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Inhibition of *Azotobacter vinelandii* Ribonucleic Acid Polymerase by Glutamyl,Tyrosyl Copolymers†

Joseph S. Krakow

ABSTRACT: Of a series of glutamyl containing copolypeptides tested, poly(Glu^1,Tyr^1) is the most effective inhibitor of RNA polymerase. Inhibition is a consequence of binding of the poly(Glu^1,Tyr^1) to the polymerase which blocks the subsequent formation of the enzyme–template complex. The preformed holoenzyme–d(A-T) complex is much more resistant to dissociation by poly(Glu^1,Tyr^1) than is the core polymerase–d(A-T) complex.

A variety of polyanionic inhibitors of RNA polymerase have been studied, including polyribonucleotides (Dubert and Hirschbein, 1969; Tissières *et al.*, 1963; Krakow and Ochoa, 1963), heparin (Walter *et al.*, 1967), and the azosulfonic acid dye, Congo Red (Krakow, 1965; Smuckler, 1972). These inhibitors bind to RNA polymerase and block the formation of the enzyme–template complex. In this paper a novel class of RNA polymerase inhibitors will be described; these are synthetic copolypeptides containing glutamyl and tyrosyl or other aminoacyl residues which act by interfering with template binding.

Methods and Materials

Tris, ATP, UTP, EDTA, and mercaptoethylamine were products of Sigma Chemical Co. Labeled and unlabeled d(A-T) and d(I-C) were prepared using *Escherichia coli* DNA polymerase I (Jovin *et al.*, 1969). Glu-Tyr-Glu was obtained from Fox Chemical Co. and the polypeptides were obtained as follows (the molecular weights listed were determined by the manufacturer): poly(aspartic acid) (mol wt 20,000–50,000) and poly(glutamic acid) (mol wt 40,000–100,000) from Pierce Chemical Co.; poly(Glu^9,Tyr^1) (mol wt 85,000) and poly(Glu^1,Tyr^1) (mol wt 22,000) from Miles Laboratories. The following were generously donated by Dr. G. Fasman, Brandeis University: poly(Glu^{95},Tyr^5), poly(Glu^{95},Trp^5), poly(Glu^9,Leu^1), and poly(Glu^3,Phe^1). [^{32}P]PP$_i$ was purchased from New England Nuclear and *p*-[^{14}C]chloromercuribenzoate from Schwarz/Mann.

Nitrocellulose membrane filters (0.45-μ pore size, 25-mm diameter) were obtained from Matheson-Higgins, Woburn, Mass. Prior to use the filters were soaked in 0.1 M KOH at room temperature for 30 min (Smolarsky and Tal, 1970) and then placed in 0.02 M Tris-HCl (pH 7.8)–0.05 M NaCl. This procedure was used to lower the blank adsorption of the labeled deoxypolynucleotides.

Azotobacter vinelandii RNA polymerase was purified by a modification of the published procedure (Krakow and Horsley, 1968). In the final step RNA polymerase holoenzyme ($\beta'\beta\alpha_2\sigma$) and core ($\beta'\beta\alpha_2$) were resolved by gradient elution from phosphocellulose (Whatman P-11) and each form was essentially homogeneous as determined by sodium dodecyl sulfate–acrylamide gel electrophoresis.

Results

Although poly(glutamic acid) and poly(aspartic acid) do not apparently affect RNA polymerase activity, the random copolymer, poly(Glu^1,Tyr^1) completely inhibits [^{32}P]PP$_i$ exchange (Table I). The extent of inhibition is related to the relative amounts of glutamyl and tyrosyl residues in the synthetic polypeptides tested; no inhibition is seen with poly(glutamic acid) or poly(Glu^{95},Tyr^5), poly(Glu^9,Tyr^1) elicits a 19% inhibition, and the poly(Glu^1,Tyr^1) elicits a 95% inhibition of the d(A-T) directed [^{32}P]PP$_i$ exchange reaction (Krakow and Fronk, 1969). The ability to inhibit RNA polymerase is not limited to those polypeptides containing glutamyl and tyrosyl residues since poly(Glu^3,Phe^1) and poly(Glu^{95},Trp^5) are also effective.

