*U. S. 50*, 355.
Iyer, V. N., and Szybalski, W. (1964), *Science 145*, 55.
Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 5*, 109.
Meselson, M. (1957), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.
Nandi, U. S., Wang, J. C., and Davidson, N. (1965), *Biochemistry 4*, 1687.
Reichmann, M. E., Rice, S. A., Thomas, C. A., and Doty, P. (1954), *J. Amer. Chem. Soc. 76*, 3047.
Skalka, A., Burgi, E., and Hershey, A. D. (1968), *J. Mol. Biol. 39*, 1.
Spatz, H.-Ch., and Crothers, D. M. (1969), *J. Mol. Biol. 42*, 191.
Summers, W. C., and Szybalski, W. (1967), *J. Mol. Biol. 26*, 107.
Szybalski, W., and Iyer, V. N. (1964a), *Fed. Proc. 23*, 946.
Szybalski, W., and Iyer, V. N. (1964b), *Microbiol. Genetics Bull. No. 21*, 16.
Thomas, C. A., and Pinkerton, T. C. (1962), *J. Mol. Biol. 5*, 356.
Vinograd, J., and Hearst, J. E. (1962), *Fortschr. Chem. Org. Naturstoffe 20*, 372.

# Studies on Analogs of Isosteric and Allosteric Ligands of Deoxycytidylate Aminohydrolase*

Mosè Rossi, Richard L. Momparler,† Roberto Nucci, and Eduardo Scarano‡

ABSTRACT: Cytosine arabinoside 5′-monophosphate (araCMP) and cytidine 5′-monophosphate (CMP), analogs of deoxycytidine 5′-monophosphate (dCMP), were deaminated at a very low rate by deoxycytidylate aminohydrolase. The addition of deoxycytidine 5′-triphosphate-Mg (dCTP-Mg), allosteric activator of this enzyme, produced a marked increase in the rate of deamination of araCMP and CMP. The kinetics of deamination of araCMP and CMP are cooperative and deoxythymidine triphosphate enhances the cooperativity. In the presence of dCTP the kinetics obeys to the Michaelis–Menten equation. At very high CMP concentrations inhibition by excess of substrate occurs and this inhibition is released by dCTP. It seems that CMP competes with dCTP for regulatory sites. Studies with analogs of allosteric effectors demonstrate the very high specificity of the allosteric sites of deoxycytidylate aminohydrolase.

The kinetic data obtained with CMP suggest the occurrence of three conformational isomers of deoxycytidylate aminohydrolase.

The activity of deoxycytidylate aminohydrolase is regulated by highly specific allosteric effectors: dCTP-Mg is the allosteric activator, dTTP-Mg is the allosteric inhibitor (Geraci *et al.*, 1967; Scarano *et al.*, 1967a). At saturating concentrations of the allosteric effectors the enzyme occurs as the enzyme–dCTP-Mg complex or as the enzyme–dTTP-Mg complex (Scarano *et al.*, 1967b). Moreover, the allosteric effectors cause specific changes of the affinity of the enzyme for competitive inhibitors (Rossi *et al.*, 1967).

The striking changes of the affinity of the enzyme for both substrate and competitive inhibitors, caused by allosteric effectors, prompted us to investigate analogous changes of the substrate specificity of the enzyme.

The present paper reports experiments of the activity of deoxycytidylate aminohydrolase toward several analogs of the substrate. In addition, studies on the specificity of the allosteric effectors of the enzyme are reported.

## Material and Methods

Homogeneous deoxycytidylate aminohydrolase was prepared as previously described (Geraci *et al.*, 1967). The activity of the enzyme toward the analogs was measured either by a spectrophotometric assay or by an isotope assay similar to those used to determine the activity toward deoxycytidylate (Scarano *et al.*, 1967a).

Samples of chemically synthesized araCMP[1] were kindly donated by G. A. Fisher of Yale University and by M. J. Wechter of Upjohn Co. AraCTP was prepared from araCMP

* From the International Institute of Genetics and Biophysics, Naples, Italy, Laboratory of Molecular Embryology, Naples, Italy, Cathedra of Molecular Biology, University of Palermo, Italy. *Received December 22, 1969.* This is the fifth paper of a series on deoxycytidylate aminohydrolase. The preceding paper is published in this journal vol. 6, p 3645.

