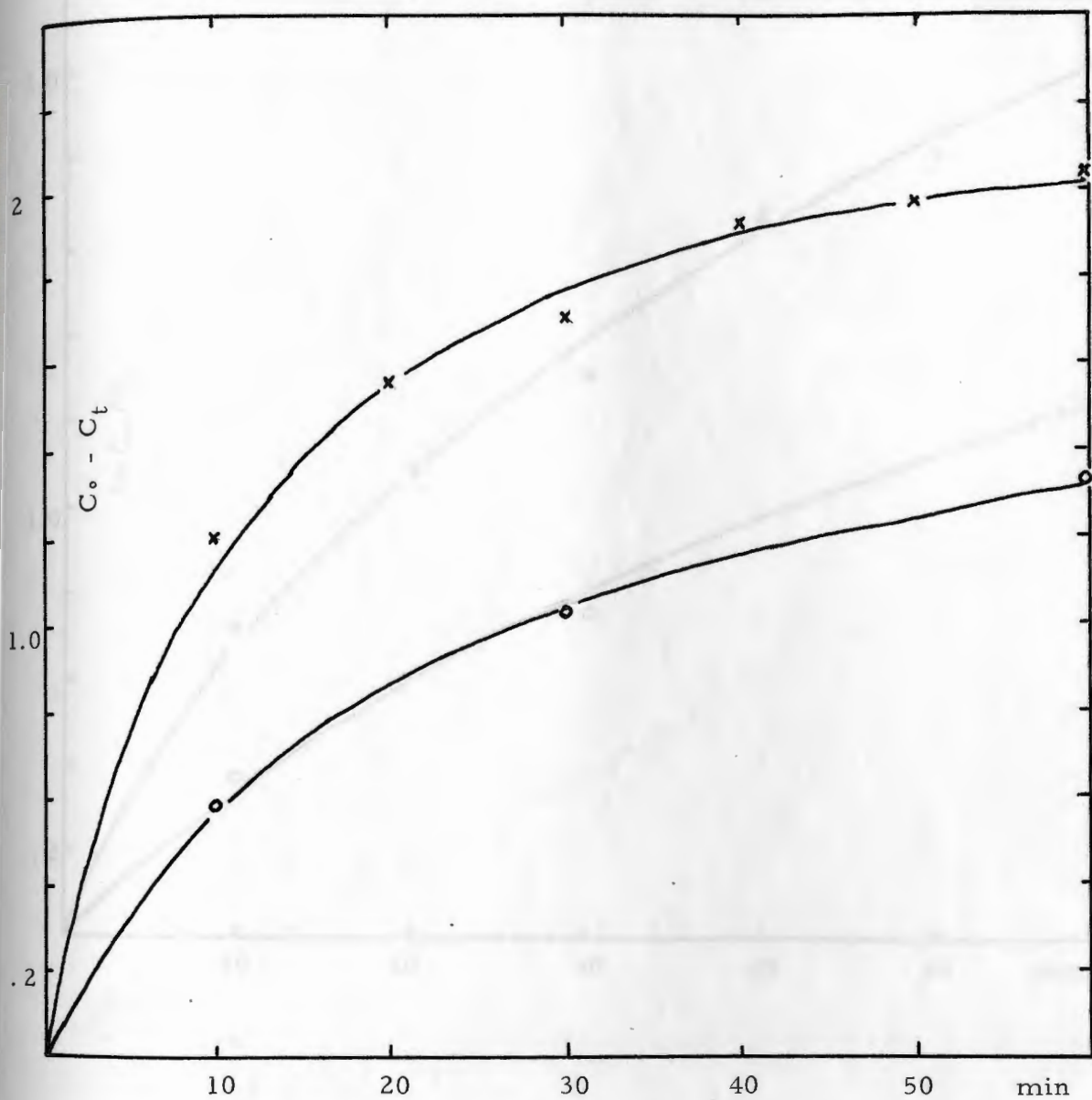
Figure 4a

Zeroth-order kinetic plot of hydrolysis of 30S ribosomal

subunits under ionic conditions identical to those used for

the protein-free rRNA. (x) 0.8, (o) 0.4 μg/ml pRNase.