At low ionic strength RNA polymerase sediments as a dimer or higher aggregate (Berg and Chamberlin, 1970) (Figure 1A); under similar ionic conditions the tRNA–enzyme complex sediments as a 12S protomer form (Figure 1B). Incubation of RNA polymerase with poly(Glu^1,Tyr^1) also results in loss of the polymerase dimer and the appearance of a protomer form sedimenting at a position identical with that of the tRNA protomer complex (Figure 1C). An ad-

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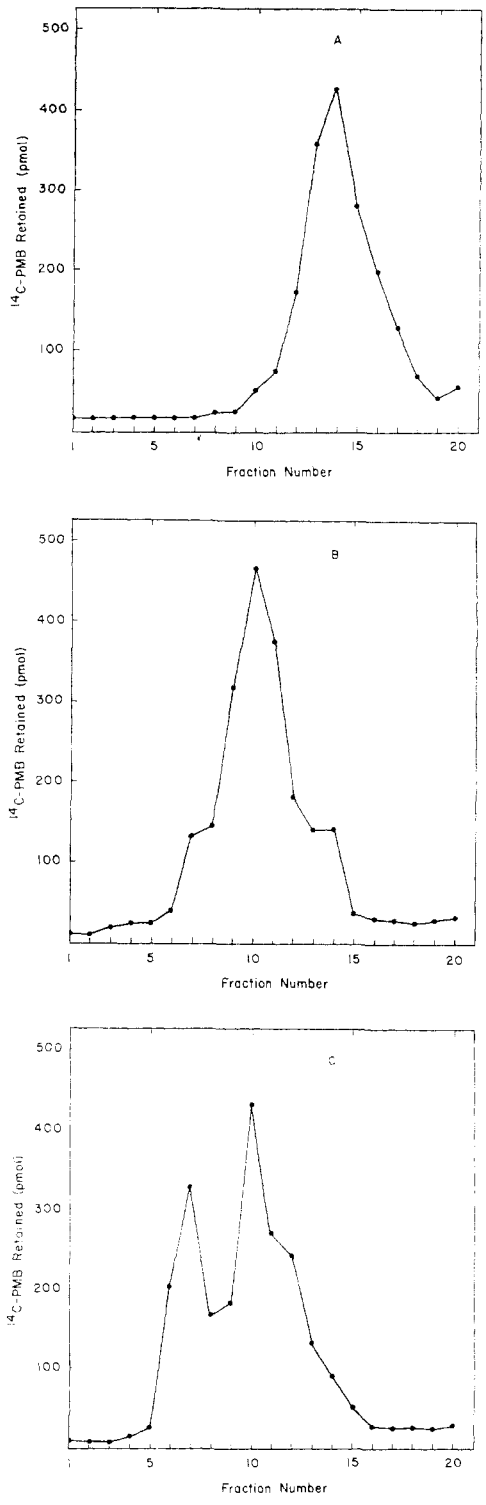

FIGURE 1: Effect of poly(Glu^1,Tyr^1 on sedimentation of RNA polymerase. Incubation mixtures (0.1 ml) were as follows: 20 mM Tris-HCl (pH 7.8), 1 mM $MnSO_4$, and 60 μg of RNA polymerase core enzyme to which were added: (A) no additions; (B) 10 μg of tRNA; (C) 10 μg of poly(Glu^1,Tyr^1). After incubation for 5 min at 37° each was layered on 5 ml of a 10–30% sucrose gradient containing 20 mM Tris-HCl (pH 7.8) and 1 mM $MnSO_4$. After centrifuging at 50,000 rpm (Spinco SW-50) for 4.5 hr at 5° fractions of 0.25 ml were collected from the top by displacement with 50% sucrose. To assay for the position of polymerase, reaction with *p*-[^{14}C]-chloromercuribenzoate was carried out. To each fraction 0.5 ml of the following mixture was added: 20 mM Tris-HCl (pH 7.8), 4 mM EDTA, 5 M urea, and 4×10^{-5} M *p*-[^{14}C]chloromercuribenzoate (2.2×10^7 cpm/μmol). After 20 min at 37° the mixture was filtered onto nitrocellulose membranes and washed with 0.02 M Tris-HCl (pH 7.8)–0.05 M NaCl.