† Postdoctoral Fellowship 7-F2-CA-29,910-01A1 of the National Cancer Institute of Public Health Service.

Present address: McGill University Cancer Research Unit, Montreal, Can.

‡ Inquires should be addressed to Professor E. Scarano, International Institute of Genetics and Biophysics, Naples, Italy.

[1] Abbreviations used are: araCMP, arabinosylcytosine monophosphate; araUMP, arabinosyluracil monophosphate; araCTP, arabinosylcytosine triphosphate; $CH_3$dCTP, 5-methyldeoxycytidine triphosphate.

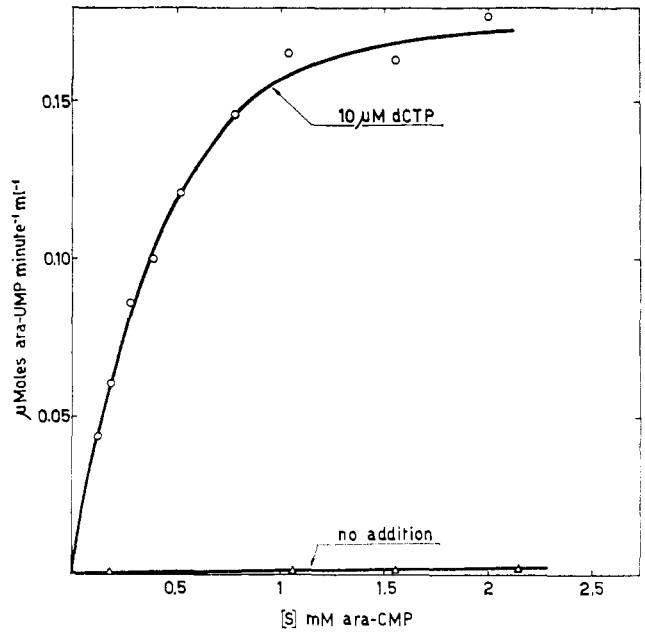


FIGURE 1: Aminohydrolysis by deoxycytidylate aminohydrolase and by the dCTP-Mg-enzyme complex of araCMP, as a function of its concentration. Spectrophotometric assay; the incubation mixture contained 0.05 M Tris, 1 mM mercaptoethanol, and 1 mM $MgCl_2$ (pH 7.5). Enzyme, 1.5 μg/ml; $T = 38°$. (△-△) No addition; (○-○) 10 μM dCTP.

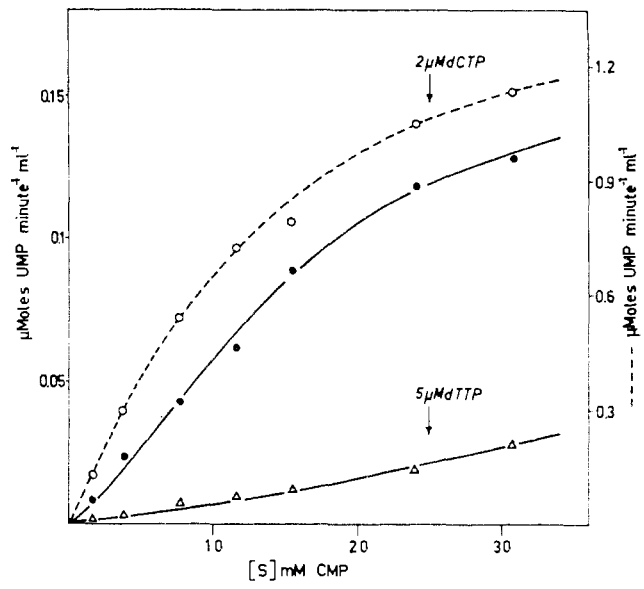


FIGURE 3: Aminohydrolysis of CMP as a function of its concentration and effect of allosteric ligands. Radioactive assay. The incubation mixture contained 0.05 M Tris, 1 mM mercaptoethanol, and 1 mM $MgCl_2$ (pH 7.5). The specific activity of CMP was 74,500 cpm/μmole. Enzyme 45 μg/ml. (●-●) No addition; (△-△) 5 μM dTTP; (○-○) 2 μM dCTP (scale on the right side). $T = 38°$.

by the method of Smith and Khorana (1958) and was purified by thin-layer chromatography on DEAE-cellulose (Grippo *et al.*, 1965). AraUMP and UMP were isolated from the enzymatic assay mixtures by thin-layer chromatography (Grippo *et al.*, 1965). [$^3$H]araCMP was prepared from the nucleoside with deoxycytidine kinase (Momparler and Fisher, 1968). [$^{14}$C]CMP was purchased from New England Corp.

