THE PROPERTIES OF RIBONUCLEASE:

A STUDY IN BINDING AND KINETICS.

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Senior Thesis
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May 9, 1967

ABSTRACT

The interaction between pancreatic ribonuclease and calf thymus DNA is studied as a function of kinetics and binding. It is found that native and denatured DNA bind strongly to RNase, and that the ratio $K_{den}/K_{nat} = 5.7$. It is also shown that at approximately equal concentrations of RNA and DNA, denatured DNA inhibits RNase hydrolysis of RNA by 83 %, and native DNA inhibits by 43 %. A gel method is found to be unsatisfactory for studying the binding of RNase to pyrimidine oligonucleotides; equilibrium dialysis is then tried, and the K_B for the interaction between RNase and dinucleoside monophosphate is found to be approximately equal to 9. The kinetics of RNase activity is discussed and an experiment proposed for studying the enzyme's mechanism.

INTRODUCTION

The interactions of proteins and DNA have been extensively studied during the past few years, inspired by the possibility that proteins have a role in regulating the performance of the biological functions of DNA--in particular, its replication and the reading of the genetic code (Felsenfeld, et. al., 1963). Ribonuclease is especially interesting, since its substrate has a structure so similar to that of DNA, and thus DNA might be expected to have similar interactions with RNase, with the same sort of specificities. It may well be, therefore, that RNase has preferred binding sites on the DNA molecule--namely pyrimidine nucleotides. Also, since RNase seems to have a destablizing effect upon the helical structure of double stranded DNA (Felsenfeld, et. al., 1963), and seems to react differently to native and denatured DNA, it seems profitable to also study the generalized aspects of the binding of ribonuclease to DNA.

The author's research falls into three broad categories, each of which shall be treated separately:

a) The binding of ribonuclease to whole calf thymus DNA and its effect on the enzymatic activity of the protein.

b) The binding of ribonuclease to oligodeoxyribonucleotides.

c) The enzymatic activity of ribonuclease.

The third topic, while not directly related to the first two, treats problems which naturally arose as a result of the methods used in the first phase of research.

I Binding to DNA; Inhibition of Ribonuclease Acitivity

Experimental--Inhibition experiments were performed in a buffer containing 0.01 F NaCl, 0.001 F Na_2HPO_4, and 0.001 F NaH_2PO_4 (pH 6.86). Binding studies were performed in 0.01 F NaCl, 0.001 F Tris (pH 7.3). Bovine pancreatic ribonuclease A (Worthington Biochemical Corp., lot RAF 6507) was used without further purification. Ribonuclease solutions contained bovine serum albumin (Pentex) in a weight ratio of 40:1 to minimize RNase adsorption to the walls of glass containers; such solutions retained 80 % of their original activity after storage for one month under refrigeration. Calf thymus DNA (Worthington lot DNA 630) was found to contain a substantial amount of RNase activity, but this was removed by phenol extracting the DNA three times. Denatured DNA was prepared by boiling a sample of native DNA for 1-3 minutes, then immediately immersing the sample in an ice bath (0° C). Yeast RNA (Calbiochem or Mann Research Laboratories) was used without further purification for the ribonuclease activity assay. All spectral measurements were performed on a Cary 14 automatic recording spectrophotometer.

The assay for ribonuclease activity was modified from the version of the Kunitz assay (1940) outlined by Felsenfeld, et. al. (1963):

> An assay mixture containing the RNase sample, 0.56 mg/ml RNA, 90 μg of BSA, and buffer made up to 1.900 ml, was allowed to react for 15 minutes at 37° C. The reaction was then terminated by adding 0.100 ml 1.5 % uranyl acetate in 25 % $HClO_4$, then letting the mixure stand at 0° C for at least ten minutes. The mixture was then centrifuged for 20 minutes at 20,000 rpm (using an SW 39 rotor in either a Model L or a Model L-2 centrifuge). The

supernatant was decanted and diluted by 1/6, and its absorbance read at 260 mμ.

Before each experiment the relative activity of the enzyme solution was determined, in order to avoid using degraded solutions in an experiment. This activity was compared to a standard increase in absorbancy (A_{260}) of 8.000 (for a sample not diluted by 1/6) for an assay mixture containing 1.18 mg/ml RNA and 0.22 μg/ml RNase from a freshly made solution.

Inhibition experiments were performed using a standard assay mixture with either native or denatured DNA added. The procedure was the same as above, except that the DNA and RNase were mixed and allowed to equilibrate at 37° C in a constant temperature bath, after which the specified amount of RNA was added and the standard assay procedure performed.

The binding of RNase to DNA was studied by boundary sedimentation. Samples were made up in a 0 - 20 % sucrose gradient, having throughout a constant concentration of DNA, RNase (2.5 μg/ml), and buffer (see above). Three tubes were prepared for each experiment--the first, a control, contained no DNA; the second contained native DNA; and the third, DNA denatured by the process described above. The sedimentation was carried out at 0° C in a Beckman Model L-2 ultracentrifuge, using an SW-50 rotor. Runs were made at a speed and time sufficient to pellet the DNA; fractions were then collected, and the pellet resuspended. The DNA content of each fraction, and of the pellet, was determined spectrophotometrically, and the fractions were then diluted so that the RNase concentration of each was low enough to assay accurately. RNase content of each fraction was determined by means of the activity assay described above, with a suitable correction made for the inhibition of

enzymatic activity by the DNA present in the fraction. Two such runs were made--one for 20 hours at 45,000 rpm, and a second for 30 hours at 36,000 rpm.

Results and discussion--The results of the inhibition experiment are shown in figure 1; the percent of activity is determined as a function of the weight ratio of DNA to RNA in the reaction mixture. The activity is determined by comparing the absorbance increase due to RNase activity in an RNA-DNA mixture to that found for a standard reaction mixture containing no DNA. Since the activity measures the rate of production of acid-soluble polyribonucleotides, what the experiment measures is the effect of the presence of DNA on the rate of hydrolysis of RNA by ribonuclease. It will be noticed that the presence of denatured DNA in the reaction mixture in about the same concentration as RNA showed an inhibition of 83 % (about 17 % of the original activity remaining), while native DNA present in about the same quantity slowed the enzymatic reaction by only 43 %. (Inhibition was not found for native DNA-RNA ratios of less than 0.750, but it is felt that these measurements, rather than the one at high DNA concentration, were the artifacts--i. e., that native DNA does indeed inhibit ribonuclease activity, but to a much smaller degree than does heat-denatured DNA.)

