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Deoxycytidylate Aminohydrolase. IV. Stoichiometry of Binding of Isosteric and Allosteric Effectors*

Eduardo Scarano, Giuseppe Geraci, and Mosè Rossi

ABSTRACT: The technique by Hummel and Dreyer (Hummel, J. R., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 95*, 1) of gel filtration was used to measure the number of isosteric and allosteric sites of deoxycytidylate aminohydrolase from donkey spleen. The enzyme binds at saturation four molecules of deoxycytidine triphosphate (dCTP) or of deoxythymidine triphosphate (dTTP) per molecule, but cannot bind at the same time both regulatory nucleotides. Magnesium ions are required for the binding of the allosteric effectors. Four molecules of deoxyadenosine monophosphate (dAMP) and four molecules of dCTP, or four molecules of deoxyguanosine monophosphate (dGMP) and four molecules of dTTP, bind at the same time per molecule of enzyme. We conclude that the molecule of dCMP aminohydrolase has four catalytic sites and either four or eight regulatory sites. It is possible that the enzyme has four regulatory sites which can bind either dCTP or dTTP, but it is also possible that dCTP and dTTP have independent binding sites on the enzyme and consequently the enzyme would possess four catalytic sites, four regulatory sites for the activator nucleotide and four regulatory sites for the inhibitor nucleotide.

The kinetic properties of homogeneous deoxycytidylate aminohydrolase (EC 3.5.4.5) (Geraci *et al.*, 1967; Scarano *et al.*, 1967; Rossi *et al.*, 1967) are compatible with a model of the enzyme molecule having multiple substrate (isosteric) sites and multiple regulatory (allosteric) sites, but no molecular mechanism can be demonstrated by kinetic experiments, and other interpretations of the kinetic data could be advanced, as for instance one based on relaxation effects of the enzyme molecule with one binding site for each ligand (Weber, 1965; Anderson and Weber, 1965). We have attempted to measure by a direct method the number of allosteric and isosteric sites of dCMP[1] aminohydrolase.

Hummel and Dreyer (1962) have described a method for measuring the binding of small molecules to proteins by gel filtration. Fairclough and Fruton (1966) have discussed the advantages of gel filtration over dialysis equilibrium in investigating complexes of bovine serum albumin with tryptophan and tryptophan derivatives.

In the present paper the method of Hummel and Dreyer was used to measure the number of allosteric and isosteric sites of dCMP aminohydrolase. For the allosteric sites no technical problems arise because of the high affinity of the enzyme for the allosteric effectors. For the measurements of the number of the substrate sites the competitive inhibitors of dCMP

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[1] Abbreviations used: dCMP, deoxycytidine monophosphate; dAMP, deoxyadenosine monophosphate; dGMP, deoxyguanosine monophosphate; dTTP, deoxythymidine triphosphate; dCTP, deoxycytidine triphosphate; dTMP, deoxythymidine monophosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)].

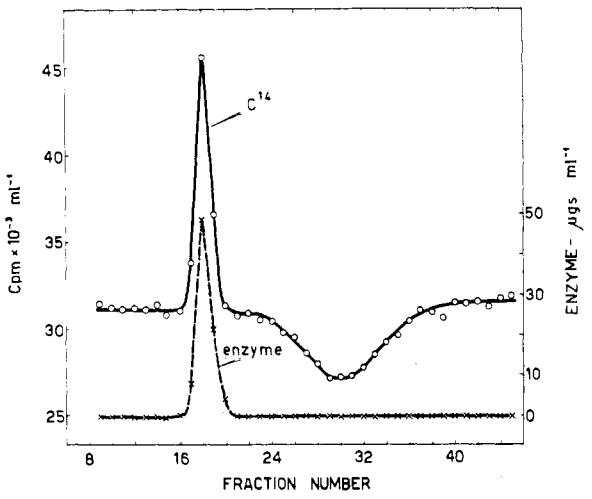

FIGURE 1: Representative elution profile for measurement of the binding of dCTP by deoxycytidylate aminohydrolase. The column was equilibrated as specified in Methods. [^{14}C]dCTP, 4 μM (sp act. 7.6 $\times$ 10^6 cpm/μmole); 22.5 μg of enzyme; enzyme recovery, 96%.

