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Sedimentation and Actinomycin D Binding Studies of Partially Denatured Crab dAT*

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ABSTRACT: That poly dAT of *Cancer antennarius* which has been heated and cooled possesses a partially denatured character has been shown by two additional methods, sedimentation analysis and actinomycin D inhibition of ribonucleic acid (RNA) synthesis. The once-melted dAT sediments more rapidly than does truly native dAT. Actinomycin inhibits RNA synthesis by *Escherichia coli* RNA polymerase less with the once-melted dAT template than with native dAT. The poly dAT component of *C. borealis* deoxyribonucleic acid (DNA) has been separated by the mercury-binding method. This dAT, which contains 2.5% guanine plus cytosine (GC) compared to 3.5% for *C. antennarius* dAT, behaves in a manner similar to that of *C. antennarius* dAT.

When melted and cooled, its T_m is lower and it melts over a wider temperature range than does unmelted *C. borealis* dAT. Its CsCl buoyant density is slightly increased and the actinomycin inhibition of RNA synthesis with the dAT template is lessened. The differences observed between the melted and unmelted forms are, however, less for the *C. borealis* dAT than those found for *C. antennarius* dAT probably because the former dAT has a lower GC content.

Sueoka (1961) has shown that poly dAT[1] is present in the DNA of crabs of the genus *Cancer*. The poly dAT component of the DNA of *Cancer borealis* contains about 3% GC interspersed in an otherwise alternating dAT structure (Swartz *et al.*, 1962). Sueoka and Cheng (1962a) isolated the dAT with a MAK column after heating and cooling the *C. borealis* DNA. This column separates the renatured dAT from the denatured typical major component DNA. The dAT component isolated in this way has been used as a template for DNA polymerase (Swartz *et al.*, 1962), for RNA polymerase (Goldberg *et al.*, 1962, 1963; Cheng and Sueoka, 1964), and for actinomycin binding studies (Goldberg *et al.*, 1962).

In a previous paper from these laboratories the development of a new dAT separation technique based on the preferential binding of Hg^{2+} to dAT was reported (Davidson *et al.*, 1965). It was further shown that *C. antennarius* dAT remains partially denatured after heating and cooling. This is probably due to the nonpairing of (mainly) G and C bases during renaturation. Differences were shown to exist between the native and once-melted forms by methods based upon optical density melting, electrophoresis, electron microscope, buoyant density, and *Escherichia coli* exonuclease I susceptibility.

Pochon *et al.* (1965) have also come to the conclusion that the once-melted dAT form is different from the original native form. They utilized optical density

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[1] Abbreviations used in this work: A, adenine; U, uracil; G, guanine; C, cytosine; dAB$\bar{U}$, alternating deoxyadenylate–deoxy-5-bromouridylate copolymer; dAT, alternating deoxyadenylate–deoxythymidylate copolymer; MAK, methylated albumin kieselguhr; σT, transition width in degrees centigrade between 20% below midpoint and 20% above midpoint of the OD melting profile; SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate); T_m, temperature at midpoint of the OD transition obtained from the OD melting profile; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

melting and optical density acid–base titration techniques with partially purified *C. pagurus* dAT.

The present paper reports two additional methods for the identification of the partially denatured character of once-melted crab poly dAT, sedimentation analysis and actinomycin inhibition of RNA synthesis supported by the dAT template. We also report the separation of *C. borealis* dAT in native form by Hg^{2+}. This poly dAT is likewise shown to remain partially denatured after heating and cooling. In this paper the following system will be employed to designate the DNA preparations used: dAT (Hg), dAT separated by the Hg^{2+} method; and dAT (column), dAT separated by the Sueoka and Cheng (1962a) procedure.

Materials and Experimental Procedures

C. antennarius DNA was isolated and the two components separated by the Hg^{2+} method described briefly by Davidson *et al.* (1965) and in more detail by Nandi *et al.* (1965). *C. borealis* DNA, the gift of Dr. T. Y. Cheng, was separated into its two components by the same method. The purities of both the dAT and the main component species were determined by analytical equilibrium CsCl density gradient centrifugation. For each such purity determination a high concentration (*ca.* 10 μg/0.7 ml) of the separated component was banded and both the photographs and densitometer tracings of the photographs were examined. Since dAT and main component DNA band at different densities, contamination of one species by the other can be discovered by this method, which as applied will detect 1% or more of such contamination. Samples containing detectable contamination were discarded. Synthetic dAT was the gift of Professor I. R. Lehman.