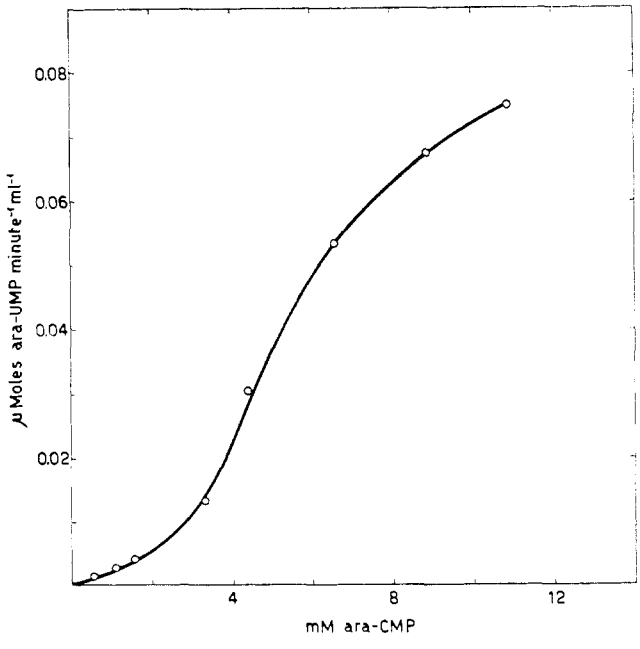


FIGURE 2: Effect of araCMP concentration on initial reaction rate of aminohydrolysis by deoxycytidylate aminohydrolase. Radioactive assay. The incubation mixture contained 0.05 M Tris, 1 mM mercaptoethanol, and 1 mM $MgCl_2$ (pH 7.5). The specific activity of [$^3$H]araCMP was 286,400 cpm/μmole. Enzyme, 16 μg/ml; $T = 38°$.

## Results

The aminohydrolysis of araCMP by deoxycytidylate aminohydrolase as a function of the concentration of araCMP is shown in Figure 1. AraCMP is deaminated at a very slow rate. By the spectrophotometric assay, an appreciable aminohydrolysis of araCMP can be observed only at 2 mM araCMP, which is the highest concentration possible for a spectrophotometric assay of the accuracy required here. A striking increase of the affinity of dCMP-aminohydrolase for araCMP occurs when the enzyme is present as the dCTP-Mg-enzyme complex. In the presence of 10 μM dCTP and Mg ions the deamination of araCMP can be easily measured by the spectrophotometric assay (Figure 1). The reaction follows the Michaelis-Menten kinetics with [S] at $(1/2)V_{max}$ of 0.6 mM.

In absence of dCTP, the kinetics of the deamination of araCMP by deoxycytidylate aminohydrolase as a function of araCMP concentration can be studied only by the isotopic assay. The data are reported in Figure 2. The Hill equation

$$v = \frac{V[S]^n}{K + [S]^n}$$

with $n = 2$ (Figure 4a) fits the data depicted in Figure 2.

The aminohydrolysis of CMP is observed when using the radioactive assay or, spectrophotometrically, in the presence of dCTP-Mg at very high enzyme concentrations.

The activatory effect of dCTP and the inhibitory effect of dTTP in the presence of Mg as a function of the substrate concentration were studied with CMP as substrate (Figure

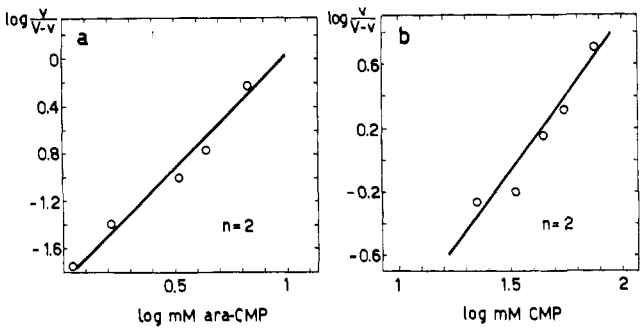


FIGURE 4: Hill plot of the dependence of the enzyme activity upon araCMP and CMP concentration in the absence of dCTP and of dTTP. Incubation mixture as reported in the legends of Figures 2 and 3.