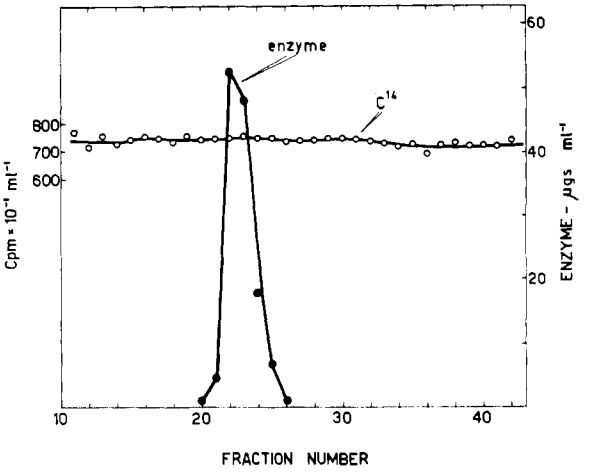

FIGURE 3: Representative elution profile for measurement of the removal of dTTP by dCTP from dCMP aminohydrolase. [^{14}C]dTTP, 4 μM (sp act. 1.7 $\times$ 10^6 cpm/μmole); dCTP, 35 μM; enzyme, 58 μg; enzyme recovery, 90%.

aminohydrolase, dAMP and dGMP, have been used. The high affinity of the dTTP–Mg–enzyme complex for dGMP and the high affinity of the dCTP–Mg–enzyme complex for dAMP (Rossi *et al.*, 1967) permit the application of the gel filtration method for measuring the number of the substrate sites. The assumption is made that the number of molecules of dAMP or of dGMP which bind to the dCTP–Mg–enzyme complex or to the dTTP–Mg–enzyme complex, respectively, measures the number of substrate sites. This assumption is based on the kinetic data already reported and on data described in the present paper.

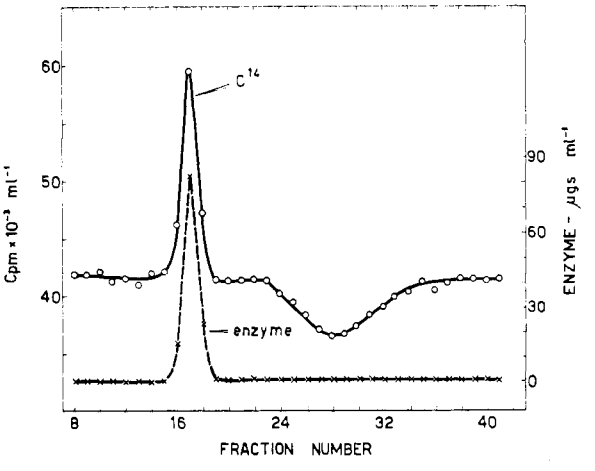

FIGURE 2: Representative elution profile for measurement of the binding of dTTP by deoxycytidylate aminohydrolase. [^{14}C]dTTP, 10 μM (sp act. 5.7 $\times$ 10^6 cpm/μmole); enzyme, 40 μg; enzyme recovery, 90%.

Materials and Methods

All reagents were analytical reagent grade and were crystallized from 10 mM Versene in quartz-redistilled water and recrystallized from quartz-redistilled water. Tris was recrystallized from 85% ethanol. Nucleotides were purchased from Calbiochem; ^{14}C- and ^{3}H-labeled nucleotides from Schwarz BioResearch. All nucleotides were purified by thin layer chromatography (Grippo *et al.*, 1965). Sephadex G-25 (bead form) was obtained from Pharmacia, Uppsala. Primary and secondary scintillators, PPO and dimethyl-POPOP, and Cab-O-Sil were purchased from Packard. Homogeneous dCMP aminohydrolase was prepared from donkey spleen as previously described (Geraci *et al.*, 1967).

Binding studies were performed at 4° on Sephadex G-25 (bead form) columns (0.4 $\times$ 100 cm). The columns were equilibrated with 50 mM Tris–2 mM $MgCl_2$–7 mM β-mercaptoethanol (pH 7.5), containing the specified nucleotides. The enzyme was added on the column as soon as the effluent had the same composition of the input solution. The rate of the effluent was 0.15 ml/min; 0.25–0.3-ml fractions were collected. Each fraction was assayed for enzyme activity and radioactivity. The amount of enzyme was calculated from its activity. The activity of the enzyme was measured as previously described (Scarano *et al.*, 1967). The assay was performed at 22° using 0.5 mM CH_3-dCMP as substrate in 0.1 M phosphate buffer containing 1 mM β-mercaptoethanol (pH 7.3). Under these conditions 1 mg of the homogeneous enzyme deaminates 233 $\pm$ 10 μmoles of CH_3-dCMP/min.