The first sedimentation run was made with a sample of native DNA with $A_{260} = 4.4$ (about 0.2 mg/ml) and a sample of denatured DNA with $A_{260} = 5.3$ (the same concentration). Unfortunately the tube containing the standard collapsed during the run, and the standard was lost. Thus fractions were not taken in the other tubes--the pellet and supernatant of each were analyzed separately. The data obtained were thus crude, but indicated that in the sample containing native DNA at least 84.5 % of the enzyme in the tube was bound to DNA, while the

denatured DNA had bound at least 92.5 % of the RNase. In the second run, the sample with native DNA had an optical density of 1.23 (about 55 μg/ml), while the sample with denatured DNA had an A_{260} = 1.59. Fractions were collected and analyzed separately for DNA content and ribonuclease activity. The data from this experiment indicated that the denatured DNA had bound essentially all the RNase present (fractions in the center of the tube showed traces of activity, but this was attributed to the binding of RNase to small fragments of DNA produced by degradation either during the phenol extraction or the relatively violent process of heat denaturation), while in the sample with native DNA 75 - 85 % of the enzyme was bound to DNA. These data, while encouraging, provide little definitive information regarding the strength of the interaction between RNase and denatured DNA. A competition study between native and denatured DNA in the electrophoresis apparatus described by Olivera, et. al. (1964) provided the needed data. In this experiment, a mixture of RNase, and native and denatured calf thymus DNA was subjected to electrophoresis until the native and denatured components of the fraction were resolved (It was possible to identify the native fraction as the leading one, as the electrophoretic mobility of native DNA has been shown to be 15 % higher than that for denatured DNA (Olivera, et. al., 1964)), and each fraction was then assayed for RNase activity. Denatured and native DNA were found to bind RNase in a ratio of about 5.7 : 1. These data, along with the sedimentation results, imply a K_m of 10.8 μg/ml (corresponding to 3.44 X 10^{-5} moles of nucleotide per liter) for native DNA and 1.9 μg/ml (or 6.05 X 10^{-6} M nucleotides).

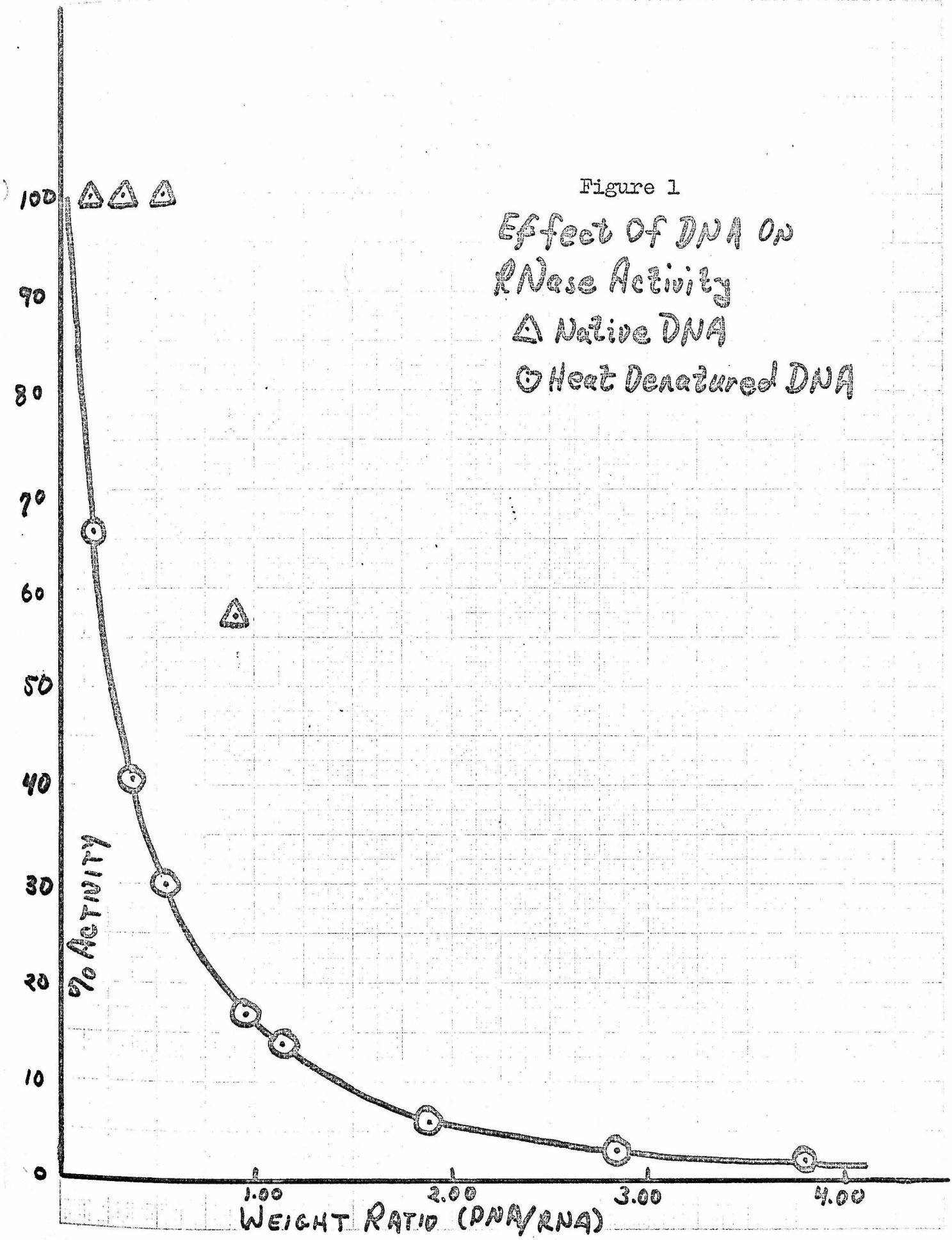
The binding and inhibition data agree reasonably well with the findings of Felsenfeld, et. al. (1963). Felsenfeld found that with a concentration of DNA equal to that of RNA in

Figure 1

Effect Of DNA On RNase Activity

△ Native DNA

⊙ Heat Denatured DNA



his standard assay (at pH 5.0), ribonuclease activity was inhibited 15 % by native DNA and 70 % by denatured DNA. These figures are lower than this author's results, but this is not surprising in view of the fact that the pH used in the present work is much closer to that reported by Kunitz (1940) as the range for optimum enzymatic activity (i. e., pH 7.6 - 7.8). Also, the value of $K_{den}/K_{nat} = 5.7$ falls within the range of 4 - 10 predicted by Felsenfeld. Finally, if one assumes the affinity of RNase to denatured DNA and to single-stranded RNA to be about equivalent, they may be compared to the electrophoretically measured (Olivera, 1966) $K_m = 700\ \mu g/ml$ for the RNA-rbonuclease T_1 complex.

From the foregoing, one infers two fairly general statements concerning the RNase-DNA interaction. Firstly, the finding that pancreatic ribonuclease has a very high affinity for both native and denatured DNA suggests that there is a large electrostatic compnent to the binding (since DNA has one negative charge per nucleotide at the pH used, and RNase is a basic protein in this range, the suggestion is particularly attractive). This idea is given further support by the data concerning T_1 ribonuclease, which binds much less strongly at this pH but is a negatively charged protein under the conditions of the experiment.

Secondly, there is an element of the binding interaction which has the gross effect of making the active site of the enzyme either totally or partially unavailable for RNA hydrolysis, and it seems likely that this element of the binding involves the recognition of one or more specific bases on the nucleic acid molecule. That this is so is implied by the fact that the binding is weaker by a factor of six for native DNA, whose bases are stacked inside the Watson-Crick helix and are thus harder to get at (and possibly

harder to recognize) than those of denatured DNA, which is essentially a single-stranded polymer. It is not clear that this "deactivation" is directly involved with the active site of the enzyme, although the idea of direct involvement is made attractive by the similarity of DNA and the enzyme's substrate, single-stranded RNA. It may be, on the contrary, that interactions at a "distant" part of the molecule have the effect of deforming or otherwise turning off the active site. Such a distinction cannot be made with present knowledge.