3). dCTP-Mg activates and dTTP inhibits the aminohydrolysis of CMP. Moreover, the reaction follows the Michaelis–Menten equation at saturating concentrations of dCTP. The data can be fitted by the same equation used for araCMP, and a value 2 of "$n$" is observed Figure 4b. In the presence of dTTP the value of $n$ becomes higher than 2. The inhibition by dTTP is reversed by dCTP.

The analysis of the data shown in Figure 3 raises the question whether using CMP as substrate the same $V_{max}$ is reached, both with and without the allosteric activator. When using deoxycytidylate as substrate the same $V_{max}$ is obtained with and without the activator (Scarano *et al.*, 1967a). Concentrations of CMP as high as 0.2 M have been used (Figure 5), and a different $V_{max}$ in the presence and the absence of dCTP has been found. In the absence of dCTP the maximum velocity is reached at 0.1 M CMP and further

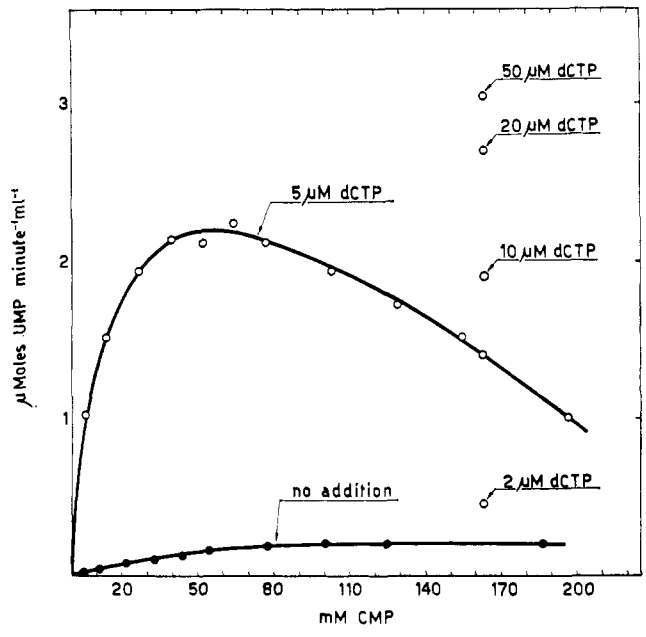


FIGURE 5: Effect of CMP concentration on initial reaction rate. Radioactive assay. The incubation mixture contained 0.05 M Tris, 1 mM mercaptoethanol, and 1 mM $MgCl_2$ (pH 7.5). The specific activity of CMP was 71,000 cpm/μmole. Enzyme, 32 μg/ml. (●–●) No addition; (○–○) μM dCTP as reported on the figure. $T$ = 38°.

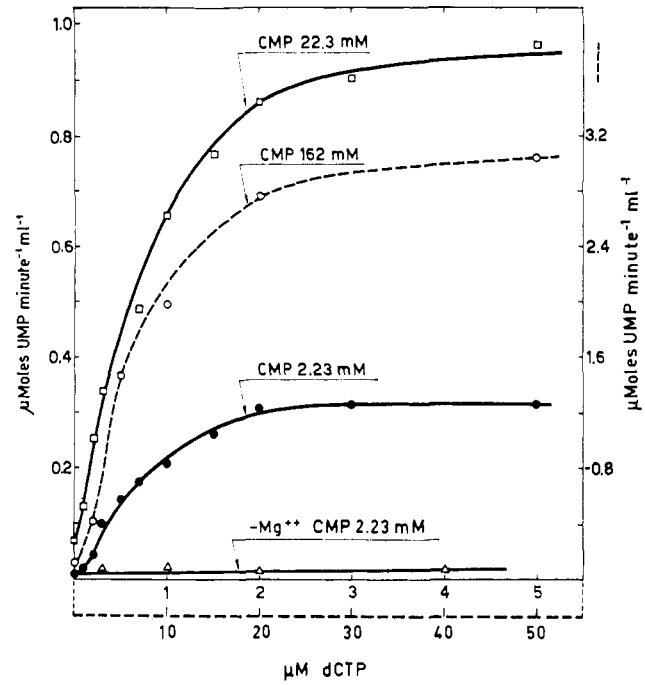


FIGURE 6: Effect of dCTP and Mg on the activity of deoxycytidylate aminohydrolase at the different CMP concentrations. The incubation mixture contained 0.05 M Tris, 1 mM mercaptoethanol, and 1 mM $MgCl_2$ (pH 7.5). Enzyme, 18 μg/ml. The concentration of dCTP must be multiplied by 10 when the curve at 162 mM CMP is considered. $T$ = 38°.