TABLE I: Inhibition of RNA Polymerase by Acidic Polypeptides.[a]

Additions	μg	[^{32}P]PP$_i$ Incorpd (nmol)
None		19.8
Poly(Asp)	10	19.5
Poly(Glu)	10	19.9
Poly(Glu^9,Leu^1)	10	19.3
Poly(Glu^{95},Trp^5)	10	16.1
Poly(Glu^3,Phe^1)	10	3.2
Poly(Glu^{95},Tyr^5)	10	22.9
Poly(Glu^9,Tyr^1)	10	16.2
Poly(Glu^1,Tyr^1)	1.5	0.4
Glu-Tyr-Glu	10	21.2

[a] The reactions contained (final volume 0.25 ml): 80 mM Tris-HCl (pH 7.8), 40 mM mercaptoethylamine, 4 mM $MgCl_2$, and 10 μg of RNA polymerase holoenzyme. After a 2-min preincubation at 37° with the indicated polypeptides the following were added: 6 nmol of d(A-T)$_n$, 4×10^{-6} M ATP, 4×10^{-4} M UTP, and 1 mM [^{32}P]PP$_i$ (360 cpm/nmol). The reactions were incubated for 5 min at 37° and [^{32}P]PP$_i$ exchange was determined by the method of Krakow and Fronk (1969).

ditional peak sedimenting at 8–9 S is also seen corresponding to subprotomeric forms of the enzyme. Since binding of poly(Glu^1,Tyr^1) results in an essentially irreversible loss of enzyme activity, the position of polymerase in the gradient fractions is determined by reaction of enzyme sulfhydryl groups with *p*-[^{14}C]chloromercuribenzoate in 5 M urea. Under these conditions polymerase sulfhydryl groups react with the mercurial and the protein–mercurial adduct is retained on nitrocellulose membrane filters (Krakow and Goolsby, 1971).

In general, those inhibitors which block template binding also cause a dimer to protomer transition; examples of this class of inhibitor are tRNA (Smith *et al.*, 1967), heparin (Walter *et al.*, 1967), and Congo Red (Krakow and von der Helm, 1970). The copolypeptides similarly inhibit RNA polymerase by interfering with template binding (Table II). While poly(Glu^1,Tyr^1) inhibits both d(A-T) binding and [^{32}P]PP$_i$ exchange (Table I) at very low concentration, poly(Glu) is without effect on [^{32}P]PP$_i$ exchange and only affects

TABLE II: Effect of Glutamyl Polypeptides on Template Binding.[a]

Polypeptide Added (μg)	[^{3}H]d(A-T) Retained (nmol)
None	3.2
Poly(Glu^1,Tyr^1), 0.2	0.1
Poly(Glu^9,Tyr^1), 2	1.0
Poly(Glu), 10	1.1

[a] The incubations contained (final volume 0.25 ml): 80 mM Tris-HCl (pH 7.8), 5 μg of RNA polymerase holoenzyme, and the polypeptides indicated. After an initial incubation of 2 min at 37° each tube received 4.5 mnol of [^{3}H]d(A-T). The mixtures were incubated for 10 min at 37° and processed as indicated in the legend to Figure 2.