An obvious "next step" appers to be an attempt to establish specifity in some way. This could be approached in several ways. First, the binding of whole DNA could be compared to the interaction of RNase and apurinic and apyrimidinic acids, or to oligonucleotides, with the aim of establishing a sort of base specifity by a process of elimination. Alternatively, one could search for an inhibitor which eliminates the electrostatic factor, such as cytosine or deoxycytosine. These bases, if competed with DNA, might hopefully inhibit DNA binding by tying up whatever portion of the enzyme molecule is involved with base recognition, while not binding to RNase electrostatically. The second method will fail, however, if the electrostatic component of the binding is so large that RNase binds only weakly to a nucleoside, and thus is not an effective competitor. There are several possible methods of investigating these possiblities, besides the obvious techniques of electrophoresis and boundary sedimentation. Two of the other alternatives will be investigated in the next section.

II Oligonucleotide Binding: the Gel Method and Dialysis

Experimental--Bovine pancreatic ribonuclease A (Worthington Biochemical Corp., Lots RAF 6JA, RAF 6JB, and RAF 6GA) was used without further purification for all experiments. It was dissolved in buffer* and stored under refrigeration. Dideoxynucleoside monophosphates and trideoxynucleoside diphosphates (pyrimidines obtained in the depurination of calf thymus DNA) were kindly provided by Miss Louise Chow. Bio-Gels P-2, and P-4, 50-150 mesh, from Bio-Rad Laboratories, were used for the gel experiments. Dialysis membranes were cut from commercial dialysis tubing (Union Carbide Corp.) which had been treated as described below.

(a) The Gel Method. Gel experiments were performed in centrifuge tubes such as the one shown in Figure 2. The lower portion of the tube (A) had a known volume (calibrated using doubly distilled water), and was used to contain the hydrated gel. The tube then had a short constricted portion with a hash mark (C) whose purpose was to minimize error in the measurements of the volume of (A). The upper sector (B) contained the supernatant fluid. The tube was readied for an experiment by adding small known amounts of gel and hydrating each portion, until the tube was filled up to the hash mark, recording the weight of gel and weight of buffer necessary to fill the tube. The gel was then cleaned by adding buffer to (B) and stirring briskly, then centrifuging lightly in a clinical centrifuge to pack the gel. The supernatant was then removed and its ultraviolet absorbance read. The cell was considered fit for use when all uv-detectable impurities had been removed.

An experiment with the gel apparatus was performed as follows:

A known volume of reaction mixture was added to sector

*The buffer used in all experiments was 0.04 F NaCl, 0.004 F Tris, pH 7.43.

(B), and the contents of the tube were stirred vigorously for three minutes to allow the mixture to equilibrate between the gell and liquid phases. The tube was then centrifuged and the ultraviolet absorption spectrum of the supernatant was read. The void volume of the gel was determined by performing the above experiment with blue Dextran, a macromolecule large enough so that it could not penetrate the gel pores, and taking the visible spectrum of the supernatant from 8000 A - 5000 A, as Dextran has a strong absorption band at 6250 A.

(b) Equilibrium Dialysis. Dialysis experiments were performed in the apparatus pictured in Figures 3a-3c. The sample chamber (A) of each sector (see Figure 3a) has a volume of 1.00 ml. A dialysis membrane, cut to size, is inserted between two identical sectors and the sectors are tightly clamped together by four screw-and-wing-nut assemblies (F) for which holes (C) are drilled in each of the sectors. (The sectors themselves are machined lucite blocks) Thus the dialysis membrane also serves as a gasket to prevent leakage. Sirring of the solutions is provided by one stirring bar (E) in each sector chamber, and the magnetic stirrer against which the cell is placed during an experiment (see Figure 3c). The chambers are filled through two screw holes (B); nylon screws were provided with the cell for the purpose of closing off these holes, but they did not porvide an effective seal, so evaporation of the solutions was prevented by taping a piece of Parafilm tightly over the top of the cell. The cell was set up for an experiment as shown in Figure 3c. A double sheet of aluminum was placed between the cell and the magnetic stirrer to dissipate the heat generated by the stirrer; it proved to be an effective heat shield, as the temperature of the cell typically remained within 0.1° - 0.3° of room temperature throughout a run. The cell was purchased from the Chemical Rubber Company.

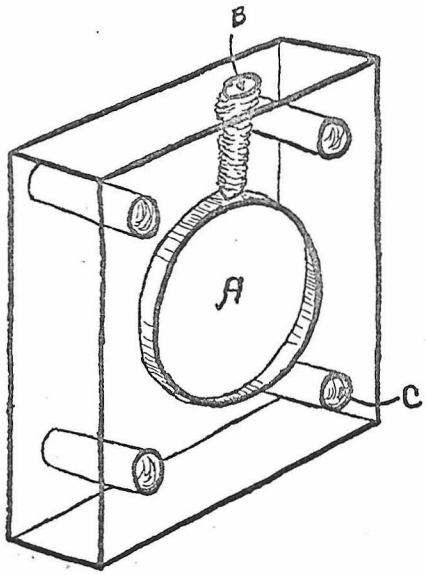


Figure 3a. Sector of dialysis cell.

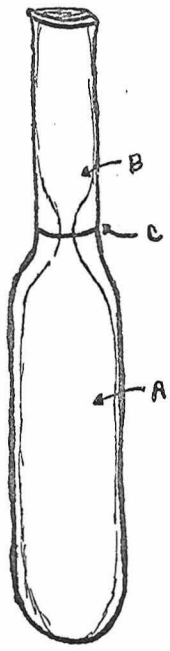


Figure 1. Centrifuge tube for gel experiments.

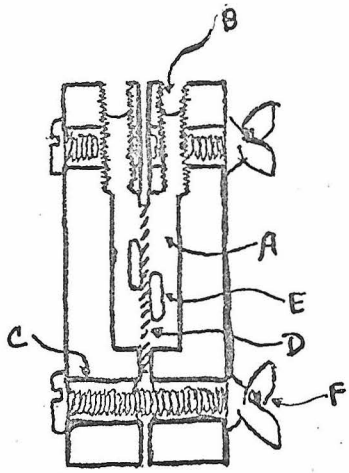


Figure 3b. Side cross section of dialysis chamber.

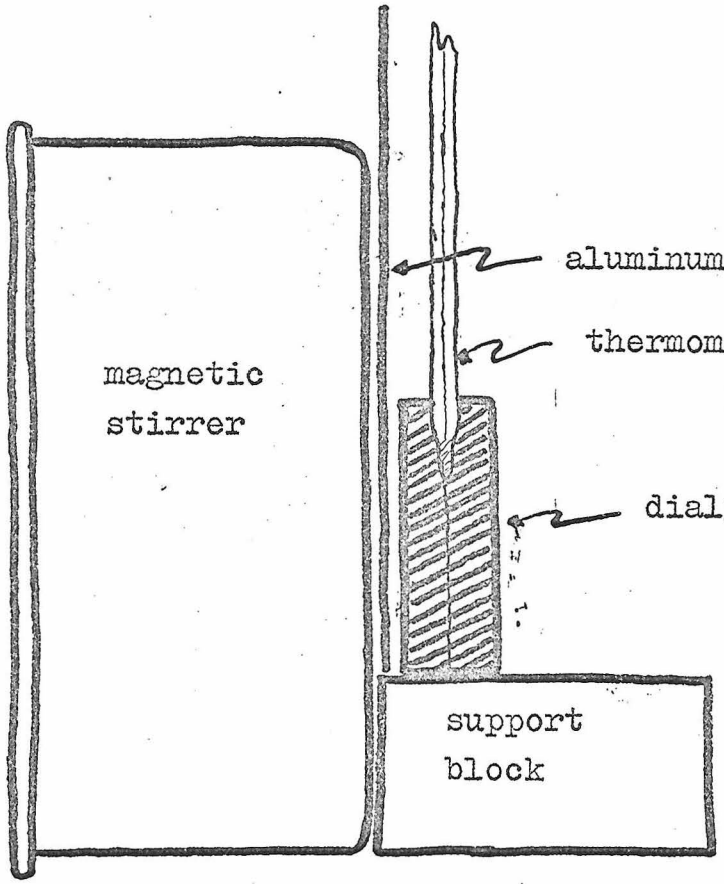


Figure 3c. Complete apparatus for dialysis experiment.