Sedimentation rates were determined using the analytical band-sedimentation method of Vinograd *et al.* (1963). *Ca.* 1.5 μg of DNA in 0.03 ml of SSC was layered on the bulk solution, 1 M NaCl or 0.9 M NaCl, 0.1 M NaOH (Studier, 1965), and centrifuged at 29,500 rpm at 20° in the Spinco Model E. The sedimentation values were corrected using the solvent correction factors determined by Studier (1965).

Fraction 4 *E. coli* RNA polymerase was prepared by the method of Chamberlin and Berg (1962). Standard reaction conditions for RNA polymerization were employed except the reaction period was 20 min and the nucleoside triphosphate concentrations were 50 mμmoles each/incubation.

The incorporation of each of the four individual bases was determined by using only one [^{14}C]nucleoside triphosphate per incubation with the other three unlabeled. Incorporation was determined by counting the trichloroacetic acid precipitable material on a membrane filter (Bac-T-Flex membrane filter for aqueous solutions type B-6, Carl Schleicher and Schuell and Co., Keene, N. H.). The molar incorporation was then calculated from the specific activities of the labeled precursors. The ^{14}C-labeled nucleoside triphosphates were purchased from Schwarz BioResearch.

The enzyme and template concentrations employed were as described in the text and were such that the RNA synthesis rates were proportional to each. Optical density melting profiles and buoyant density values in CsCl were determined as described by Davidson *et al.* (1965). DNA samples were melted routinely in SSC by heating in a boiling water bath for 10 min and then were cooled in ice.

Results and Discussion

Sedimentation Studies of dAT. Sedimentation rates give information concerning the size and shape of the molecules in question. Studier (1965) has shown that DNA which has been denatured with base (and the solution then neutralized) sediments faster in 1 M salt than when native. Thus there are the following forms in which DNA may be investigated: (1) native DNA, unbranched double helices; (2) DNA in 0.1 M NaOH (alkaline DNA), extended single strands; (3) denatured DNA at neutral pH, single strands condensed by nonregular, intrastrand base–base interactions. Studier has derived formulas for converting sedimentation coefficients to molecular weights for the three DNA forms listed above.

Synthetic and crab dAT, however, act differently than usual DNA since, after strand separation caused by heating or by alkaline pH, the single strands apparently fold back on themselves and form hydrogen-bonded double-helical structures (Schachman *et al.*, 1960; Inman and Baldwin, 1962; Davidson *et al.*, 1965). The synthetic dAT molecules are slightly branched both before and after heating as shown by electron microscopy, but other tests show that such molecules are completely hydrogen bonded (Davidson *et al.*, 1965). Micrographs by the same authors show that native crab dAT molecules are typical linear strands, but become very compact, highly branched structures after heating and cooling. *Ca.* 93% of the original base pairs re-form upon cooling in this case. Sedimentation experiments should be able to corroborate these structures.

Since all DNA is assumed to be in an extended single-stranded conformation in alkali, sedimentation of the macromolecules under this condition will give an accurate estimate of the size of the covalent structure. Comparison of the *s* values in neutral and alkaline pH will reveal conformational information about the DNA at neutral pH. Alkaline conditions are assumed to destroy only noncovalent interactions and not to degrade the covalent structure.

The DNA preparations were centrifuged under four conditions: (1) original DNA at neutral pH, to determine *s* of original molecules; (2) original DNA in 0.1 M NaOH, to determine *s* of single strands; (3) heated and cooled DNA at neutral pH, to determine *s* of single strands after renaturation; (4) heated and cooled DNA in 0.1 M NaOH, to determine *s* of heated and cooled single strands.

