PHYSICAL AND BIOLOGICAL STUDIES OF CRAB DEOXYRIBONUCLEIC ACID

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ABSTRACT

The native dAT components of \underline{C} . antennarius and of \underline{C} . borealis DNA have been separated in pure form by a new mercury binding technique.

Two further DNA species, not heretofore described, are apparently also present in <u>C</u>. <u>antennarius</u> DNA. They have melting points and buoyant densities intermediate between those of dAT and of the major DNA component.

A series of tests using optical density melting, electrophoresis, buoyant density, electron microscopic, band sedimentation, exonuclease I susceptibility and actinomycin D inhibition techniques are employed to show that melted and renatured <u>C</u>. antennarius dAT remains partially denatured. After strand separation and recombination approximately 7% of the base pairs do not reform. These unpaired bases are predominantly guanine and cytosine (G and C) since exonuclease I hydrolysis assays and RNA synthesis inhibition by actinomycin D give the results which would be expected on this basis. The <u>C</u>. antennarius dAT contains 3.5% G and C bases. <u>C</u>. borealis dAT with 2.5% GC behaves similarly to <u>C</u>. antennarius dAT, except that the denatured structure is less evident, apparently because of the lower GC content.

When the aforementioned tests are applied to synthetic dAT, which contains no G or C bases, the results indicate that this alternating dAT copolymer is fully hydrogen bonded both

before and after melting.

dAT is present to the extent of 10-11% in another Cancer species, C. anthonyi.

dAT is found in all \underline{C} . antennarius tissues and is localized in the cell nucleus.

Studies with the \underline{E} . $\underline{\operatorname{coli}}$ RNA polymerase system show that dAT is copied at a rate 2.6 times as fast as is the main DNA component. dAT also has a higher affinity for the enzyme.

Actinomycin D inhibits the transcription of native primers more than it does that of melted ones. Incorporation of GC into RNA is preferentially inhibited.

Protamine, histones Ib, IIb, III and IV, listed in order of decreasing effectiveness, inhibit the RNA synthesis supported by DNA. These basic proteins inhibit incorporation of A and U preferentially indicating preferential binding of the inhibitors to AT rich regions of the template.

The RNA synthesized from the dAT template by \underline{E} . \underline{coli} RNA polymerase is double-stranded and melts at 67°C. This rAU has an S value of 5-6 after ribonuclease T_1 treatment. This result is due to the low G content of the dAT, 1.8%.

Actinomycin D inhibition studies with crab hepatopancreas tissue indicate that rAU is not synthesized at an appreciable rate <u>in vivo</u> if at all.

The RNA from this tissue also contains no rAU as shown by melting and by T_1 treatment. The rAU if present would

melt sharply and be detectable and would show up as 5-6 S material in sucrose density gradient centrifugation of the hepatopancreas RNA after T_1 treatment.

Chromatin is less active as template for RNA synthesis than is whole crab DNA and the chromatin dAT component is particularly repressed.

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ABBREVIATIONS

A = Adenine

A₂₆₀ = Absorbance measured at 260 mµ

C = Cytosine

dAT = Alternating copolymer of deoxyadenylate-deoxythymidylate

EDTA = Ethylenediaminetetraacetic acid

G = Guanine

MAK = Methylated albumin kieselguhr

muM = Millimicromolar

mum = Millimicromoles

NTP = Nucleoside triphosphate

P = Density

rAU = Alternating copolymer of riboadenylate-ribouridylate

S = Sedimentation coefficient

S_{20,w} = S corrected to the value expected in a solution having the density and viscosity of water at 20°C

 $S_{20,w} = S_{20,w}$ found at zero concentration of sedimenting material

σ T = Transition width in degrees C between 20% below midpoint to 20% above midpoint of the O.D. melting profile

SLS = Sodium lauryl sulfate

T = Thymine

TCA = Trichloroacetic acid

Tm = Melting temperature obtained from the O.D. melting profile (midpoint of O.D. transition)

Tris = Tris (hydroxymethyl) aminomethane

U = Uracil

INTRODUCTION

The DNA of the sperm of <u>Cancer borealis</u> and <u>C. irroratus</u> has been found by Sueoka (1) and Sueoka and Cheng (2) to contain a minor component. This component, which makes up about 30 per cent of the <u>C. borealis</u> DNA and 10 per cent of the <u>C. irroratus</u> DNA, was characterized by a low melting temperature and low buoyant density indicating a low GC content.