Samples for radioactivity measurements were prepared by mixing 100 μl of each fraction from the gel filtration column with 20 ml of a liquid scintillator, which was prepared by adding 300 ml of methanol

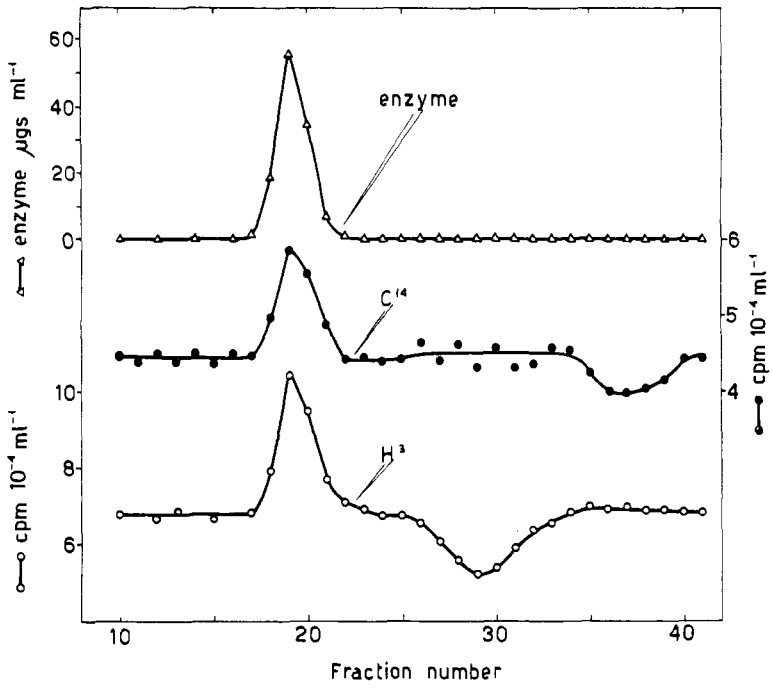

FIGURE 4: Representative elution profile for measurement of the simultaneous binding of dGMP and dTTP by deoxycytidylate aminohydrolase. [^{3}H]dTTP, 4 μM (sp act. 16.9 $\times$ 10^6 cpm/μmole); [^{14}C]dGMP, 5.85 μM (sp act. 7.6 $\times$ 10^6 cpm/μmole); enzyme, 47 μg; enzyme recovery, 96%.

and 1000 ml of Cab-O-Sil powder to 700 ml of toluene containing 2.8 g of PPO and 70 mg of dimethyl-POPOP. A Packard Model 4312 Tri-Carb scintillation spectrometer was used.

Results

All the data of stoichiometry of nucleotide binding to dCMP aminohydrolase are referred to a molecular weight of 1.2 $\times$ 10^5, which was reported in a previous paper (Geraci *et al.*, 1967).

The results of a typical experiment for measuring the number of molecules of dCTP–Mg, which bind per molecule of enzyme, are reported in Figure 1. When the column was equilibrated with 4 μM [^{14}C]dCTP, four molecules of dCTP were bound per molecule of enzyme. Doubling the concentration of dCTP does not cause any increase in the binding ratio. It is not possible to remove dCTP from the enzyme by addition of dTMP, tested up to a concentration of 6 mM. The same ratio dCTP:enzyme of 4 is obtained when the experiment is performed in the presence of 4 μM [^{14}C]-dCTP and 6 mM dTMP, but no dCTP binds to the enzyme when the column is equilibrated with 5 μM [^{14}C]dCTP and 50 μM dTTP.

With dTTP at 4 and 10 μM concentration, 5.1 molecules of nucleotide were found/molecule of enzyme. The data of one experiment are shown in Figure 2. In the presence of 6 mM dTMP and 4 μM [^{14}C]dTTP the ratio between dTTP and enzyme decreases from 5.1 to 3.5. The addition of 35 μM dCTP causes total removal of [^{14}C]dTTP from the enzyme as demonstrated in Figure 3. It is evident from Figure 3 that no accumulation of radioactivity occurs in the fractions containing the enzyme.

The binding experiments described below demonstrate the changes of the affinity of deoxycytidylate aminohydrolase for competitive inhibitors caused by allosteric effectors.