The dialysis tubing was prepared for use by alternate boiling for approximately 30 minutes in distilled water, and the a solution which was about 1 F NaCl and 0.025 F disodium ethylenediaminetetraacetate (EDTA). The tubing was thoroughly rinsed, inside and out, with distilled water after each boiling. This process was repeated until no uv-impurities were detected in buffer solutions in which the membranes had soaked for at least 24 hours. The tubing was then stored under refirgeration in 0.05 F EDTA. Eighteen hours before use, a piece of membrane was cut to size, rinsed, soaked for 12 hours in 2 F NaCl to remove traces of EDTA, then rinsed thoroughly with both distilled and doubly distilled water, and soaked in the buffer to be used for the experiment for at least two hours.

The stirring bars and the cell itself, were cleaned in a similar manner, with the exception that neither the cell nor stirring bar were placed in boiling water (the cell was boiled once, but it was feared that subsequent boiling would deform it). Both were cleaned after each use by soaking in a mixture of 2 F NaCl and 0.05 F EDTA, then rinsed and allowed to soak in 1 F NaCl for one hour, then rinsed again and allowed to soak in the buffer to be used in the experiment. After a final thorough rinsing, the stirring bars were dried with a Kimwipe and the cell with filtered compressed air. Both were then stored in dry sterile beakers until they were used. All handling of the cells, stirring bars, and membranes was done while wearing surgical gloves, in order to minimize the danger of contamination.

During an experiment, readings were taking by removing samples from the cell with a Pasteur pipette. Sample of approximately equal volume were removed from both cells at the same time, in order to minimize concentration changes from migration of buffer solution across the cell membrane. Samples

were then returned to the cell and the experiment resumed.

Theory: (a) The Gel Method. The gel beads themselves are a compolymer of acrylamide and bisacrylamide, whose relative concentrations rather carefully control pore size. In general, for a molecule which is not adsorbed onto the gel surface, one would expect

(1) $V_s C_{ob} = V_s C_{fb} + KV_g C_{fb} + V_v C_{fb}$, for which

C_{ob} is the original concentration of species b; i. e., its concentration in the supernatant solution which is added to the gel mixture,

V_s = the volume of the supernatant solution,

V_g = the volume inside the gel which is available to entering molecules,

V_v = void volume; i. e., the solution volume between gel beads in sector (A) of the tube,

C_{fb} = the equilibrium concentration of the macromolecule in V_s and V_v,

K_b = the partition coefficient of species b between the gel and the solution phases, so that

$K_b C_{fb}$ = the concentration of b inside the gel.

Equation (1) may be rearranged to give the two more useful forms

(1a) $$K_b V_g + V_v = V_s \left(\frac{C_{ob}}{C_{fb}} - 1 \right), \text{ and}$$

(1b) $$\frac{V_s}{K_b V_g} = \frac{C_{fb}}{C_{ob} - C_{fb}} \left(\frac{V_v}{K_b V_g} + 1 \right).$$

If the species b is larger than the pore size of the gel, so that $K_b = 0$, we have

(2) $$\frac{C_{ob}}{C_{fb}} = \frac{V_s + V_v}{V_s}.$$

If we assume $K_b \neq 0$ and we introduce a larger molecule c with $K_c = 0$, we then may describe the situation by

(3) $V_s C'_o = V_s (C'_f + C'_b) + K_b V_g C'_f + V_v (C'_f + C'_b)$,

if we also assume that species c complexes with species b. Since b is the only species with whose concentration we are concerned we have dropped the second subscript on the C's. Here we now have

C'_o = initial concentration of b,

C'_b = concentration of b which is bound to species c,

C'_f = concentration of b which is not bound.

Since the concentration we measure is that in the supernatant, which is $C'_s = C'_b + C'_f$, we rearrange (3) to obtain

(3a) $$\frac{C'_f}{C'_f + C'_b} = \frac{V_s}{K_b V_g}\left[\frac{C'_o - (C'_f + C'_b)}{C'_f + C'_b}\right] = \frac{V_v}{K_b V_g}$$, and then

substitute (1b) to obtain

(3b) $$\frac{C'_f}{C'_f + C'_b} = \left(1 + \frac{V_v}{K_b V_g}\right)\frac{\left[\dfrac{C'_o - (C'_f + C'_b)}{C'_f + C'_b}\right]}{\dfrac{C_o + C_f}{C_f}} - \frac{V_v}{K_b V_g}$$

$$\cong \frac{\dfrac{\delta C'}{C'}}{\dfrac{\delta C}{C}} + \frac{V_v}{K_b V_g}\left(\frac{\dfrac{\delta C'}{C'} - \dfrac{\delta C}{C}}{\dfrac{\delta C}{C}}\right),$$

where $$\frac{\delta C'}{C'} = \frac{C'_o - (C'_f + C'_b)}{C'_f + C'_b}, \quad \frac{\delta C}{C} = \frac{C_o - C_f}{C_f}.$$

We still need to know V_g, since not all the volume that the gel occupies is available to the solution and species b. But since we know, the weight of the gel added, and that of the buffer used to hydrate it, and the density of the buffer, and since we

can easily find V_v, we can easily find V_g from the relation

$$(4) \qquad \frac{\text{Wt.}_{\text{gel + buffer}} - \text{Wt.}_{\text{gel}}}{\text{Density of Buffer}} - V_v = V_g \ .$$

We now have all the quantities we need.

(b) Equilibrium Dialysis. If we assume that one enzyme molecule binds to one oligonucleotide molecule, we may then adopt the expression given by Hammes and Schimmel (1965) for the binding constant of ribonuclease:

$$(5) \qquad K_B = \frac{1}{K_D} = \frac{C}{(E - C)(P^* - C)} \ , \text{ in which}$$

K_B is the apparent binding constant at equilibrium, which is the reciprocal of the dissociation constant of the enzyme-oligonucleotide complex K_D, C is the equilibrium concentration of the complex, E the stoichiometric concentration of enzyme, and P* "pseudostoichiometric" concentration of ligand. We say pseudostoichiometric, since at equilibrium, there is on the "blank" side of the dialysis membrane a concentration of ligand equal to that of "free" ligand on the "sample" side, so that in reality we have

$$(6) \qquad P = P_b + 2P_f = C + 2P_f, \text{ so that}$$

$$(6a) \qquad P^* = C + P_f = P - P_f.$$

If we now define $\beta \equiv P^*/C$, we may rearrange (5) and substitute K_D for $1/K_B$, we obtain

$$(5a) \qquad \beta = 1 + \frac{K_D}{E + P^*} \ .$$

Now the optimum condition for measuring K_D is to have the concentration of the ligand equal to the K_m; i. e., to have $\frac{1}{2}$ the substrate bound, so that experimentally, we would like to have $\beta = P^*/C = 2$, so that $E + P^* = K_D$.