These workers (2,3) separated the two DNA components by a method based upon the ability of the minor component to "renature" after heating and fast-cooling. The "renatured" minor component is then easily separated on a MAK column from the major denatured fraction which adsorbs more strongly. The MAK column can be used also to separate the main component in its native form since the column adsorbs native low GC DNA more strongly than native GC rich DNA. The low GC DNA cannot be purified completely in this way without repeated chromatographic fractionation. Thus all the previous work done with the light DNA component has apparently been done with "renatured" material (4,5,6).

The minor component was found by Swartz, Trautner, and Kornberg (4) to contain about 3 per cent GC. Nearest neighbor studies showed that the A and T residues are almost completely in an alternating sequence. The major component,

however, is a typical DNA containing 36 per cent GC.

Since the minor component, which will be called dAT hereafter, is predominantly an alternating AT polymer, it is evident why it "renatures" easily. The dAT structure and behavior make it very similar to synthetic dAT which is made up only of alternating A and T residues and which melts and renatures reversibly (7).

Smith (8) reported that the DNA of four additional <u>Cancer</u> species contains from 10 to 30 per cent dAT depending on the species and Pochon, Massoulie and Michelson (9) found dAT in yet another species, <u>C. pagurus</u>.

The studies presented in this dissertation began as a search for the biological function of this unusual DNA. Since most DNA functions as a genetic determinant, the dAT of crabs might also function in this way. There are numerous ways in which the function of isolated genes can be studied and several approaches to the matter are discussed below.

During the course of experimentation, some interesting physical properties of the "renatured" <u>C</u>. <u>antennarius</u> dAT became evident, properties which conflicted with those reported by earlier workers as well as with their assumptions concerning the nativeness of the "renatured" material. These findings led to the development of several tests to determine more clearly the differences between native and melted dAT. Synthetic dAT was also studied because of the

structural and behavioral similarities between it and crab dAT. Part of the results of this work have been published previously (10).

These studies were made possible by the discovery of a separation technique reported by Davidson, Widholm, Nandi, Jensen, Olivera and Wang (10) which separates the crab DNA components without prior denaturation. The method relies on the selective binding of mercury to AT rich DNA. This produces a density differential between the dAT and main component DNA great enough to allow them to be separated by centrifugation in a cesium sulfate density gradient. Silver can also be used to separate the two components as it binds more strongly to the higher GC DNA.

The mercury binding technique has been applied to the DNA of both \underline{C} . antennarius and \underline{C} . borealis and elegant separations were obtained in each case.

The biological studies consider the distribution of the dAT in crab tissues and its localization within the cell.

Experiments were also carried out with dAT as template for <u>E. coli</u> RNA polymerase. The effects of actinomycin D and of basic proteins upon the template activity of dAT were determined. The properties of the RNA product synthesized by the RNA polymerase with dAT as template have been studied. On the basis of these properties experiments were then designed (and executed) for detection of rAU in crab tissues.

The natural nucleoprotein (chromatin) has been isolated

from \underline{C} . antennarius hepatopancreas tissue and its properties investigated.

The DNA species used throughout this study will be identified as follows: whole crab DNA, the total extracted crab DNA; dAT[0], the native crab dAT not separated from the main component DNA; dAT[1], the mercury separated crab dAT; dAT[2], crab dAT isolated by the Sucoka and Cheng procedure; dAT[3], melted dAT[1]; synthetic dAT, the dAT synthesized by DNA polymerase in vitro (7); main component DNA[0], the major crab DNA component not separated from dAT; main component DNA[1], the mercury separated major crab DNA component; main component [2], the major crab DNA component separated by the Sucoka and Cheng column procedure without prior denaturation.

This dissertation is divided into two sections. Section I is concerned with physical studies and Section II with biological studies.

I. PHYSICAL STUDIES OF CRAB dAT

A. Methods Employed for Physical Studies

Sperm were collected from the vas deferens and spermatheca of the crab, <u>Cancer antennarius</u>, and frozen immediately. The DNA was isolated from the sperm, after thawing, by the procedure of Smith (8), except that all the operations were carried out in the cold and a ribonuclease treatment was included after the isopropanol precipitation (11). This was followed by another isopropanol precipitation. The resulting whole crab DNA was dissolved in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.5) or DSC (SSC/10) and stored frozen. No RNA was detectable in electrophoresis experiments or by banding analytically in cesium chloride.