Figure 4 shows the data of an experiment in which the column was equilibrated with 4 μM [^{3}H]dTTP and 6 μM [^{14}C]dGMP. dTTP (4.5 molecules) and dGMP (4 molecules) are bound at the same time per enzyme molecule, but, when the column was equilibrated with 6 μM [^{14}C]dGMP and 35 μM dCTP, no more than 1.7 molecules of dGMP bind per molecule of enzyme. When the equilibrating solution contained 7 μM [^{3}H]dCTP and 10 μM [^{14}C]dAMP, 4.7 molecules of dCTP and 4.6 molecules of dAMP bind at the same time per enzyme molecule (Figure 5). However, with 7 μM dTTP and 10 μM dAMP, less than one molecule of dAMP binds per molecule of enzyme.

In experiments planned to measure the binding of dGMP to the enzyme in the absence of allosteric effectors, 1.4 molecules of dGMP bind/molecule of active enzyme; however, in this case, where dGMP was the only nucleotide present, 85% of the enzyme was inactivated during the experiment. If the bound dGMP is referred to the total protein a ratio dGMP:enzyme of 0.1 is obtained. At present it is not possible to distinguish between the following two possibilities: the nucleotide binds only to the active enzyme with a

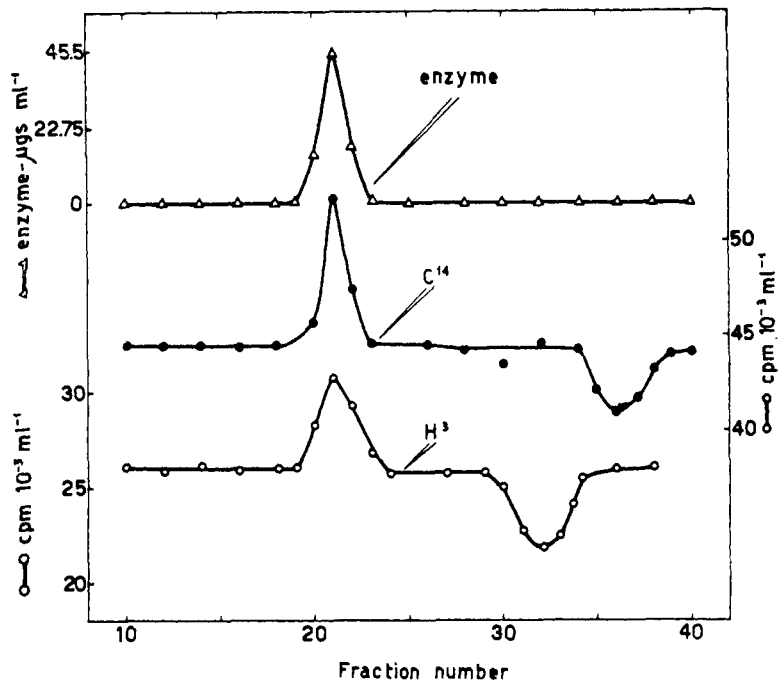

FIGURE 5: Representative elution profile for measurement of the simultaneous binding of dAMP and dCTP by deoxycytidylate aminohydrolase. [^{3}H]dCTP, 7 μM (sp act. 3.7×10^6 cpm/μmole); [^{14}C]dAMP, 10 μM (sp act. 4×10^6 cpm/μmole); enzyme, 30 μg; enzyme recovery, 80%.

ratio of 1.4 or it binds to the total protein, active and inactive enzyme, with an average ratio of 0.1.

In Tables I and II are listed all the results of the experiments of binding of isosteric and allosteric ligands. The data of experiments planned to search for heterotropic effects caused by isosteric ligands are reported in Table II. dAMP at 15 mM concentration decreases the affinity of the enzyme for dTTP. With dGMP up to a concentration of 15 mM it was not possible to find any effect on the binding of dCTP. When no Mg^{2+} is present in the equilibrating solution allosteric effectors do not bind to the enzyme and consequently no cooperative binding with competitive inhibitors is observed.

Discussion

The experiments of stoichiometry of binding of the allosteric effectors demonstrate that dCMP aminohydrolase can bind four molecules of the allosteric activator (dCTP–Mg) or four molecules of the allosteric inhibitor (dTTP–Mg). The two allosteric effectors at saturating concentrations cannot bind at the same time on the enzyme molecule. This means either that they bind at identical sites or that although they might have independent binding sites on the enzyme molecule, the binding of one regulator causes a conformational change of the binding site of the other one, which does not permit its binding.