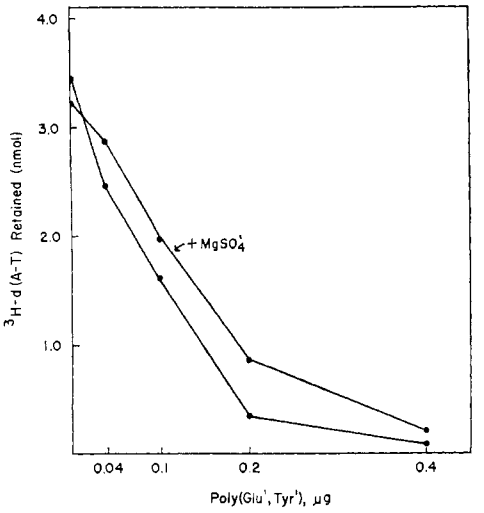

FIGURE 2: Inhibition of d(A-T) binding by poly(Glu^1,Tyr^1). The incubations contained (final volume 0.25 ml): 80 mM Tris-HCl (pH 7.8), 0.4 mM EDTA, 5 μg of RNA polymerase holoenzyme, and, where indicated, 20 mM $MgSO_4$. Poly(Glu^1,Tyr^1) was added as indicated and, following an initial incubation of 2 min at 37°, 4.5 nmol of [^{3}H]d(A-T) was added. After 10 min at 37° 2 ml of 0.02 M Tris-HCl (pH 7.8)–0.05 M NaCl was added and the mixtures were filtered onto nitrocellulose membranes and washed with 5 ml of 0.02 M Tris-HCl (pH 7.8)–0.05 M NaCl.

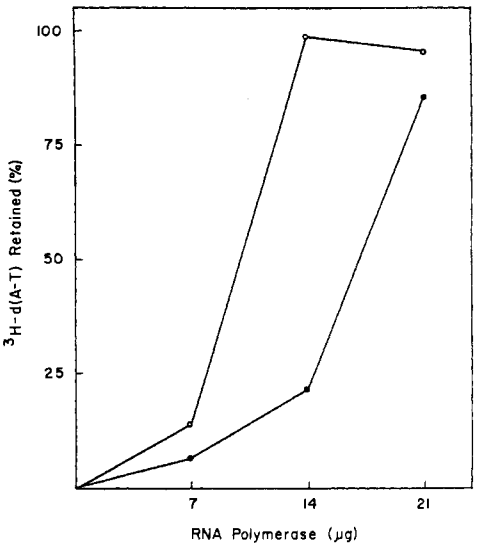

FIGURE 3: Titration of poly(Glu^1,Tyr^1) with RNA polymerase. The incubations contained (final volume 0.25 ml): 80 mM Tris-HCl (pH 7.8), 0.4 mM EDTA, 0.3 μg (○) or 0.6 μg (●) of poly(Glu^1,Tyr^1), and the amounts of RNA polymerase holoenzyme indicated. Following a 2-min incubation at 37°, 4.5 nmol of [^{3}H]d(A-T) was added. After 10 min at 37° the assays were processed as indicated in the legend to Figure 2.

template binding at high poly(Glu) to polymerase ratio. Poly(Glu^9,Tyr^1) shows an intermediate effect on template binding and [^{32}P]PP_i exchange activity.

Of the several acidic polypeptides tested, the most effective inhibitor of RNA polymerase is poly(Glu^1,Tyr^1). The high affinity of this polypeptide for the enzyme is shown by the ability of an approximately stoichiometric amount of poly(Glu^1,Tyr^1) to inhibit d(A-T) binding (Figure 2). The presence of 20 mM $MgSO_4$ during the formation of the polymerase–poly(Glu^1,Tyr^1) complex has only a slight effect when [^{3}H]d(A-T) is subsequently added for the binding assay. Incubation of 0.4 μg of poly(Glu^1,Tyr^1) with 7 μg of the RNA polymerase holoenzyme (one–two molecules of polypeptide per enzyme protomer) results in a complete inhibition of template binding.

The data thus far indicate that poly(Glu^1,Tyr^1) inhibits RNA polymerase by binding to the enzyme and preventing the formation of the enzyme–template complex necessary for subsequent phosphodiester bond formation (*i.e.*, PP_i exchange). Because of the tight binding between poly(Glu^1,Tyr^1) and RNA polymerase it is possible to titrate out a fixed amount of the polypeptide by addition of increasing amounts of the enzyme (Figure 3) leaving free enzyme which will bind [^{3}H]d(A-T). Addition of 0.3 μg of poly(Glu^1,Tyr^1) inhibits d(A-T) binding by 7 μg of polymerase, while no effect is seen with 14 μg of the enzyme. Similarly with 0.6 μg of the polypeptide up to 14 μg of the polymerase is titrated until, on addition of 21 μg of polymerase, one observes 85% of the input [^{3}H]d(A-T) retained on the filter by free enzyme. The relation between the amount of polymerase and poly(Glu^1,Tyr^1) rules out the possibility that binding of the polypeptide to d(A-T) could be responsible for the observed inhibition of template binding by RNA polymerase.