Main component DNA[2] and dAT[2] were isolated by the MAK column method of Sueoka and Cheng (3).

The two DNA components were also separated by the mercury binding method of Davidson et al. (10). Approximately 0.1 moles of mercury as $HgCl_2$ were added per mole of whole crab DNA phosphate in a solution of cesium sulfate (Gallard Schlesinger). The density of the solution was 1.50 and the pH was buffered at 9.2 by 0.005 M borate. The DNA was added in 0.01 M sodium sulfate solution instead of SSC in order to keep the final chloride ion concentration low,

since high Cl concentrations can displace the DNA complexed mercury. DNA concentrations of A₂₆₀ of 5.0 were usually Two ml were placed in an SW-39 centrifuge tube employed. which was then filled with three ml of mineral oil and centrifuged for 48 hours at 31,000 rpm at 25°C, or 8 ml were put into an SW-25 tube and filled with 22 ml of mineral oil and centrifuged for 72 hours at 25° at 25,000 rpm in the Spinco Model L Ultracentrifuge. The tubes were punctured, fractions collected, and the A₂₆₀ of each fraction after dilution was determined with a Hitachi Perkin-Elmer model 139 spectrophotometer. All the centrifuge tubes from the isolations could be dripped into the same collecting tubes since the two DNA bands are very viscous and serve as markers. The fractions were dialyzed against three changes of 500 ml of 1 M NaCl, 0.005 M cacodylate, pH 6.2 for 24 hours and then against SSC or DSC. The dialysis tubing was treated with EDTA solution before use, as described by Chamberlin, Baldwin and Berg (12).

The DNA buoyant densities were determined in cesium chloride by analytical equilibrium density gradient centrifugation (13). The densities were calculated by the method of Schildkraut et al. (14) using Micrococcus lysodeikticus DNA as reference (ℓ = 1.731 g cm⁻³).

The purity of each preparation was determined analytically by the banding in cesium chloride of an excess of the DNA, about 10 μg , and observing if there is any contaminating

DNA present. Approximately 0.1 µg will form a detectable band so that the level of detectable contamination is about 1 per cent in these experiments. Fractions containing detectable impurity were not used.

DNA samples in SSC were routinely melted by heating in a boiling water bath for 10 minutes and then were cooled in ice.

Optical density melting profiles of DNA were obtained by following the absorbancy at 260 mm (A_{260}) as a function of temperature in a Beckman DK-2 spectrophotometer with a heating attachment or with a Beckman DU spectrophotometer equipped with a Gilford Multiple Absorbance Recorder attachment with a heating attachment (10). The results obtained with both of these instruments were identical to those determined with the heating cell of a Cary spectrophotometer described previously by Dove and Davidson (15). The heating rate was about 0.5 degrees per minute. The cooling rate was about twice the heating rate.

DNA was also isolated from <u>C. antennarius</u> spermatheca by lysing the material directly in cesium chloride solution of density 1.70 and then banding preparatively. Three ml of this solution with two ml of mineral oil on top were placed in an SW-39 centrifuge tube and centrifuged at 35,000 rpm for 48 hours at 25°C. The same was carried out with 12 ml of the solution plus 18 ml of mineral oil in an SW-25 head and centrifuged for 72 hours at 25,000 rpm at 25°C. Fractions

were collected and the A_{260} of each was determined. The fractions containing the DNA were dialyzed against saline-EDTA (0.1 M NaCl, 0.05 M EDTA, pH 8.0) and then against SSC.

Electrophoretic mobilities were determined using the method of Olivera et al. (16).

DNA samples were prepared for electron microscopy by the method of Kleinschmidt (17). The micrographs were taken with a Phillips 200 electron microscope after the preparations were shadowed with platinum-carbon.

Sedimentation rates were determined using the analytical band sedimentation method of Vinograd et al. (18). Approximately 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 and centrifuged at 29,500 rpm at 20°C in the Spinco Model E. The sedimentation values were corrected using the solvent correction factors determined by Studier (19).

Exonuclease I treatment was carried out as described by Lehman (20) with 40 enzyme units in each 0.6 ml incubation mixture containing about 35 mpm of DNA. The reaction rate was followed by measuring A_{260} as a function of time. Exonuclease I and synthetic dAT were gifts of I. R. Lehman.