From the experiments of stoichiometry of binding of the competitive inhibitors dAMP and dGMP it appears plausible to conclude that the enzyme has four catalytic sites. Indeed, the enzyme–$(dCTP)_4$ complex binds four molecules of dAMP and the enzyme–$(dTTP)_4$ complex binds four molecules of dGMP per molecule. Thus, at least eight specific binding sites occur on the enzyme molecule. The following points indicate that dAMP and dGMP bind to the catalytic sites of dCMP aminohydrolase. The two deoxynucleotides inhibit the enzyme with a clear kinetics of competition for the substrate sites (Rossi *et al.*, 1967); they bind to the enzyme together with an allosteric effector. Thus, on the assumption of the existence of only four sites for dCTP and dTTP only the catalytic sites would be available for the binding of dGMP or dAMP, but on the assumption that dCMP aminohydrolase has four binding sites for dCTP and four independent binding sites for dTTP, one might argue that dGMP in the presence of dTTP would bind to the four dCTP enzyme sites and conversely that dAMP in the presence of dCTP would bind to the four dTTP enzyme sites. The latter hypothesis appears unlikely. First, there is no evidence for independent binding sites for dCTP and for dTTP. Second, it would be difficult to understand how dAMP behaves as an activator at low substrate concentrations when using the enzyme and how it is a competitive inhibitor at all substrate concentrations when using the enzyme–$(dCTP)_4$ complex (Rossi *et al.*, 1967).

In some experiments more than four molecules of a single nucleotide bind per molecule of enzyme, but when the recovery of the enyzme is near 100%, the

TABLE I: Binding of Isosteric and Allosteric Ligands by Deoxycytidylate Aminohydrolase.

Nucleotides in the Equilibrating Solution (μM)	Moles of Labeled Nucleotides/Mole of Enzyme			
	dCTP	dTTP	dGMP	dAMP
a [^{14}C]dCTP (4 or 10)	4.0			
b [^{14}C]dTTP (4 or 10)		5.1		
c [^{14}C]dTTP (4) + dCTP (35)		0		
d [^{14}C]dGMP (4)			1.4	
e dTTP (6) + [^{14}C]dGMP (4)			3.9	
f [^{3}H]dTTP (4) + [^{14}C]dGMP (6)		4.5	4.0	
g dCTP (35) + [^{14}C]dGMP (6)			1.7	
h [^{3}H]dCTP (7) + [^{14}C]dAMP (10)	4.6			4.7
i [^{3}H]dTTP (7) + [^{14}C]dAMP (10)		3.9		1

number is near four within the limits of the error of the methods. It is pertinent to discuss some specific instances. The binding of 5.1 molecules of dTTP on the enzyme in expt b of Table I might be explained assuming that the substrate sites have some affinity for dTTP. This interpretation is supported by the data of expt i, which show that in the presence of dAMP only four molecules of dTTP bind. It is more difficult to understand how 4.7 molecules of dCTP and 4.6 molecules of dAMP bind per molecule of enzyme (expt h of Table I). The following three explanations can be discussed. (1) The extra molecules of nucleotides bound might be apparent, because the enzyme is determined by its activity and due to its 80% recovery the inactivated enzyme, which might still bind nucleotides, is not taken into account; (2) some unspecific binding sites on the enzyme molecule might bind the extra nucleotides; and (3) on the assumption that dCTP and dTTP have independent binding sites, the enzyme would have four substrate sites, four sites for dCTP and four sites for dTTP. Thus, the extra nucleotides might bind on some free regulatory sites.

TABLE II: Changes of the Affinity of Allosteric Sites Caused by the Binding of Isosteric Ligands.