In the preceding experiments the polypeptides were incubated with RNA polymerase before addition of [^{3}H]d(A-T); when the holoenzyme is incubated with d(A-T) prior to additon of poly(Glu^1,Tyr^1) one finds that the preformed holoenzyme–template complex is refractory to even a high concentration of the polypeptides (von der Helm and Krakow, 1972). In the absence of $MgSO_4$ only 20% of the holoenzyme [^{3}H]d(A-T) complex is dissociated by 10 μg of poly(Glu^1,Tyr^1) (Figure 4) and as will be shown this probably corresponds to the amount of core polymerase in the preparation. Poly(Glu^1,Tyr^1)-induced dissociation of the holoenzyme–d(A-T) complex occurs in the presence of 40 mM $MgSO_4$; after 10 min of incubation in the presence of 10 μg of the copolypeptide only 15% of the enzyme–template complex remains. This effect is not restricted to $MgSO_4$ (or other divalent cations) but is a response to increasing the ionic strength of the medium. The results shown in Table III indicate that the holoenzyme–[^{3}H]d(I-C) complex is resistant to dissociation by poly(Glu^1,Tyr^1) in the standard reaction mixture. Addition of either 20 mM $MgSO_4$ or 0.16 M KCl results in dissociation of the holoenzyme–template complex by the copolypeptide.

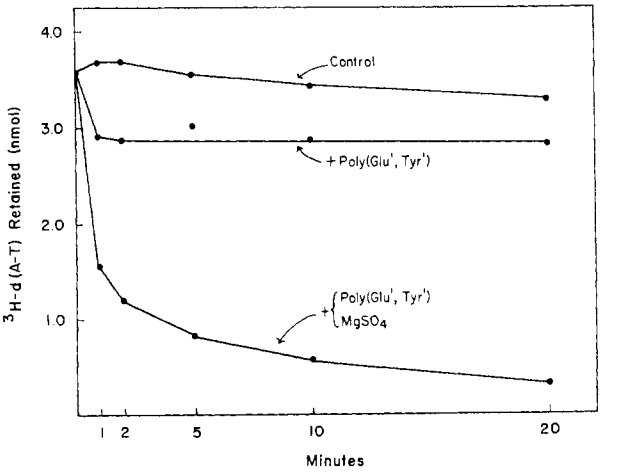

FIGURE 4: Dissociation of holoenzyme–d(A-T) complex by poly(Glu^1,Tyr^1). The incubations contained (final volume 0.25 ml): 80 mM Tris-HCl (pH 7.8), 0.4 mM EDTA, 4.5 nmol of [^{3}H]d(A-T), and 5 μg of holoenzyme. After an initial incubation of 10 min at 37° one set was brought to 40 mM $MgSO_4$ and each received 10 μg of poly(Glu^1,Tyr^1) as indicated. After incubation at 37° for the times indicated the assays were processed as indicated in the legend to Figure 2.

TABLE III: Effect of Added $MgSO_4$ or KCl on Dissociation of the Holoenzyme–d(I-C) Complex by Poly(Glu^1,Tyr^1).[a]

Additions (M)	[^{3}H]d(I-C) Retained (nmol)	
		+ Poly(Glu^1,Tyr^1) (10 μg)
None	4.11	3.88
$MgSO_4$, 0.02		0.41
$MgSO_4$, 0.04	4.05	0.23
KCl, 0.08		2.96
KCl, 0.16	3.92	0.37

[a] The incubations contained (0.25 ml final volume): 80 mM Tris-HCl (pH 7.8), 0.4 mM EDTA, 4.5 nmol of [^{3}H]d(I-C) (1490 cpm/nmol), and 5 μg of RNA polymerase holoenzyme. After an initial incubation of 10 min at 37° the components listed above were added as indicated and incubated for 10 min at 37°. The assays were processed as indicated in the legend to Figure 2.