B. Results of Physical Studies

1. Properties of Crab DNA and the Mercury Binding Separation Technique

Crab DNA generally has been characterized with the use of buoyant density and optical density melting techniques. Sueoka (1) and Sueoka and Cheng (2) investigated <u>Cancer borealis</u> and <u>C. irroratus</u> and Smith (8) studied four other <u>Cancer species including <u>C. antennarius</u>, which is the species studied here. These two methods were likewise utilized repeatedly in the work described in this dissertation.</u>

Pigure 1 shows a microdensitometer tracing of a DNA preparation from <u>C</u>. <u>antennarius</u> sperm and spermatheca banded in cesium chloride. The smaller peak represents dAT with a density of 1.677 g cm⁻³. The area under the peak indicates that this component makes up 23 per cent of the whole crab DNA. The main component peak banded at a density of 1.701. Smith (8) found 26 per cent dAT and buoyant densities of 1.677 and 1.700 for the components of C. antennarius DNA.

A typical optical density melting profile is shown in Figure 2. Tm values of 67.7°C and 85.6°C for the dAT and main component DNA, respectively, were determined and 25 per cent of the hyperchromicity was due to the absorbance increase of the dAT component. Smith (8) found 30 per cent dAT by this method and Tm values of 65.8°C and 84.0°C for

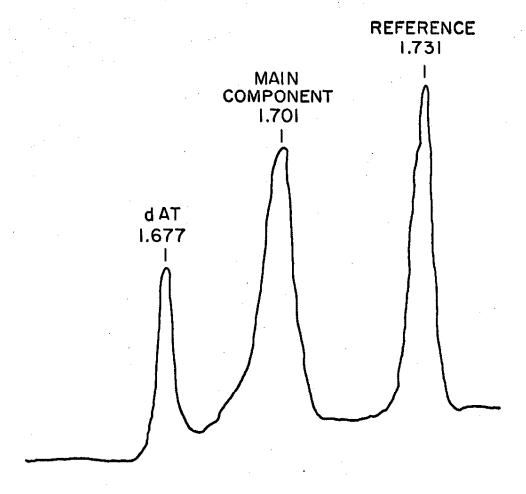


Figure 1. Densitometer tracings of <u>C</u>. <u>antennarius</u> whole crab DNA banded in CsCl. Reference DNA is <u>Micrococcus lysodeikticus</u> (*f*=1.731). The dAT peak contains 23% of the total crab DNA.

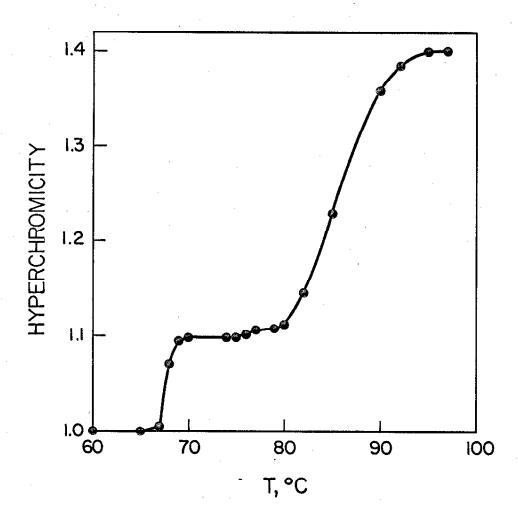


Figure 2. Optical density melting profile of <u>C</u>. <u>antennarius</u> whole crab DNA in SSC.

the two components.

The two components were separated in pure form by the mercury binding method developed by Davidson et al. (10). Figure 3 shows the absorbances of the combined fractions collected from three isolations done with SW-39 rotors. The results noted in Figure 3 are typical and the recovery was 90 per cent in this case (73 of 81 optical density units put in were recovered).

The fractions were dialyzed free of Hg*+ and Cs2SO4 and then banded in cesium chloride to determine the composition of each fraction. Figure 4 shows tracings from these analytical centrifuge experiments carried out with DNA from each fraction. Fractions 4 and 8 contain excess DNA in order to make contaminants visible even if present in minute amounts. Purity is thus assured to about 99 per cent if no contaminating DNA band is visible. Fraction 4 is seen to be pure dAT and fraction 8 pure main component DNA. Fraction 5 contains besides dAT a small proportion of main component DNA and probably some material between. Fraction 6 contains more main component and definitely some material banding between dAT and the main component DNA. Fraction 7 continues the trend and contains more main component and intermediate material beside dAT.