Figure 4b

First-order kinetic plot of the hydrolysis of 30S ribosomal
subunits with pRNase under ionic conditions identical to those
used for the protein-free rRNA. (x) 0.8, (o) 0.4 μg/ml pRNase.
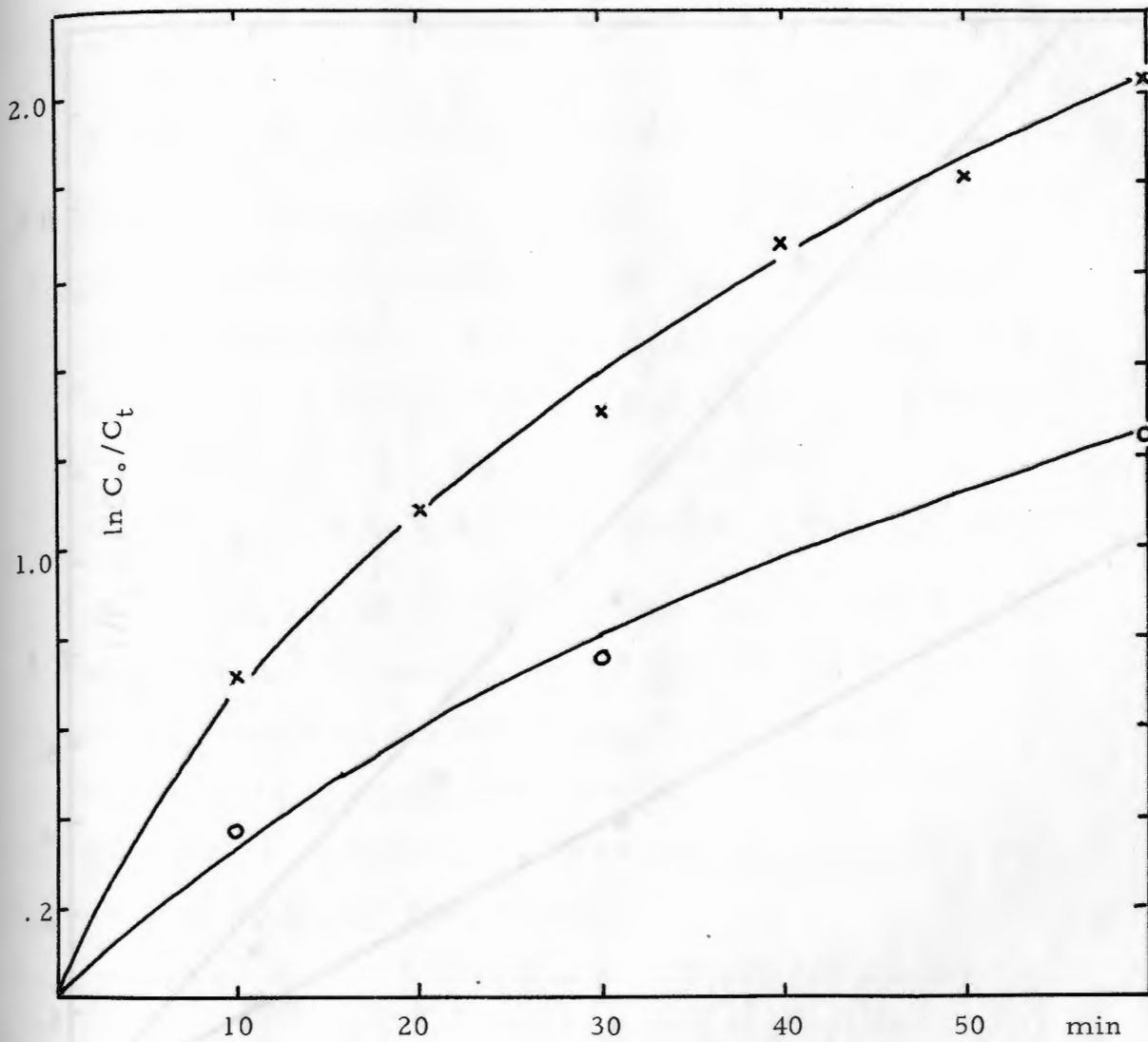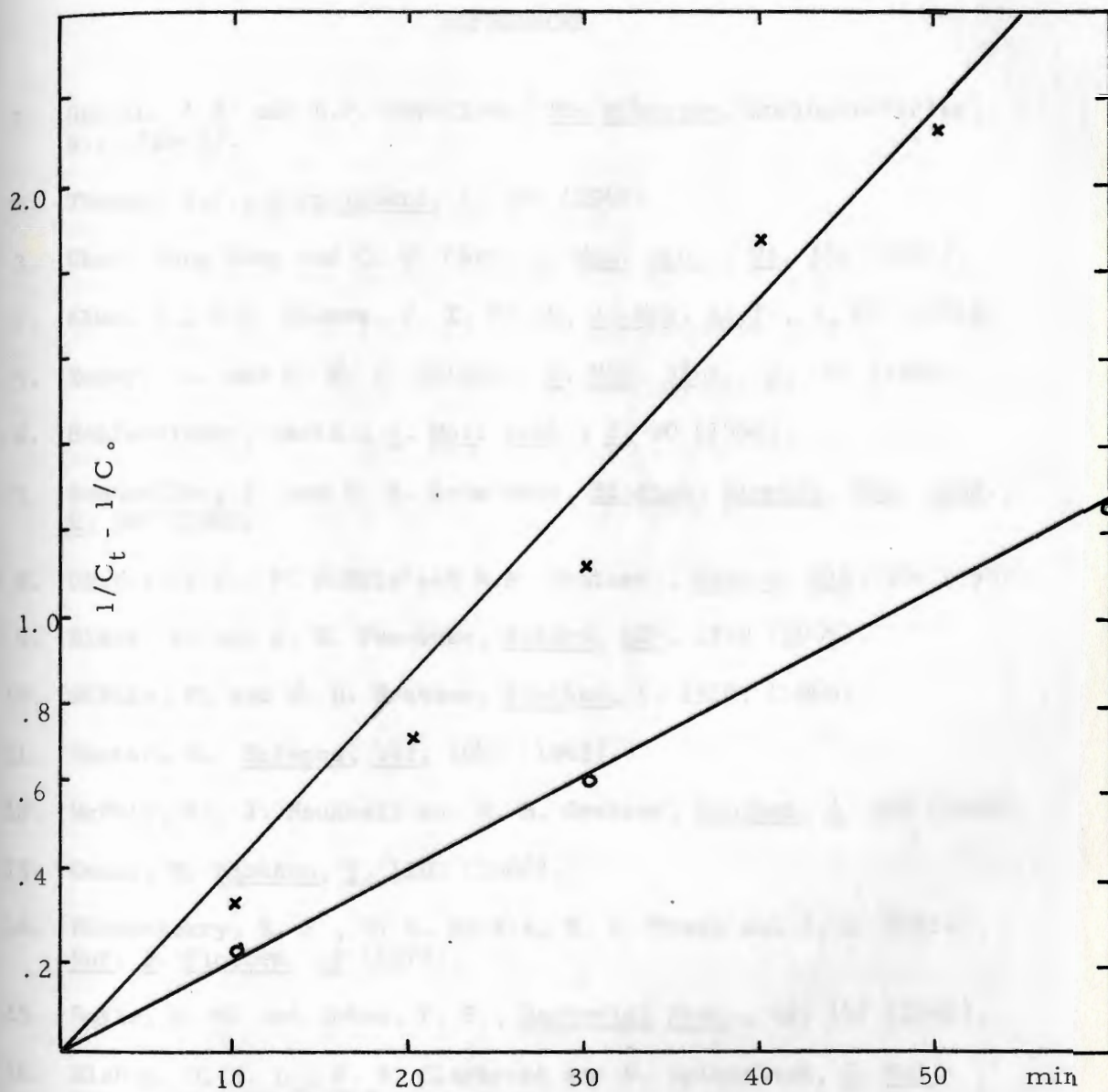
Figure 4c