The data presented in Figure 4 indicated that approximately 20% of the polymerase–d(A-T) complex was rapidly dissociated by poly(Glu^1,Tyr^1) even in the absence of $MgSO_4$. This is probably a reflection of the relative amount of holoenzyme and core enzyme (either lacking σ or containing a nonfunctional σ) in the polymerase preparation used. In contrast to the resistance of the holoenzyme–d(A-T) complex, the core enzyme–d(A-T) complex is readily dissociated by 3 μg of poly(Glu^1,Tyr^1) even in the absence of added salt (Figure 5); addition of $MgSO_4$ has only a minor effect. It would appear that in the holoenzyme–template complex σ acts either by tightening the enzyme–template complex or by preventing access of poly(Glu^1,Tyr^1) to its binding site(s) on the enzyme surface.

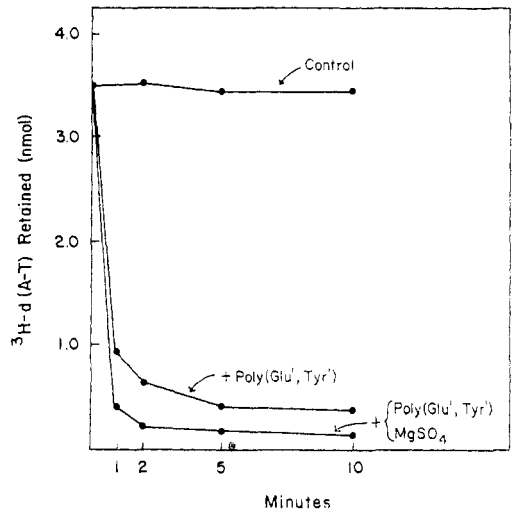

FIGURE 5: Dissociation of core enzyme–d(A-T) complex by poly(Glu^1,Tyr^1). The incubations contained (final volume 0.25 ml): 80 mM Tris-HCl (pH 7.8), 0.4 mM EDTA, 4.5 nmol of [^{3}H]d(A-T), and 5 μg of core enzyme. After an initial incubation of 10 min at 37° one set was brought to 40 mM $MgSO_4$ and each received 3 μg of poly(Glu^1,Tyr^1) as indicated. After an incubation at 37° for the times indicated the assays were processed as indicated in the legend to Figure 2.

Discussion

The ability of certain of the acidic polypeptides to bind to and inhibit RNA polymerase provides an additional example of a general class of template binding inhibitor. These include template analogs such as tRNA, poly(I), poly(U), etc., which under appropriate *in vitro* conditions may act as a template to direct the synthesis of complementary RNA sequences. The sulfate containing mucopolysaccharide, heparin, presumably acts by binding to basic amino acid residues in or near the template site(s) of the enzyme; the sulfonic acid dye, Congo Red, may inhibit by binding to similar sites. The inhibitors described in this paper are interesting in that their affinity for RNA polymerase is determined by acidic groups, glutamyl residues, along with another amino residue which is not acidic, tyrosyl, phenylalanyl, and to a lesser extent tryptophanyl among the obviously incomplete survey carried out in this study. The most effective inhibitor of RNA polymerase studied is the random copolymer containing an equimolar ratio of glutamyl and tyrosyl residues. The high affinity of the poly(Glu^1,Tyr^1) for RNA polymerase is shown by the low concentration necessary for inhibition and also by the ability of this copolypeptide to dissociate the protomeric structure of the enzyme (Figure 2C). This effect is also seen after incubation of RNA polymerase with Congo Red (Krakow and von der Helm, 1970). The present use of these copolypeptides stems from the work of Sela (1962) who found that poly(Glu^1,Tyr^1) and poly(Glu^1,Phe^1) were potent inhibitors of ribonuclease (below pH 6); however, poly(aspartic acid) and poly(Glu^9,Tyr^1) were poor inhibitors of ribonuclease. Although RNA polymerase is completely inhibited at pH 7.8 (the standard pH used in this study) where ribonuclease is not affected the results do agree qualitatively in terms of the relative effectiveness of the synthetic polypeptides as inhibitors of these two enzymes. Sela proposed that inhibitory efficiency is related to interaction between aromatic amino acids in the polypeptide and groupings in ribonuclease.