increase of the CMP concentration does not cause appreciable changes in the velocity. An [S] at $(1/2)V_{max}$ of 10 mM is obtained. When dCTP is present at a concentration of 5 μM, inhibition of the enzyme reaction occurs at a concentration of CMP higher than 80 mM. The inhibition by high concentration of CMP (Figure 5) can be released by an increase of the dCTP concentrations.

TABLE I: Kinetic Parameters for Substrates and for Analogs of the Substrate of Deoxycytidylate Aminohydrolase.

| Substrate | Allosteric Activator (dCTP) (μM) | [S] at $(1/2)V_{max}$[a] (mM) | $n$ Hill | Turnover[b] No. ($\times 10^{-2}$) |
|---|---|---|---|---|
| dCMP | 0 | 0.48 | 2 | 1150 |
| dCMP | 0.3 | 0.15 | 1 | 1150 |
| $CH_3$dCMP | 0 | 0.05 | 1.6 | 360 |
| $CH_3$dCMP | 2.5 | 0.025 | 1 | 260 |
| AraCMP | 0 | ~10 | 2 | 11 |
| AraCMP | 10.0 | 0.6 | 1 | 210 |
| CMP | 0 | 80 ± 10 | 2 | 8.2 |
| CMP | 50.0 | 10 | 1 | 82 |

[a] Substrate concentration when velocity of reaction is equal to one-half the maximal activity. [b] Moles of product formed per minute per mole of enzyme at 38°.

TABLE II: Analogs of Allosteric Effectors.[a]

| Substrate | μmoles Deaminated/min mg of Enzyme |
|---|---|
| dCMP (0.1 mM) | 2.4 |
| dCMP (0.1 mM) + dCTP (0.4 μM) | 10 |
| dCMP (0.1 mM) + $CH_3$dCTP (0.5 μM) | 9.6 |
| dCMP (0.1 mM) + araCTP (0.5 μM) | 2.4 |
| dCMP (0.1 mM) + araCTP (25 μM) | 2.7 |
| dCMP (0.1 mM) + araCTP (100 μM) | 2.7 |
| dCMP (0.1 mM) + CTP (0.5 μM) | 2 |
| dCMP (0.1 mM) + CTP (10 μM) | 2.4 |

[a] Incubation mixture: 0.05 M Tris (pH 7.5), 1 mM $MgCl_2$, 1 mM mercaptoethanol, and 0.1 mM dCMP. Enzyme, 0.25 μg/ml. Incubation mixture and nucleotides as specified. $T = 38°$. Spectrophotometric assay.

The activation of deoxycytidylate-aminohydrolase by dCTP at various concentrations of CMP is depicted in Figure 6. At 2.23 and 22.3 mM CMP the enzyme is half-saturated by 0.5 μM dCTP. However, at a concentration of 162 mM CMP, ten times more dCTP is necessary to half-saturate the enzyme. A requirement for $Mg^{2+}$ ions to obtain the dCTP effect is also demonstrated in Figure 6.

Table I lists the [S] at $(1/2)V_{max}$, the turnover number, and the $n$ of the Hill equation for different substrates of deoxycytidylate aminohydrolase. In every case the enzyme–Mg-dCTP has a higher affinity for the substrates than the enzyme.

In Table II is reported the effect of several analogs of the allosteric activator dCTP on the rate of deamination of deoxycytidylate by the enzyme. dCTP and $CH_3$dCTP produce almost identical activation (Scarano *et al.*, 1967a). On the other hand, araCTP and CTP, up to concentration of 0.1 mM produce no activation of deoxycytidylate aminohydrolase.