Second-order kinetic plot of the hydrolysis of 30S ribosomal
subunits with pRNase under ionic conditions identical to
those used for the protein-free rRNA. (x) 0.8, (o) 0.4 μg/ml
pRNase.

REFERENCES

1. Spirin, A.S. and L.P. Gavrilova, The Ribosome, Springer-Verlag, N.Y.,(1969).

2. Thomas, G.J., Biopolymers, 7, 325 (1969)

3. Choi, Yong Sung and C. W. Carr, J. Mol. Biol., 25, 331 (1967).

4. Klug, A., K.C. Holmes, J. T. Finch, J. Mol. Biol., 3, 87 (1961).

5. Zubay, G., and M. H. L. Wilkins, J. Mol. Biol., 2, 105 (1960).

6. Schlessinger, David., J. Mol. Biol., 2, 90 (1960).

7. Bonhoeffer, F. and H. K. Schachman, Biochem, Biophys. Res. Comm., 2, 366 (1960).

8. Cotter, R.I., P. McPhie and W.B. Gratzer , Nature, 216, 864 (1967).

9. Blake, A. and A. R. Peacocke, Nature, 208, 1319 (1965).

10. McPhie, P. and W. B. Gratzer, Biochem, 5, 1310, (1966).

11. Santer, M. Science, 141, 1049 (1963).

12. McPhie, P., J. Hounsell and W. B. Gratzer, Biochem, 5, 988 (1966).

13. Gould, H. Biochem, 5, 1103 (1966).

14. Eickenberry, E. F., T. A. Bickle, R. R. Traut and C. A. Price Eur. J. Biochem. 12 (1970).

15. Boyle, S. M. and Cohen, P. S., Bacteriol Proc., 69, 147 (1969).

16. Bishop, O. H. L., J. R. Claybrook and S. Spiegelman, J. Mol. Biol., 26, 373 (1967).

17. Hartman, K. A., J. Amaya, E. M. Schachter, Science (1970).

18. Teraoka, H., J. Mol. Biol., 48, 511 (1970).

19. Spencer, M., W. J. Pigram and J. Littlechild, Biochem., Biophys., Acta, 179, 348 (1969).

APPENDIX

It is well known that monovalent cations, in cooperation with divalent cations, are important in the association and function of ribosomes (1, p. 31). I thought it interesting to ask whether there are any changes in the fragment pattern of the mild hydrolysis for the 30S ribosomal subunit as a function of the potassium ion concentration.

E. coli D10 30S ribosomes, obtained and concentrated as described in section II of this work, were digested in 0.005 M KCl, 0.005 M Mg(Acetate)$_2$ and 0.005 M Tris-Cl (pH = 7.4), using 0.1 - 0.4 μg/ml of pRNase. The electrophoretic analysis of the RNA products showed a prominent band (30') which reached maximum height toward the end of the first stage of hydrolysis (figure 1 of appendix).

The products from 30S subunits hydrolyzed in 0.06 M KCl, 0.001 M Mg(Acetate)$_2$ and 0.005 M Tris-Cl (pH = 7.4) were analyzed in a 3.75% polyacrylamide gel after stopping the reaction with redistilled phenol and 0.1% SDS. The 2600 A - absorbance pattern is shown in figure 2 of this appendix. Outstanding features of this pattern are a) the evident accumulation of a least three different fragments, and b) the appearance of band 30A' which is absent from the other ribosomal fragment patterns.

It has been shown in section III of the thesis that mild hydrolysis of 30S ribosomes in the absence of of K^+ yields fragments which do not accumulate. The preliminary data presented in this appendix argue that significant changes are introduced in the fragment pattern of the 30S subunit by varying the concentration of KCl. An immediate conclusion

is that perhaps the ribosome undergoes conformational transitions,

and even exposes different sites to the RNase, at the measure that K^+

is being added or subtracted. It has been suggested that a particular

concentration of monovalent cations, such as K^+ and NH^+_4 may be responsible

for the conformation adopted by the 50S subunit (18). A possible

explanation of this phenomenon could be found in the ability of monovalent

cations to exchange with the divalent cations that cross-link the RNA

chain.