To correctly interpret the results one must know if the heated dAT (synthetic or crab) single strands fold back and hydrogen bond intramolecularly when cooled

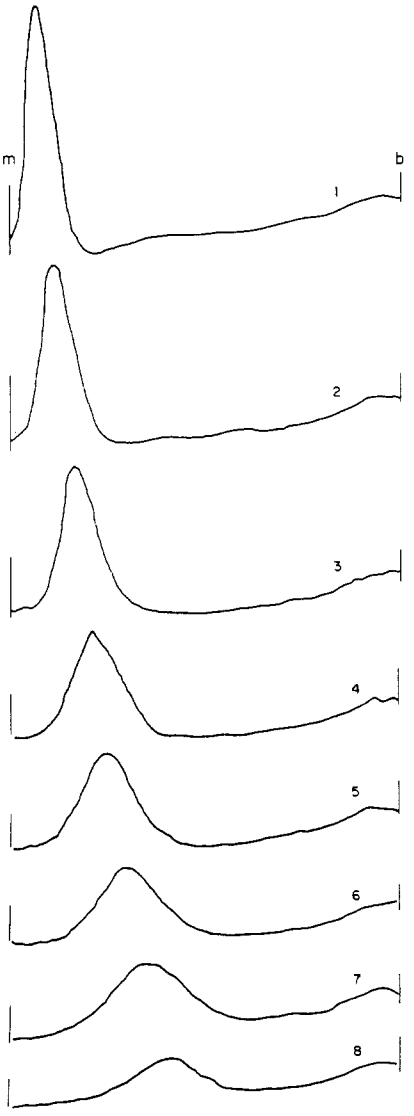

FIGURE 1: Densitometer tracings of photographs taken of a typical dAT band sedimentation pattern obtained in 1 M NaCl. The interval between each photograph was 8 min.

or if the hydrogen bonding is at least partially intermolecular. Intermolecular hydrogen bonding or entangling would result in the formation of aggregates which would sediment faster than the structures formed from only one strand.

Inman and Baldwin (1962) have shown that intermolecular hydrogen bonding does not occur unless the DNA is present at a high concentration (30 OD units/ml and higher) and the cooling rate is very low in the melting temperature range. These workers measured hybridization and entangling between synthetic dAT and synthetic dA$\overline{\text{BU}}$. They concluded that unless the DNA concentration is high the molecules with their alternating base sequence fold back on themselves instead of uniting with other strands.

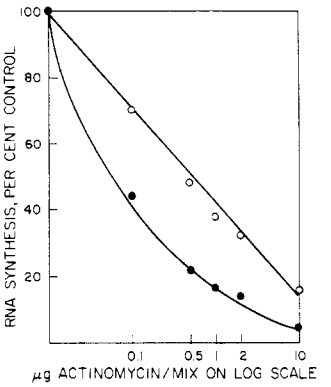

FIGURE 2: Inhibition of RNA synthesis by actinomycin D using *C. antennarius* native (●) and melted (○) main component DNA as templates with *E. coli* RNA polymerase. The four [^{14}C]nucleoside triphosphates were incorporated separately and the total RNA synthesis was determined. DNA (0.5 mμmole) was incubated for 20 min with 3 enzyme units. With no actinomycin present, 1.0 and 1.1 mμmoles of RNA were synthesized from the native and melted templates, respectively. Reaction mixture contained, for each 0.25 ml, 10 μmoles of Tris buffer (pH 8), 1 μmole of $MgCl_2$, 0.25 μmole of $MnCl_2$, 3 μmoles of β-mercaptoethanol, and 50 mμmoles of each of the four riboside triphosphates

Thus one would not expect intermolecular aggregation under the conditions employed in the experiments reported here (DNA concentration of 1 OD unit/ml and fast cooling rate), but the possibility was, nevertheless, investigated. If intermolecular hydrogen bonding or entangling does occur it should be more prevalent with high DNA concentrations. Thus, *C. antennarius* dAT of concentrations from 1 to 500 μg/ml (*ca.* 0.02–10.0 OD units/ml) in SSC were melted and fast cooled

TABLE I: Sedimentation Rates of *C. antennarius* dAT Samples Melted and Fast Cooled at Various Concentrations.

dAT Concn[a] (μg/ml)	Neutral ($s_{20,w}$)	Alkaline ($s_{20,w}$)
1	24.2 ± 0.6	17.5
25	26.4 ± 0.2	19.7
50	28.6 ± 1.0	20.0
100	26.1 ± 0.1	20.9
200	26.4 ± 1.8	19.3
500	23.7 ± 0.1	19.6

[a] The *C. antennarius* dAT was melted and fast cooled in SSC at the concentrations listed and then either diluted or concentrated to a final concentration of 50 μg/ml before analysis.