## Discussion

The effect of allosteric ligands on the affinity of the enzyme for competitive inhibitors: dGMP, dAMP, dTMP, and dUMP was reported previously (Scarano *et al.*, 1967b; Rossi *et al.*, 1967). Preferential binding of competitive inhibitors to the enzyme in the activated or the inhibited form was found. For example, dAMP has more affinity for the dCTP-Mg–enzyme and dGMP has more affinity for the dTTP-Mg–enzyme. These results, confirmed by experiments of stoichiometry of binding, indicate that the binding of allosteric effectors modifies the structure of the catalytic sites so that variation of affinity for inhibitors is observed.

All the analogs of the substrate tested have a higher apparent affinity for the dCTP-Mg–enzyme complex than for the enzyme with no ligands. The inhibition of the amino hydrolysis of cytidylate by excess of substrate observed with the dCTP-Mg–enzyme complex does not occur with the nonactivated enzyme. The more plausible interpretation is that CMP, at high concentrations, competes with dCTP for the activatory sites of the enzyme. In contrast to the inhibition by high concentrations of CMP, no inhibition occurs even at concentrations as high as 0.2 M when deoxycytidylate is used as a substrate for the dCTP-Mg–enzyme complex. This suggests that CMP does not induce the activated conformation of the enzyme while deoxycytidylate does. That deoxycytidylate induces the activated conformation is shown by the fact that the same velocity of deamination is observed at high concentrations of deoxycytidylate whether dCTP is present or not. Moreover, it is necessary to assume that CMP can be deaminated both by the conformation of the enzyme with no ligand and by the enzyme dCTP-Mg–complex, with two different $V_{max}$. This implies that three conformational isomers of deoxycytidylate aminohydrolase occur: the conformation of the enzyme with no ligands, the conformation of the enzyme–dCTP-Mg complex, and the conformation of the dTTP-Mg–enzyme complex. Direct evidence for the existence of three conformational isomers of the enzyme has been already presented (Rossi and Scarano, 1969). Kirtley and Koshland (1967) on the basis of an analysis of kinetic experiments (Geraci *et al.*, 1967; Scarano *et al.*, 1967a) have also suggested a three conformation model for deoxycytidylate aminohydrolase.

An interpretation of the data of Table I is at present impossible. It is not clear whether the enzyme recognizes only the position of the atoms in position 2′,3′ of the substrate, or also the position of the base with respect to the glycosylic bond. dCMP, $CH_3$dCMP, and CMP do occur both in the *anti* and in the *syn* configuration, while araCMP can only have the *anti* configuration (Haschemeyer and Rich, 1967). The higher affinity of the enzyme for araCMP as compared with CMP could be explained on the basis that araCMP has the OH groups of the 2′ and 3′ positions in *trans* conformation and thus resembles deoxycytidylate more than CMP does.

The data of Table II demonstrate that only a substitution in the 5 position of the pyrimidine ring is compatible with the regulatory action of dCTP. Other changes in the sugar structure lead to compounds which have no activatory effects. For instance, araCTP and CTP have no regulatory effect on deoxycytidylate aminohydrolase. This shows the very high specificity of the regulatory effectors.

The presence of an amino or of a hydroxyl group in position 6 of the pyrimidine ring determines the activatory or inhibitory action of the regulatory nucleotides as shown by the fact that $CH_3$dCTP is an activator as is dCTP and dUTP (Scarano *et al.*, 1967a) is an inhibitor as is dTTP.

## Acknowledgments

We thank Dr. Edward P. Whitehead for helpful discussion of the work and Mr. Sepe, Mr. Vaccaro, and Mr. Limongelli for their very skillful and enthusiastic technical assistance.

## References

Geraci, G., Rossi, M., and Scarano, E. (1967), *Biochemistry 6*, 182.

Grippo, P., Iaccarino, M., Rossi, M., and Scarano, E. (1965), *Biochim. Biophys. Acta 95*, 1.

Haschemeyer, A. E. V., and Rich, A. (1967), *J. Mol. Biol. 27*, 369.
Kirtley, M. E., and Koshland Jr., D. E. (1967), *J. Biol. Chem. 242*, 4192.
Momparler, R. L., and Fisher, G. A. (1968), *J. Biol. Chem. 243*, 4298.
Rossi, M., Geraci, G., and Scarano, E. (1967), *Biochemistry 6*, 3640.
Rossi, M., and Scarano, E. (1969), *4th Meeting Federation European Biochem. Soc., Madrid.*
Scarano, E., Geraci, G., and Rossi, M. (1967a), *Biochemistry 6*, 192.
Scarano, E., Geraci, G., and Rossi, M. (1967b), *Biochemistry 6*, 3645.
Smith, M., and Khorana, H. G. (1958), *J. Amer. Chem. Soc. 80*, 1142.