Nucleotides in the Equilibrating Solution	Moles of Labeled Nucleotides/Mole of Enzyme	
	dCTP	dTTP
a [^{3}H]dTTP (4 μM)		5.0
b [^{3}H]dTTP (7.5 μM)		4.9
c [^{3}H]dTTP (4 μM) + dAMP (15 mM)		1.1
d [^{3}H]dTTP (7.5 μM) + dAMP (15 mM)		3.7
e [^{3}H]dTTP (4 μM) + dGMP (15 mM)		4.2
f [^{3}H]dCTP	4.3	
g [^{3}H]dCTP (4.1 μM) + dGMP (15 mM)	4.7	

The specificity of the nucleotide binding sites of dCMP aminohydrolase is suggested by the following. (1) dTTP or dCTP does not bind to the enzyme in the absence of divalent cations; (2) when experiments are done in the presence of [^{14}C]dTTP and cold dCTP, or [^{14}C]dCTP and cold dTTP, no accumulation of radioactivity was found in the enzyme peak; (3) dTTP increases the affinity of dGMP, and dCTP increases the affinity of dAMP for the enzyme; and (4) a consistent pattern of specific correlation between allosteric and isosteric sites can be inferred from the analysis of both the kinetic experiments and the binding experiments; for instance, the experiments of stoichiometry of binding support the conclusion, reached on the basis of kinetic experiments, about the specific changes of the affinity of the catalytic sites for dAMP and dGMP caused by the allosteric effectors.

The data in Table II demonstrate the heterotropic effects caused by isosteric ligands on the allosteric sites. Thus, dCMP aminohydrolase shows all the possible homotropic and heterotropic effects between catalytic and isosteric sites.

Acknowledgment

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A Method for the Hybridization of Nucleic Acid Molecules at Low Temperature*

James Bonner, Grace Kung, and Isaac Bekhor

ABSTRACT: Ribonucleic acid (RNA) may be hybridized to denatured deoxyribonucleic acid (DNA) at temperatures of 0–24° rather than the usual *ca.* 66°, provided that the hybridization reaction mixture contains an adequate concentration of formamide. The formamide concentration which yields maximum hybridization of RNA to DNA depends upon temperature and salt concentration. Maximum hybridization was achieved at 0° in a salt concentration of 0.16 M in 30 vol. % formamide, and at 24° in 0.32 M salt in the same formamide concentration. Under these two conditions the fraction of DNA hybridized with RNA at saturation is identical with the fraction of RNA hybridized at saturation under the usual hybridization conditions at elevated temperature. Advantages of the use of aqueous formamide over the use of high temperature include increased retention of DNA by membrane filters and lessened nonspecific absorption of RNA to such filters, as well as lessened chain scission of RNA during the long periods of incubation required for hybridization to saturation of complex RNAs to DNA.

We have found that RNA may be hybridized to denatured DNA at low temperature in the presence of appropriate concentrations of formamide. The procedure was originally developed during search for conditions under which RNA and protein might simultaneously be annealed to DNA. It has now been found, however, that the method has advantages over the use of heat for routine hybridization. We describe below the method and its merits.

The general procedures are based upon those of Gillespie and Spiegelman (1965) in which RNA in solution is hybridized to denatured DNA which is immobilized on nitrocellulose filters. Deproteinized pea DNA was further purified by treatment with RNase, pronase (preincubated to remove nuclease activity) to remove the RNase, and finally phenol extraction. It was then denatured in alkali and applied to 25-mm nitrocellulose filters (Schleicher & Schuell B-6) as described by Gillespie and Spiegelman (1965).

^{14}C-Labeled RNA was made by the transcription of pea cotyledon chromatin prepared according to the methods of Bonner *et al.* (1963). Such chromatin, which possesses a template activity 0.3 that of deproteinized pea DNA, was incubated under template-limiting conditions with purified *Escherichia coli* RNA polymerase (f_4 of Chamberlin and Berg, 1962) and ^{14}C-labeled ATP in the standard RNA polymerase reaction mixture of Chamberlin and Berg (1962). Incubation was for 2 hr at 30°. The RNA synthesized, which amounted to 40–90 times the amount of template used, was purified by phenol extraction, DNase (electrophoretically purified) treatment, and finally phenol extraction. The specific activity of the [^{14}C]ATP[1] used was adjusted so that the final RNA possessed a specific activity of approximately 75,000 cpm/OD_{260}. The RNA possessed an average sedimentation coefficient of 14 S in 0.1 M potassium acetate buffer.

In general, duplicate DNA-containing filters and one blank filter were incubated in 1 ml of RNA made up in salt of the required concentration and contained in a scintillation counting vial. Incubation was at various temperatures and for various periods of time detailed below. At the end of the incubation period the filters were removed, washed, treated with RNase, rewashed, and dried as recommended by Gillespie and Spiegelman (1962). The dried filters were then counted in a scintillation spectrometer.

When labeled RNA is incubated with DNA-con-

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[1] Abbreviations used: ATP, adenosine triphosphate; SSC, standard saline citrate.