TABLE II: DNA Sedimentation Rates and Calculated Molecular Weights.

		Mol Wt × 10^{-6}		
	$s_{20,w}$	Native[a]	Alkaline[b]	Neut Denatured[c]
Main component DNA				
Native	28.9 ± 0.8	19		
Alkaline	15.3 ± 0.3		1.4	
Heated and cooled	19.9 ± 1.7			0.93
Alkaline	14.4 ± 1.3		1.2	
Synthetic dAT				
As synthesized	20.5 ± 2.0	(6.9)[d]		
Alkaline	22.4 ± 1.7		3.7	
Heated and cooled	15.8 ± 0.5	(3.3)		(0.61)
Alkaline	18.8 ± 1.4		2.4	
dAT (Hg)				
Native	28.6 ± 0.6	18		
Alkaline	25.6 ± 0.4		5.2	
Heated and cooled	29.2 ± 1.8	(19)		(1.9)
Alkaline	20.8 ± 0.1		3.1	
dAT (column)				
As isolated	27.0 ± 0.6	(15)		(1.6)
Alkaline	18.5 ± 0.8		2.3	
Heated and cooled	20.7 ± 1.4	(7.1)		(1.0)
Alkaline	17.6 ± 0.2		2.0	

[a] Calculated using formula for native DNA. $s^0_{20,w} = 0.0882\ M^{0.346}$. [b] Calculated using formula for alkaline DNA. $s^0_{20,w} = 0.0528\ M^{0.400}$. [c] Calculated using formula for neutral denatured DNA. $s^0_{20,w} = 0.0105\ M^{0.549}$. [d] The values in parentheses were calculated using the formula for the DNA conformation indicated, but the actual conformation is unknown.

and the sedimentation rates determined using equal amounts of DNA (1.5 μg) in each centrifuge cell.

The *s* values of these preparations, shown in Table I, do not increase with dAT concentration. The largest variations are found for the samples melted at 1 and 500 μg/ml. The sample melted at 1 μg/ml sediments more slowly than the other samples because of a smaller covalent-strand size. The smaller strand size is indicated by the low *s* value obtained when this preparation is sedimented under alkaline conditions. The reason for the low *s* value for the sample melted at 500 μg/ml is unclear since the alkaline *s* value is comparable to that found for the other samples.

The experimental evidence presented here and that of Inman and Baldwin (1962) indicate that there is no apparent intermolecular aggregation under the melting conditions employed in these experiments. Thus we feel that when melted DNA samples are sedimented we measure the sedimentation rates of single covalently linked chains.

Table II shows the experimental results for the native and melted DNA preparations sedimented in neutral solution and in 0.1 M alkali. The molecular weight values listed are calculated from the sedimentation values using Studier's (1965) formulas. The results are not of the highest precision, since as shown in Figure 1, the band of sedimenting DNA becomes broad and disappears about half-way down the cell making accurate measurement difficult. This spreading is due apparently to the size heterogeneity of the DNA preparations.

The results listed in Table II show that the main component DNA acts as expected on the basis of Studier's (1965) results. The melted DNA sediments more rapidly in neutral solution than in alkali showing that the structure is very compact in neutral solution. The molecular weights calculated from the *s* values found in alkali before and after melting and for the melted sample in neutral solution are very similar. Apparently many single strand breaks are present in the original preparation since the calculated *M* decreases markedly when the strands are separated by alkali or by heating.