Equilibrium is not attained instantaneously in a dialysis experiment; the kinetics of the transport of a species

into a chamber may be given by

(7) $$\frac{C_e - C_t}{C_e} = e^{-t/\tau}, \text{ in which}$$

C_e is the concentration of the species in the chamber at equilibrium, C_t its concentration at time t, and τ a constant characteristic of the species, the membrane, and the composition of the solution, having the units of time. τ will therefore be referred to as the characteristic time for the reaction. It may be determined for the dialysis of a species through a membrane in a solution of a given ionic strength and pH by placing a solution of the species in one chamber and pure buffer in the other, and measuring the rate of appearance of the species in the blank chamber as a function of time. In this case $C_e = \frac{1}{2}C_o$, where C_o is the initial concentration of the species in the sample chamber, and Equation (7) becomes

(7a) $$\frac{C_o - 2C_t}{C_o} = e^{-t/\tau}, \text{ or}$$

(7b) $$\tau = -\frac{t}{\ln\left(\frac{C_o - 2C_t}{C_o}\right)} .$$

It should be noted that

(8) $$1 - e^{-t/\tau} = C_t/C_e = \phi, \text{ where}$$

100 ϕ is the percentage of completion of the reaction at time t.

Results and discussion--Bio-Gel P-2 appeared to exclude trinucleoside diphosphate, even though the published exclusion limit of the gel (Bio-Rad manual) was 1600, and the largest trinucleoside has a molecular weight of about 850. However, it should be recalled that the exclusion limits were calculted for polypeptide chains, and that a polynucleotide of

the same molecular weight is quitely likely to be much more rigid, and since it is highly charged, it is likely to have solvent and/or buffer molecule associated with it. Both of these could plausibly be the cause of a substantially larger effective radius than would be possessed by a polypeptide of comparable weight. It was concluded therefore that P-2 could quite possibly be excluding the trinucleoside, so it was decided to try experiments using P-4 (with an exclusion limit of 3600) and dinucleoside monophosphate, which have an average molecular weight of 531 (assuming equal amounts of each of CpC, CpT, TpC, and TpT).

These experiments were equally unsuccessful. Two independent experiments gave widely divergent values for the K_b of the dinucleoside, and one binding experiment gave a value for $C_f'/(C_f' + C_b') > 1$, which is clearly impossible. To explain these anomalous results, we should first recount the technical difficulties which were encountered.

The volume which the gel occupied was found to depend critically on the speed at which it was centrifuged for packing, and neither the speed nor the time at speed could be controlled closely. Also, if the speed of centrifugation was above a certain critical speed, the gel was compressed, but began to expand again after centrifugation was stopped. Finally, it was observed that once a gel had been packed in this manner, it frequently exhibited a tendency to expand over a period of a day or two to a volume larger than that which it occupied before any packing at all had taken place.

There appeared to be two explanations possible for the first two observations:

(a) The force could squeeze gel beads together and possibly deform them, thereby eliminating portions of the space between beads; i. e., shrinking V_v.

This however should have no effect on the binding determinations, or on K_b, since $V_v + V_s$ = const., and a change in V_v should have no effect on the distribution of b between the gel and solution phases.

(b) Alternatively, the force could simply compress and deform the beads in such a manner as to make the effective pore size smaller and/or to reduce V_g by a considerable amount. Such an effect could well explain the anomalous results.

It is not obvious what caused the last phenomenon; however, it could be that the gels were not sufficiently hydrated before their first use. At any rate, it has become apparent for a number of reasons that this adaptation of gel filtration is not a satisfactory method for measuring the binding between RNase and oligonucleotides.

Myer and Schellman (1962), in developing an equilibrium dialysis technique for measuring the binding between RNase and 5' adenosine monophosphate, found that since ribonuclease is a small protein, appreciable amounts of it dialyze across a membrane during a long experiment; thus their membranes were acetylated to prevent enzyme transport and thus increase the accuracy of their measurments. However, since their equilibration times were on the order of 50-75 hours, and since the transport of dinucleoside monophosphate was 99 % complete after 10 hours under the conditions used here (see Figure 4; the curve was determined by the method described in the theoretical section above), it was decided to try an untreated (i. e., unacetylated) membrane first.

Experiments similar to those described in the theoretical section above were performed to determine the relative speeds of transport of RNase, dinucleosides, and trinucleosides. The