Appendix

Figure 1

Fragment pattern of 30S ribosomal subunits digested in
0.005 Molar KCl. Deproteinization carried out with
0.2% SDS and RNA fragments analyzed in a 3% polyacrylamide
gel. Input ribosomes gave a single band in the position
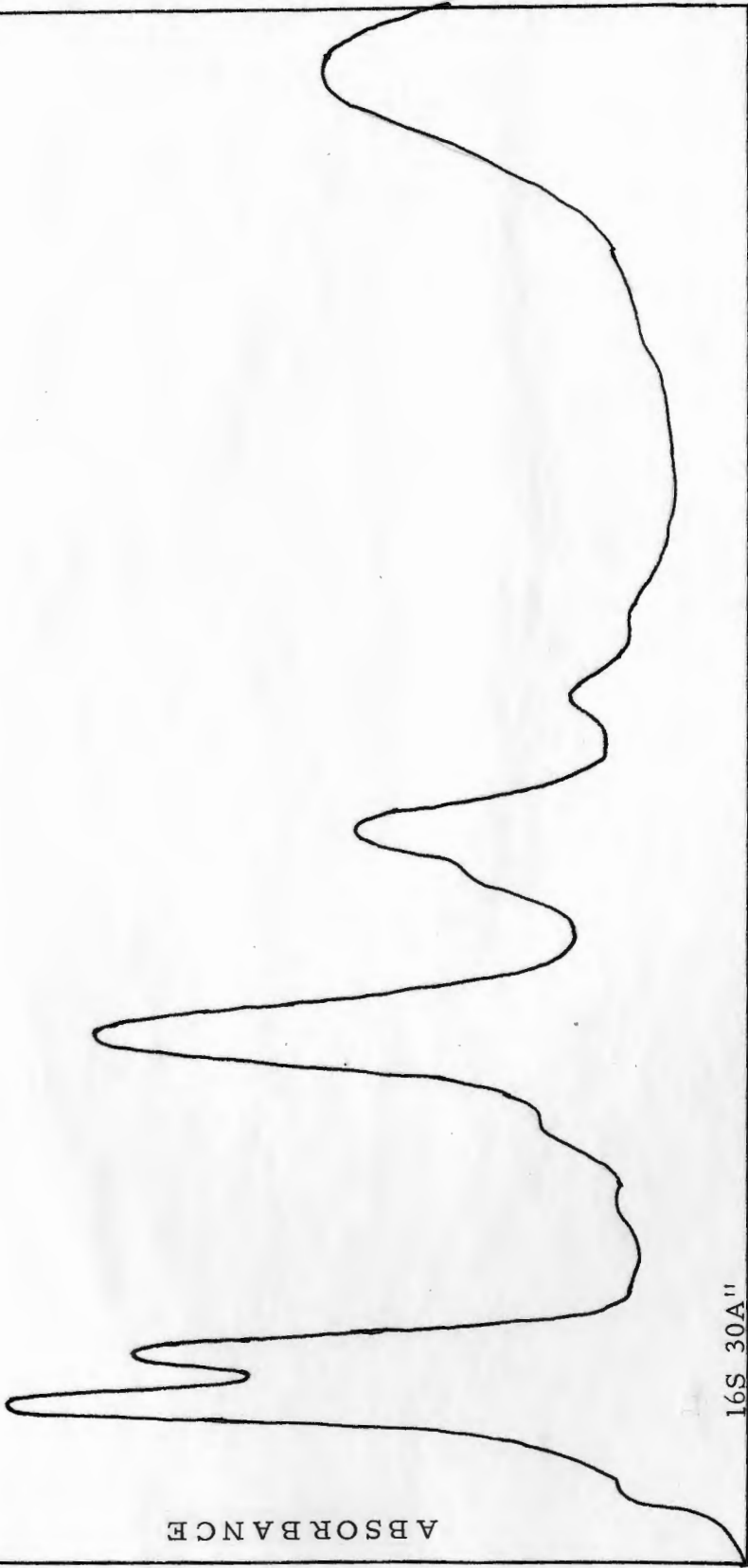of the 16S RNA.