The data for synthetic dAT in Table II shows that the dAT structure in neutral solution is much like that of native DNA (fully hydrogen bonded and extended) since the *s* value in neutral solution both before and after melting is lower than the *s* of the same preparation in alkali. Typical DNA, which does not hydrogen bond appreciably after heating, sediments faster than alkaline DNA because of a collapsed single-stranded structure. The *M* calculated for synthetic dAT using the formula for native DNA is somewhat higher than the *M* calculated from the alkaline *s* value. This finding is con-

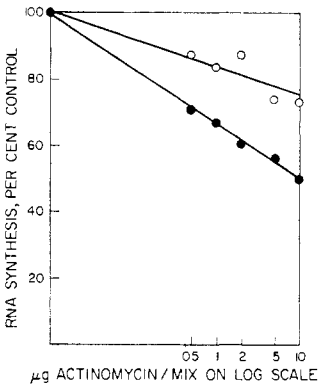

FIGURE 3: Inhibition of RNA synthesis by actinomycin D using *C. anteannrius* dAT (Hg) (●) and melted dAT (Hg) (O) as templates with *E. coli* RNA polymerase. The four [^{14}C]nucleoside triphosphates were incorporated separately and the total RNA synthesis was determined. DNA (1.4 mμmoles) was incubated for 20 min with 3 enzyme units. With no actinomycin present, 7.5 and 6.5 mμmoles of RNA were synthesized from the native and melted templates, respectively.

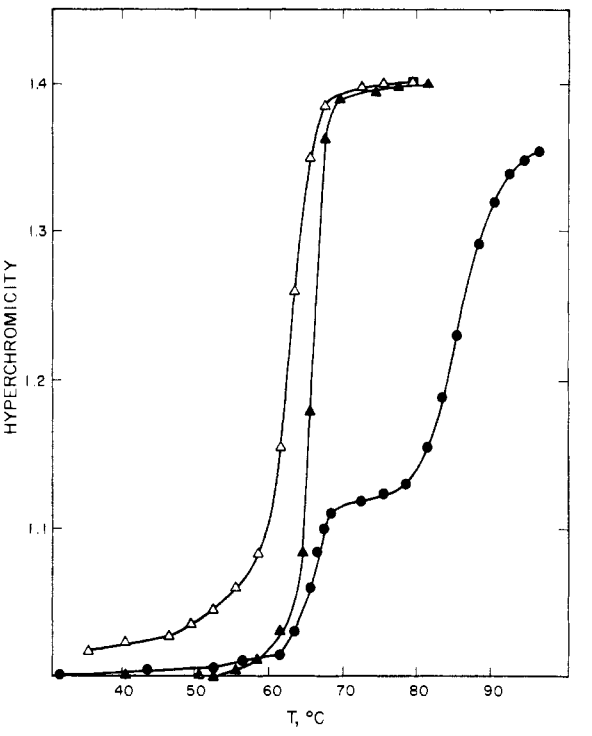

FIGURE 4: Optical density melting profiles of *C. borealis* whole crab DNA (●) and dAT (Hg) (heating ▲, cooling Δ) in SSC.

sistent with a slightly branched structure as DNA with a branched structure would be expected to sediment somewhat faster than completely linear extended double helical DNA of the same molecular weight. Comparison of the alkaline *s* values of synthetic dAT before and after heating indicates that the heating does produce some single strand breaks.

The calculated *M* of dAT (Hg), which is known to be native, is 18×10^6. The *M* calculated for the same DNA as single strands in alkali is 5.2×10^6 indicating that many single strand breaks are present in the native mercury-isolated crab dAT. Comparison of the *s* values after melting in neutral and alkaline conditions shows that the neutral form sediments more rapidly. This behavior is in marked contrast to that of synthetic dAT and points to a very compact structure for melted crab dAT in neutral solution. As shown by Davidson *et al.* (1965), melted crab dAT molecules are composed predominantly of hydrogen-bonded double helices, but are highly branched and compact. The heating of crab dAT apparently introduces some strand breakage as seen by a comparison of the sedimentation rate of the alkaline forms before and after heating.