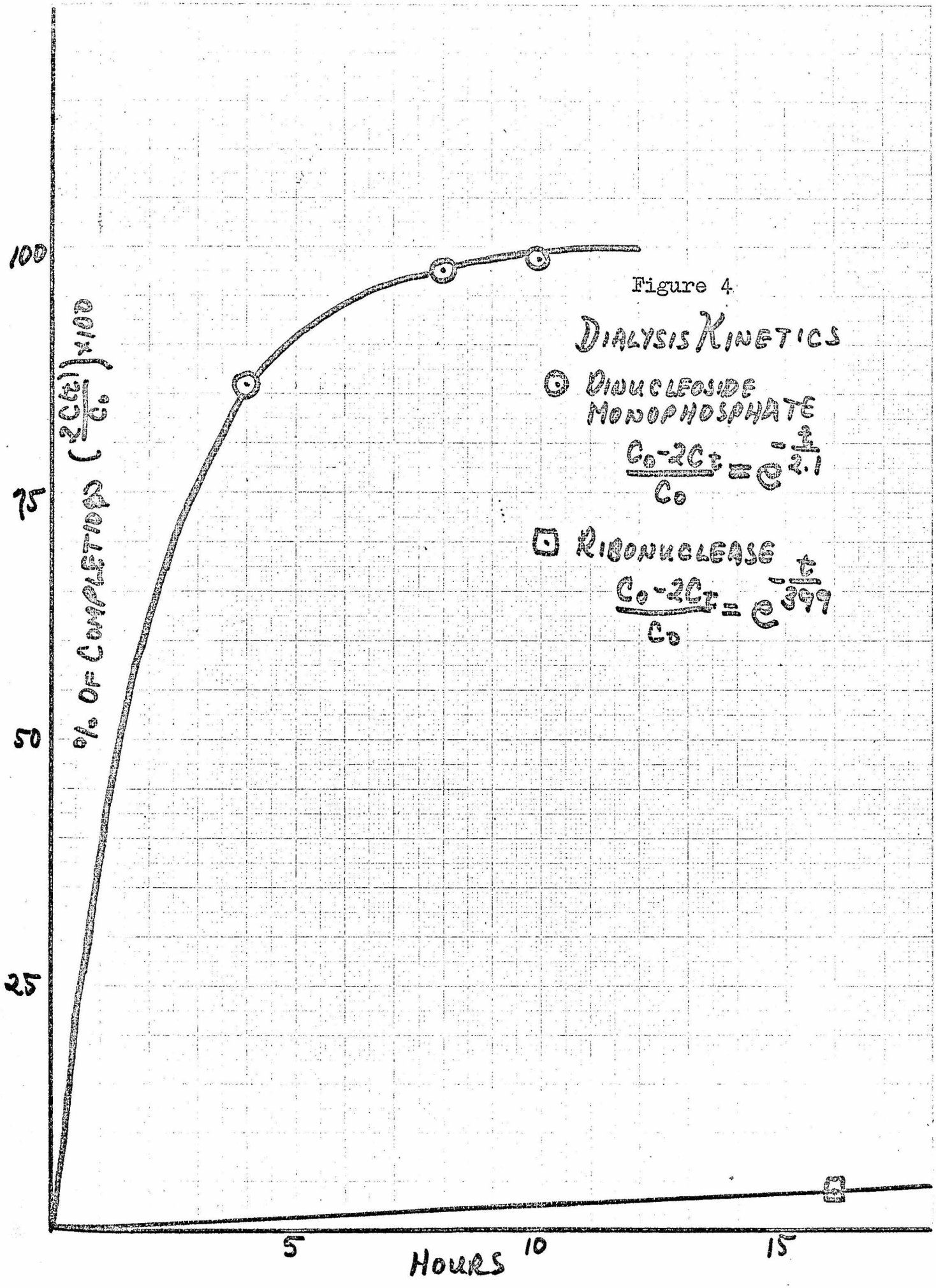


Figure 4

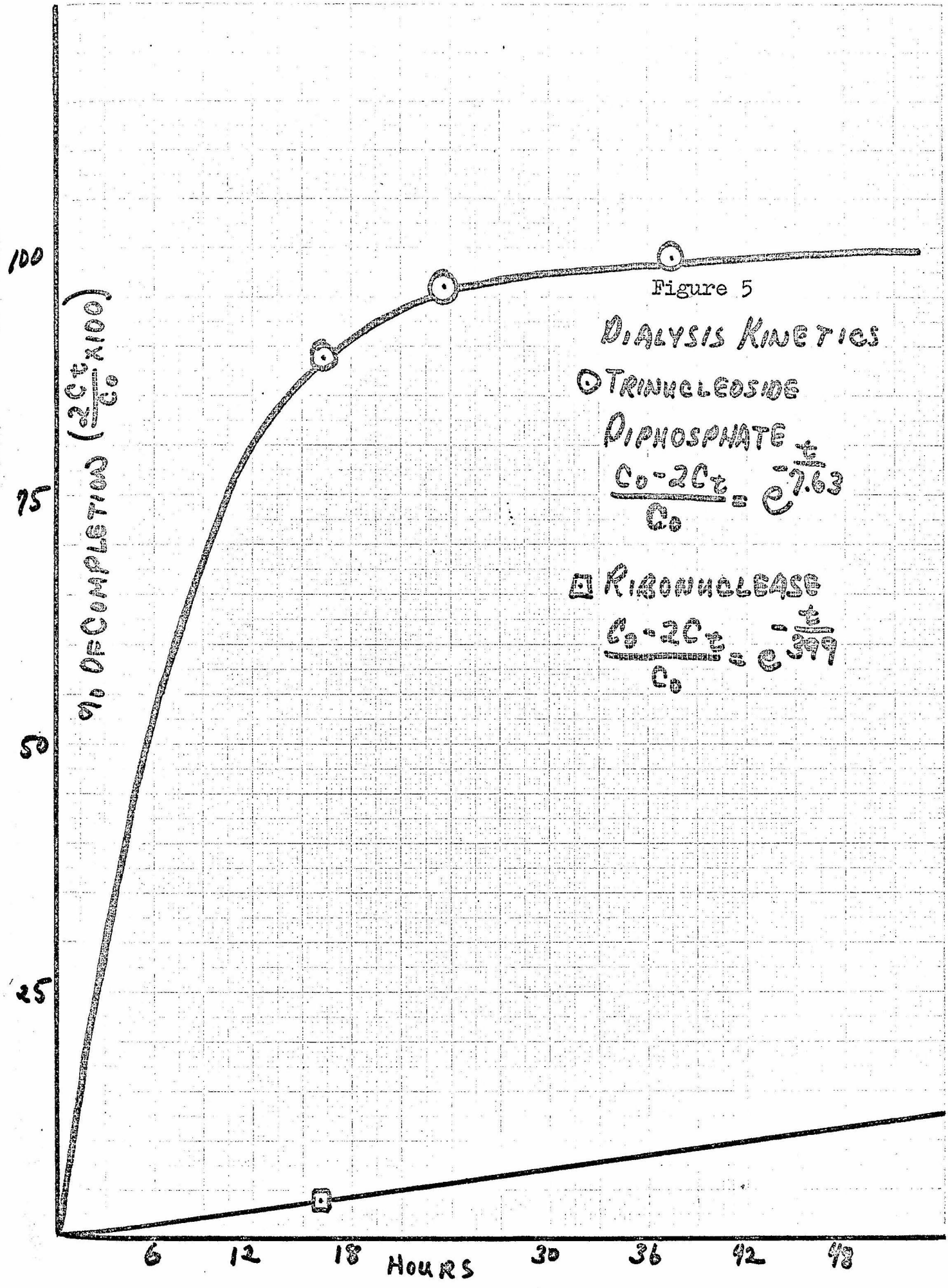
DIALYSIS KINETICS

○ DINUCLEOSIDE MONOPHOSPHATE

$$\frac{C_0 - 2C_t}{C_0} = e^{-\frac{t}{2.1}}$$

□ RIBONUCLEASE

$$\frac{C_0 - 2C_t}{C_0} = e^{-\frac{t}{3.99}}$$


100
75
50
25
% OF COMPLETION $\left(\frac{2C_t}{C_0} \times 100\right)$
6
12
18
HOURS
30
36
92
48
Figure 5
DIALYSIS KINETICS
⊙ TRINUCLEOSIDE DIPHOSPHATE
$\frac{C_0 - 2C_t}{C_0} = e^{-\frac{t}{7.63}}$
⊡ RIBONUCLEASE
$\frac{C_0 - 2C_t}{C_0} = e^{-\frac{t}{379}}$

concentration of RNase was determined spectrally, as were the concentrations of the pyrimidine oligonucleotides. For the nucleotides, the absorbance peak at 267 mμ was used, while RNase concentration was determined from the height of the peak at 278 mμ. The results of these kinetics experiments are shown in Figures 4 and 5. Given the curve for RNase dialysis, it was decided that dinucleoside experiments were certainly practical, and that a trinucleoside experiment of 35 or so hours could be performed if one were willing to tolerate a systematic error of about 5 % in the determination of a binding constant.

Binding experiments were run with the pyrimidine dinucleosides, taking as a model the data of Hammes and Schimmel (1965) on the RNase-3' cytidine monophosphate complex; i. e., assuming the dinucleoside behaved rather as a mononucleotide than as a long DNA chain. Under this assumption, K_B should have been about 630 at pH 7.4 (reckoning from a linear extrapolation of Hammes' curve). The most useful concentration of dinucleoside for these experiments corresponds to an A_{267} in the range 1.0 --2.0 for P, which means A_{267} should be not greater than 1.7 for P*, or $P^* \leq 10^{-4}$. Under these conditions, we should then expect to find values of β corresponding to those in Table 1 below (recall Equation (5a)).