It has been shown by Zillig *et al.* (1970) that the σ subunit is the most acidic subunit of RNA polymerase and it is possible that there may be a relation between the manner in which σ binds to the core polymerase and that by which the acidic copolypeptides bind to the enzyme. Assays for σ-like effects of several of these copolypeptides on core enzyme have been carried out (stimulation of T7 DNA directed transcription, stimulation of [^{32}P]PP_i exchange directed by d(A-T) at low ATP on GTP levels (Krakow and Fronk, 1969)) with negative results.

Acknowledgment

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Differences in *in Vivo* Methylation Patterns of Tyrosine and Histidine Transfer Ribonucleic Acids from Rat Liver and Novikoff Hepatoma†

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ABSTRACT: The methylation of tRNA from rat liver and Novikoff hepatoma has been studied using *in vivo* labeling of the tRNAs with [*Me*-^{3}H]methionine. Tyrosine tRNA and histidine tRNA have been purified from both tissues by means of chromatography on DEAE-Sephadex and benzoylated DEAE-cellulose. Essentially no difference in the methylation of unfractionated tRNAs from these two tissues was found. However, a clear difference in the methylated base patterns appeared when tyrosine tRNA and histidine tRNA from Novikoff hepatoma were compared to the same specific tRNAs from normal rat liver. The possible correlation of these findings with previous observations on alterations in chromatographic profiles of tRNAs (Baliga, B. S., Borek, E., Weinstein, I. B., and Srinivasan, P. R. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 899) and in tRNA methyltransferase activity (Sharma, O. K. (1973), *Biochim. Biophys. Acta 299*, 415) in those tissues is discussed.

A high level of tRNA methyltransferase activity has been observed in all tumor tissues which have been studied up to now (for a review, see Borek and Kerr, 1972). However, there is no conclusive evidence concerning the actual level of methylation in tumor tRNA. Some authors reported a very high content of methylated bases in tRNA from neoplastic tissues (Bergquist and Matthews, 1962; Craddock, 1969; Viale *et al.*, 1967). Other investigators found qualitative differences in the methylation patterns of tumor tRNAs (Inose *et al.*, 1972). Analysis of tRNA from tumor cell in culture (Iwanami and Brown, 1968) or from brain tumors (Randerath *et al.*, 1971) failed to demonstrate any significant variation, either qualitative or quantitative, of the methylation patterns. It thus appears that the activity of the tRNA methyltransferase has no direct relationship with the overall level of methylation of the tRNAs.

On the other hand, many investigators have reported alterations in chromatographic patterns of tumor specific tRNAs compared to their normal counterparts. (See, for instance, Taylor *et al.*, 1967; Gonano *et al.*, 1971; Srinivasan *et al.*, 1971; Hayashi *et al.*, 1973.) In each case, the differences, either quantitative or qualitative or both, were restricted to a few tRNA species; most specific tRNAs showed the same chromatographic profiles in tumor and in normal tissue.

Since it is known that methylation may alter the chromatographic behavior of a tRNA (Capra and Peterkofsky, 1968), there is a possibility that some of the alterations observed in tumor tRNAs correspond to modifications in their methylation pattern. According to this hypothesis, the elevated tRNA methyltransferase activity in tumors would not be correlated to an overall hypermethylation, but rather to aberrant methylation of some specific tRNAs; this would not give rise to significant differences when methylation of bulk tRNA is examined.

In order to test this hypothesis, we decided to analyze the methylation of tyrosine tRNA and histidine tRNA, whose chromatographic profiles on MAK columns[1] have been shown to differ in normal rat liver and in Novikoff hepatoma (Baliga *et al.*, 1969). The tRNA was labeled *in vivo* with [*Me*-^{3}H]-methionine, the specific tRNAs were isolated, and their methylated base content was determined. This system was chosen for several reasons. First, Novikoff hepatoma is a very fast growing tumor, where high label incorporation may be expected. Second, the only specific tRNAs which differ significantly in this tumor from the normal ones are tyrosine,

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[1] Abbreviations used are: MAK, methylated albumin kieselguhr; BD-cellulose, benzoylated DEAE-cellulose.