As expected, column-isolated crab dAT (which is melted before column isolation) shows sedimentation behavior like that of melted dAT (Hg). The melted dAT sediments faster in neutral solution than in alkali. This is expected since it is known that melted crab dAT and column-isolated crab dAT have structures which are identical or at least very similar (Davidson *et al.*, 1965).

The sedimentation studies show that crab dAT molecules after melting are very compact and sediment more rapidly than the same molecules in alkali. Thus melted crab dAT acts like typical melted DNA as both sediment more rapidly in neutral solution than in alkaline solution. Melted synthetic dAT is, however, appreciably extended in neutral solution and thus has a lower *s* value under this condition than when in alkali.

Actinomycin Inhibition of RNA Synthesis. Actinomycin D is a potent inhibitor of DNA-dependent RNA synthesis *in vitro* and inhibits the synthesis to an extent roughly proportional to the G content of the DNA template (Goldberg *et al.*, 1962; Kahan *et al.*, 1963). These studies indicate that the G residues are involved in the binding of actinomycin to the DNA template and that actinomycin must bind to be effective. Also, actinomycin binds less strongly to denatured DNA and inhibits RNA synthesis less strongly with the denatured primer than with native DNA primers. This is presumably caused by poorer binding of the actinomycin to the G residues of single-stranded DNA.

A useful technique for identification of a difference between native and melted dAT can be based upon the fact that actinomycin is a less effective inhibitor of RNA synthesis supported by a single-stranded primer than of such synthesis supported by a double-stranded primer. That this method might be expected to be especially sensitive is suggested by the fact that the *E. coli* exonuclease I hydrolysis data (Davidson *et al.*, 1965) indicate that it is largely the GC base pairs which do not re-form after renaturation of the dAT molecules.

The results of a control experiment using *C. antennarius* native and melted main component DNA tem-

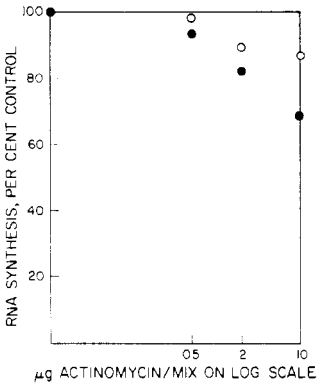

FIGURE 5. Inhibition of RNA synthesis by actinomycin D using *C. borealis* dAT (Hg) (●) and melted dAT (Hg) (○) as templates with *E. coli* RNA polymerase. The four [^{14}C]nucleoside triphosphates were incorporated separately and the total RNA synthesis was determined. DNA (1.1 mμmoles) was incubated for 20 min with 3 enzyme units. With no actinomycin present, 4.8 and 4.1 mμmoles of RNA were synthesized from the native and melted templates, respectively. Reaction mixture contained, for each 0.25 ml, 10 μmoles of Tris buffer (pH 8), 1 μmole of $MgCl_2$, 0.25 μmole of $MgCl_2$, 3 μmoles of β-mercaptoethanol, and 50 mμmoles of each of the four riboside triphosphates.

plates are shown in Figure 2. RNA synthesis by *E. coli* RNA polymerase with main component DNA as template (40% GC content as determined by base incorporation) is inhibited quite markedly by actinomycin. Greater inhibition by all actinomycin concentrations is noted, however, with native DNA than with melted DNA. Thus, RNA synthesis with the largely single-stranded melted primer is less inhibited by actinomycin.

The results of similar experiments carried out with native and melted *C. antennarius* dAT templates are shown in Figure 3. In this case the inhibition is much less than that observed with the higher G-containing main component DNA. This is expected since the inhibition is known to be a function of the G content.

Figure 3 also shows that RNA synthesis with the native dAT template is inhibited to a greater degree than is such synthesis with the melted form. This difference is quite evident at all actinomycin concentrations employed. It is clear therefore that at least many of the GC base pairs of crab dAT do not re-form after heating and cooling.

Characteristics of Melted C. borealis dAT. Since *C. antennarius* dAT renatures incompletely after melting, one would expect *C. borealis* dAT to behave in a similar way. A sample of *C. borealis* DNA (obtained from Dr. T. Y. Cheng) was separated into its two components by the mercury-binding method. The two components separated elegantly and were recovered in pure form.