Table 1

RNase conc. (mg/ml)	1.38	2.76	13.87
" " (mole/l)	1×10^{-4}	2×10^{-4}	1×10^{-3}
β	9.4	5.2	1.84
C_e (in A_{267} units)**	0.944	0.892	0.589

**Assuming $P = C_o = 2.00$ A_{267} units; i. e., $P^* = 0.948$, 1.108, and 1.410.

The experimental data, however, are somewhat different. In practice, the dinucleoside concentrations (P) were around $A_{267} = 1.5$, but this should have given essentially similar values of C_e. Instead, no binding was observed at all for RNase concentrations less than 10 mg/ml. Two runs were made at higher enzyme concentrations, and the following were observed (all dinucleoside are in absorbance units at 267 mμ):

E (RNase)	10.125 mg/ml	13.26 mg/ml
	7.36×10^{-4} M	9.62×10^{-4} M
P*	0.550	0.7435
C	0.004	0.007
β	137.5	113.3
P* (moles/l)	0.33×10^{-4}	0.44×10^{-4}
K_B	9.53	8.34
Dialysis time	10 hours	10 hours

Since the absorbance differences are so small, these measurements are not to be taken too seriously. However, one may assert with some confidence that the binding constant is certainly of the order of 10 or less, and is at least a factor of 600 lower than what might be expected from the data of Hammes and Schimmel (1965).

It is not nearly as easy to explain this phenomenon. At first glance, it might appear as though the addition of a second nucleoside without the concomitant addition of a phosphate group lowers the charge to mass ratio to an extent that the electrostatic binding is drastically cut. Put another way, let us imagine RNase to have a structure essentially similar to that of lysozyme; i. e., we shall say that there is one binding site for each monomer unit in a long chain. Then one binding site can happily accept a 3'-pyrimidine nucleotide, but the site below it is now asked to bind to a pyrimidine without a 3'-phosphate, and it is conceivable that the second-site binding might be

substantially weaker, and thus destabilized relative to the binding of a mononucleotide, a dinucleotide with a 3'-phosphate (pPypPy), or even a trinucleoside diphosphate. Hence the significantly smaller K_B. The answer could also involve some sort of steric recognition, but this is difficult to evaluate in any more than a vague qualitative sense, especially when considered relative to the electrostatic compnent of the binding. At any rate, obvious future experiments are comparisons of these data with those obtained for 3'-pyrimidine dinucleotides and trinucleoside diphosphates, to see if either the addition of a second negative charge, or of another pyrimidine nucleotide, makes an oligodeoxynucleotide look more like a substrate molecule to the ribonuclease.

III The Enzymatic Activity of Ribonuclease

The problems and experiments discussed in this section arose largely in the course of an attempt to develop a workable assay for ribonuclease activity. At first, the assay procedure tried was that given by Felsenfeld, et. al. (1963), which was carried out at pH 5.0 in a buffer that was 0.064 F acetate. All attempts to perform an assay in which a substantial amount of acid-soluble polyribonucleotides were produced failed. The reason is still not known, but it was decided to change the pH to 6.86 (which was closer to the pH optimum of 7.6-7.8 reported by Kunitz (1940) for RNase activity) and to lower the ionic strength to 0.014 M. A rate study was done at 37° C, with the above ionic conditions, and an RNase concentration of 0.264 g/ml, and an RNA concentration of 1.06 mg/ml. The assay was performed as described on pp. 2-3 of this work, but for varying incubation times. The hydrolysis (production of acid-soluble material) is given in Figure 6. It was decided that 15 minutes was sufficiently close to the linear portion of the rate curve to give a workable assay, and thus a test assay was performed, using a range of concentrations of RNase which would be employed in future experiments. As Figure 7 shows, the conditions chosen do provide a workable assay for which the rate of hydrolysis (i. e., the amount of acid-soluble material produced) is proportional to the first power of the RNase concentration. This assay procedure was consequently used for all further experiments described in section I.

As a natural consequence of the RNase-DNA binding studies, the possibility of doing such a sedimentation experiment to determine the strength of RNA-RNase binding aroused considerable interest. As a preliminary test of the idea, an