Appendix

Figure 2

Fragment pattern of 30S subunits digested in 0.06 M KCl.
The RNA was recovered by the phenol method, and products
analyszed in a 3.75% polyacrylamide gel. Input ribosomes
gave a single band in the position of the 16S RNA.
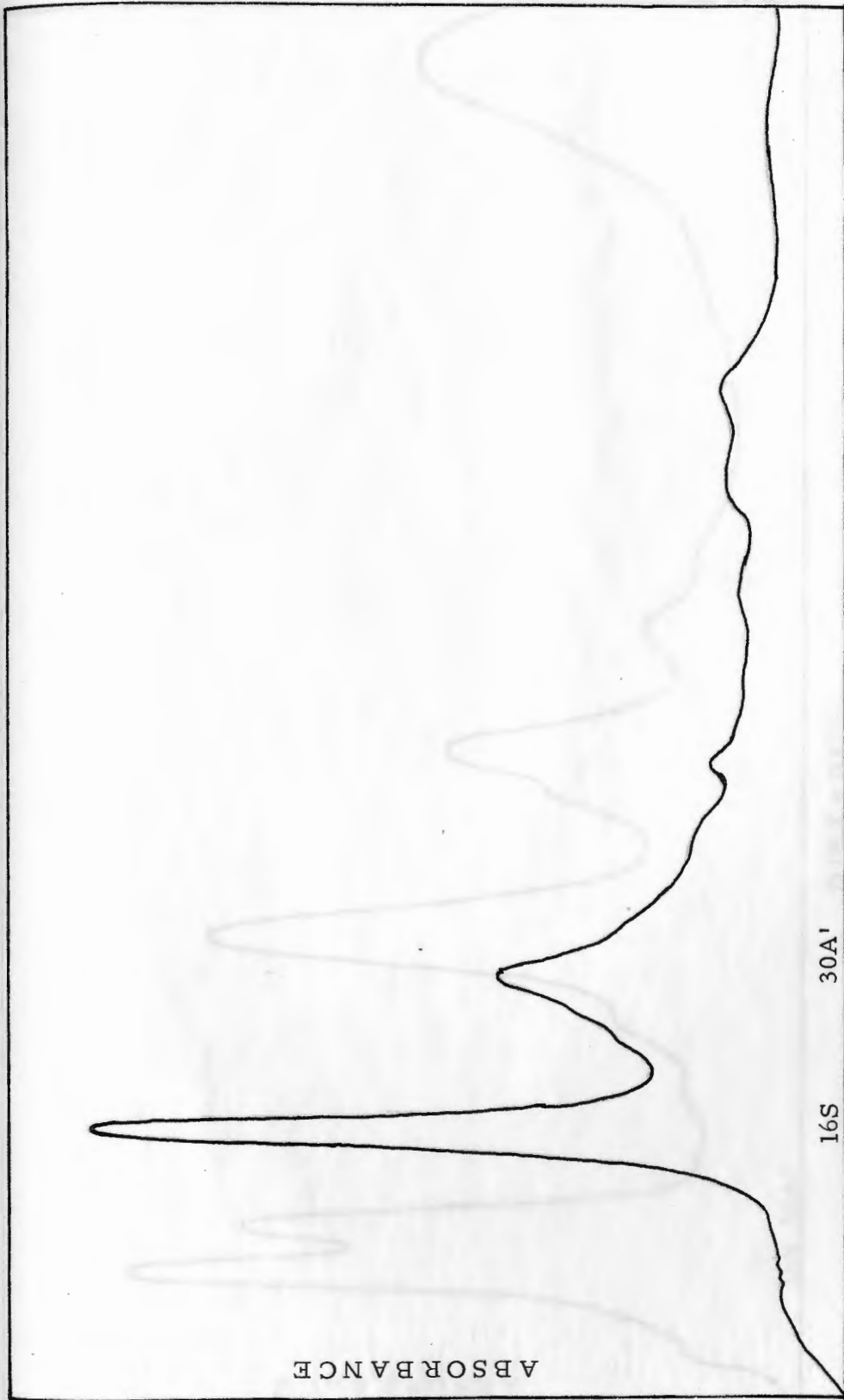
DISTANCE

Figure 1 (Appendix)

DISTANCE

Figure 2 (Appendix)