# Multiple Ribonucleic Acid Polymerases and Ribonucleic Acid Synthesis during Sea Urchin Development*

Robert G. Roeder† and William J. Rutter‡

ABSTRACT: Ribonucleic acid (RNA) synthesis during early sea urchin development has been examined at three levels of biological organization. The multiple RNA polymerases which are present in isolated nuclei have been solubilized and separated by chromatographic procedures at various stages of development. The total level of polymerase I, presumed to be associated with ribosomal RNA synthesis, increases in proportion to cell division so that the level per cell is nearly constant. In contrast, the levels of polymerases II and III per embryo increase only slightly; thus, the levels per cell actually decline severalfold. Polymerases II and III account for the bulk of the activity and at least one of these (polymerase II) is believed to be associated with the synthesis of deoxyribonucleic acid like (DNA-like) RNA. The decline in the level of total polymerase per cell parallels the decline in the apparent rate of RNA synthesis in the intact embryo. Optimal conditions have been established for the measurement of template-bound RNA polymerase activity in isolated nuclei. Under all of the metal ion and ionic strength conditions tested and at all stages of development examined, the RNA synthesized is DNA like as determined by partial base ratio and nearest-neighbor frequency analyses; and in this respect, the RNA resembles that synthesized *in vivo* during short labeling periods. The low ionic strength (intact nuclei) activity declines severalfold from the early blastula to the late gastrula stages on a per nucleus basis, in general agreement with the observations on the rates of RNA synthesis per cell in intact embryos. Rates of RNA synthesis *in vivo* were calculated after determining the uridine triphosphate specific activity and the incorporation of radioactivity into RNA during short labeling periods with [$^3$H]uridine. The apparent rate per cell declines about two- to threefold from the early blastula to the late gastrula stages. These observations suggest the involvement of the multiple RNA polymerases in the regulation of ribonucleic acid synthesis. Additional indirect observations suggest that other factors may be involved in the control of the activity of these enzymes.

The patterns of RNA synthesis during sea urchin development have been qualitatively described. The synthesis of DNA-like RNA[1] begins very early in development (Nemer and Infante, 1965) and is apparent at all stages examined thereafter (Glisin and Glisin, 1964; Gross *et al.*, 1964; Emerson and Humphreys, 1970). In contrast, earlier studies (Nemer, 1963; Comb *et al.*, 1965; Giudice and Mutolo, 1967) suggested that ribosomal RNA synthesis is not initiated until gastrulation. Recently, however, Emerson and Humphreys (1970) have demonstrated ribosomal RNA synthesis at the blastula stage and have, furthermore, suggested that the rate of accumulation of rRNA per cell might be constant from the blastula to the postgastrula stages, while the rate of accumulation of DNA-like RNA decreases severalfold.

It is apparent that there are changes in the relative rates of synthesis and accumulation of the major classes of RNA during development, as well as changes in the populations of DNA-like RNA molecules formed during this interval (Whiteley *et al.*, 1966; Glisin *et al.*, 1966). Elucidation of the mechanisms regulating the synthesis of the various species of RNA remains a formidable problem. Studies on the chromatin found in the cells of higher organisms suggest

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received December 12, 1969.* Supported by National Institutes of Health Grant HD-02126 and National Science Foundation Grant GB 6407. A preliminary report of this work has been presented (Roeder and Rutter, 1969a).

† Predoctoral fellow of the National Institutes of Health (f-F01-GM-31,539-04). Present address: Carnegie Institution of Washington, Department of Embryology, Baltimore, Md. 21210.

‡ Present address: University of California at San Francisco, Department of Biochemistry and Biophysics, San Francisco, Calif. 94122.

[1] Abbreviations used are: DNA-like RNA, RNA having a base composition similar to that of DNA; TGMED buffer is 0.05 M Tris-HCl (pH 7.9), 25% (v/v) glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM dithiothreitol.