Figure 6

RNase Kinetics
Study pH 6.86
37°C

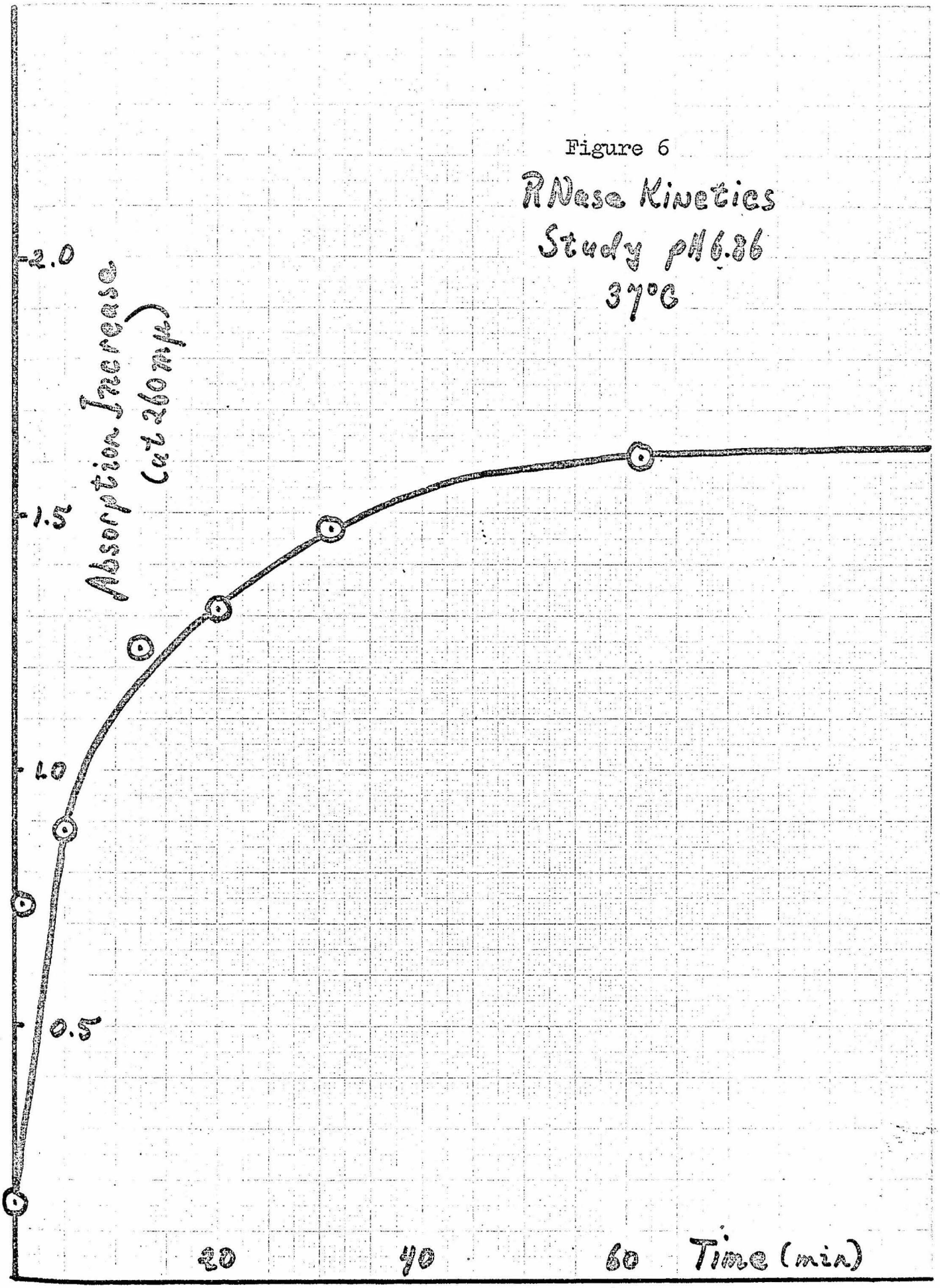
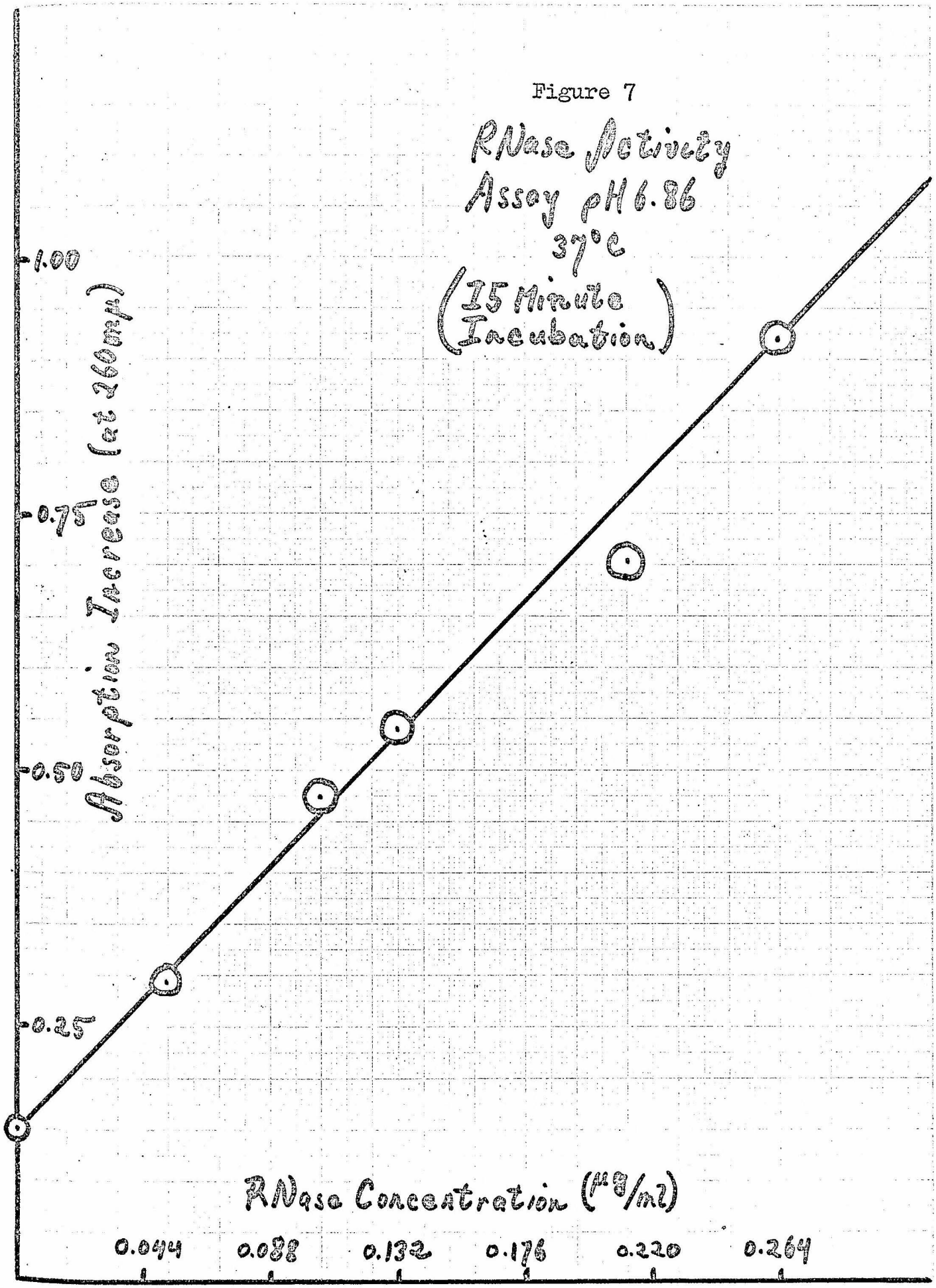


Figure 7


RNase Activity
Assay pH 6.86
37°C
(15 Minute
Incubation)
1.00
0.75
0.50
0.25
Absorption Increase (at 260mμ)
RNase Concentration (μg/ml)
0.044
0.088
0.132
0.176
0.220
0.264

assay similar to that described above was run at 0° C, again for varying times of incubation. It was found that the rate of production of acid-soluble material was 10 % of that at 37° C, which indicates that a sedimentation run which would take on the order of ten hours, is quite unfeasible. This does not, however, rule out the possibility of an electrophoretic determination of the binding constant, since the nucleic acid band should still be quite distinct, even though it will be broadened somewhat as a result of RNase hydrolysis.

Let us return to the activity assay for a moment. It will be noticed that the assay measures the production of acid-soluble polyribonucleotides, with the implicit assumption that this is equivalent to, or at least directly porportional to the rate of hydrolysis. It is not at all clear that this is so, and whether it is or not depends on the mode of action which the enzyme molecule pursues. To illustrate, let us imagine a solution of macromolecules as depicted in Figure 8, and assume that an RNase molecule enters this solution and attacks an RNase molecule at point A, splits the molecule and releases it. If the hit is close to an end, an acid-soluble particle is produced; if not, we just have one more big molecule. Now if the RNase, releases the RNA and diffuses randomly, the tertiary structure of the RNA may cause a distant part of the same molecule, or even a part of another molecule, to be closer to the enzyme than a "close" part of the chain it had just hit, say sites B or C. Thus B or C are hit in preference to D, and an acid-soluble

Figure 8.

particle is not formed. In fact, it may take several hits before an acid-soluble particle is formed. Clearly, under these conditions, and with one or a most a few enzyme molecules per RNA molecule, the rate of production of acid-soluble material is not proportional to the rate of hydrolysis. On the other hand, if the RNase molecule, after a hit travels systematically down the chain either a certain length (this does not seem too likely) or until it finds another hydrolysis site (this idea is more attractive), an acid-soluble particle should be produced with each hit.

In order to differentiate between the two cases, the following experiment is proposed:

A sample of homogeneous RNA would be subjected to RNase hydrolysis until a given average number of hits per molecule had occurred (this may be determined by calculating the pH change which should occur with each hit, then performing the hydrolysis in an unbuffered solution and monitoring the pH change), then stopping the reaction and looking at the size distribution of the molecules present. If the first explanation is correct, the size distribution should conform to a most probable distribution which may be calculated beforehand. The second alternative would cause the peak to shift to the left of the peak expected from the calculated distribution, and would cause the concentrations of the smaller polynucleotides to be correspondingly larger. The size distribution could then be determined experimentally by either analytical or preparative sedimentation, or by paper chromatography.

The author would like to thank Dr. Norman Davidson for the inspiration and invaluable guidance he provided, and Dr. Baldomero Olivera, for his patient instruction in experimental technique, the electrophoretic data he so kindly provided, and for many enlightening and encouraging coffee breaks and breakfasts after particularly trying experiments. The author would also like to thank Drs. J. Vinograd and M. Raftery for permitting the use of their equipment on various occasions.

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