As shown in Figure 4, the melting behavior of *C. borealis* dAT is similar to that of *C. antennarius* dAT (Davidson *et al.*, 1965); the material melts sharply when heated for the first time and melts at a temperature higher than the cooling and remelting temperatures. In this case the cooling rate was *ca.* 1.0°/min. The original T_m in SSC is 65.7° with the σT value of 1.3°. The cooling T_m is 62.8° and the subsequent melting T_m value is also 62.8°, with a σT value of 4.0°. The absorbance returns very nearly to the value before melting, but *ca.* 4% of the absorbance increase does not disappear after cooling.

C. antennarius dAT melts the first time at 67.5° with a σT value of 0.5° and cools and remelts at 62.5° with a σT value of 3.5°. Seven per cent of the absorbance increase does not disappear after cooling (Davidson *et al.*, 1965).

The buoyant density of the native *C. borealis* dAT in CsCl was 1.680, but increased to 1.682 after melting. Sueoka (1961) and Sueoka and Cheng (1962a,b) give values of 1.681 both for the native and the melted material when calculated using the standard density for *E. coli* DNA of 1.710.

The buoyant density of *C. antennarius* dAT increases from 1.677 when native to 1.680 after melting. (The values listed by Davidson *et al.* (1965) are in error due to the use of an incorrect magnification factor.) The respective buoyant density values for the *C. antennarius* DNA preparations before and after melting should be: synthetic dAT, 1.677 (1.678); dAT component in whole crab DNA, 1.677 (1.680); dAT (Hg), 1.677 (1.680); dAT (column), 1.680 (1.680); main component DNA, 1.701 (1.714).

The results of experiments on actinomycin inhibition of RNA synthesis with native and melted *C. borealis* dAT as templates are given in Figure 5. It is clear that the RNA synthesis with native dAT as primer is inhibited more strongly by actinomycin D than is RNA synthesis with melted dAT (Hg) as primer. The differences between the melted and native dAT inhibition are not as large, however, as with *C. antennarius* dAT.

Incorporation studies using *E. coli* RNA polymerase and [^{14}C]nucleoside triphosphates showed that the RNA synthesized from the *C. borealis* dAT template has the following base composition: A, 48.0%; U, 49.5%; G, 1.2%; C, 1.3%. Thus the *C. borealis* dAT GC content is 2.5%. Swartz *et al.* (1962) found a value of 2.7% with *C. borealis* dAT and *E. coli* DNA polymerase and Goldberg *et al.* (1962) reported 2.6% with the same template and *E. coli* RNA polymerase.

The RNA synthesized from the dAT component of *C. antennarius* had the following base composition: A, 46.8%; U, 49.7%; G, 1.8%; C, 1.7%. In this case the GC content is 3.5%.

This GC content difference may explain the quantitative differences noted in the behavior of the dAT's obtained from the two species before and after melting. Since the *C. antennarius* dAT has the higher GC content, it might be expected to renature less, and the changes caused by melting should thus be greater in this case than in the case of *C. borealis* dAT.

We do indeed find that the differences observed are larger for *C. antennarius* dAT than for that of *C. borealis* in all cases: 5 *vs.* 3° for the differences between the T_m values for the first and subsequent meltings, a buoyant density increase of 0.003 compared with 0.002, and a larger differential in the actinomycin D inhibition of RNA synthesis.

Other facts also indicate that *C. antennarius* dAT has a higher GC content. Its original T_m is higher, 67.7 *vs.* 66.2°, and the RNA synthesis primed either by dAT (Hg) or melted dAT (Hg) is inhibited to a greater extent by actinomycin D than is that by the comparable *C. borealis* dAT forms. The buoyant densities of the two are, however, the reverse and would indicate that *C. borealis* dAT has the higher GC content.

It is evident that *C. borealis* dAT, like *C. antennarius* dAT, possesses a partially denatured structure after heating and cooling. The extent of denaturation seems to be greater for the dAT from the latter species, apparently because this dAT has a higher GC content.

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