Only about 10 per cent of the total DNA was usually recovered as purified dAT even though the original DNA

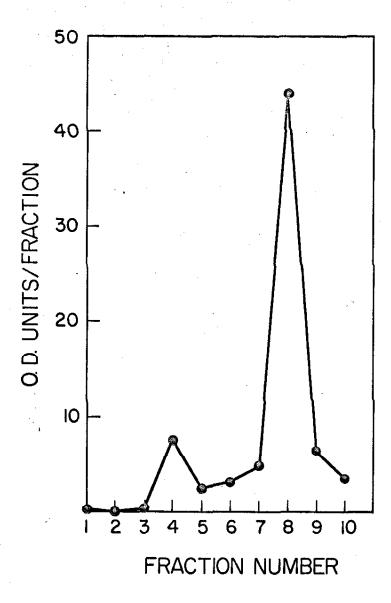


Figure 3. Optical density units (260mµ) recovered using mercury binding separation technique with <u>C</u>. <u>antennarius</u> whole crab DNA. Equilibrium centrifugation in Cs₂SO₄ (mean P=1.50) with the mercury to DNA phosphate ratio of O.1. Nine SW-39 tubes were collected together.

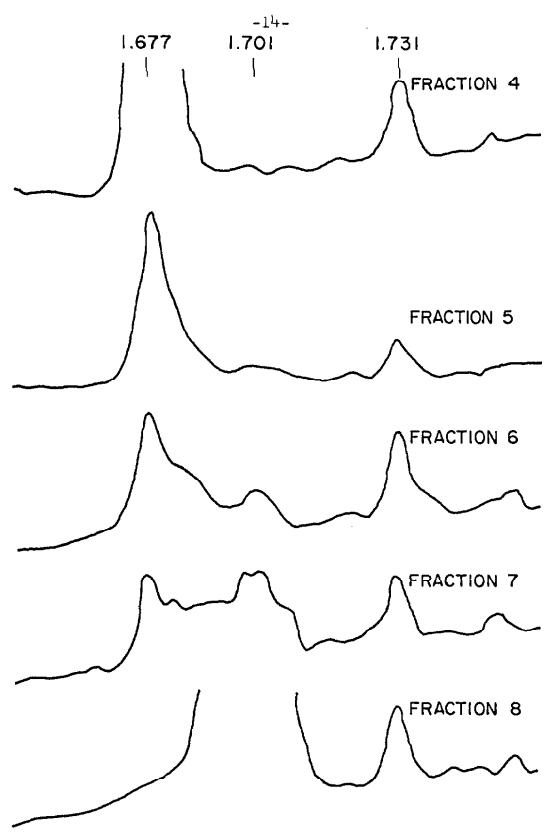


Figure 4. Tracings of DNA from each fraction obtained during the mercury separation of whole crab DNA, banded in CsCl.

mixture contained about 23 per cent of this component.

It is evident from the tracings in Figure 4 that there occurs some smearing of the dAT component into fractions 5, 6 and 7 which may be due to crosslinking caused by Hg⁺⁺. An estimate of the amount of dAT in these fractions from Figures 3 and 4 shows about half of the total dAT in the tube is found in fractions 5, 6 and 7 and the other half in fraction 4.

The intermediate fractions from a number of these preparations were pooled and recentrifuged in cesium sulfate with mercury to purify further the DNA banding between dAT and main component DNA in cesium chloride. The A₂₆₀ pattern from one of these preparations is shown in Figure 5. There is definitely DNA banding in a position between the small dAT peak and the large main component peak.

Fractions 10, 11 and 12 were pooled, dialyzed and then analyzed in the ultracentrifuge in cesium chloride. The tracing of the run in Figure 6 indicates that a substantial proportion of the DNA in these fractions is the intermediate material.

The melting profile of this material is shown in Figure 7 which shows also that there is some DNA present that melts at a temperature between that of dAT and that of the main component DNA. The dAT has a buoyant density of 1.677 and Tm of 67.7°C and the respective values for the main component DNA are 1.701 and 85.6°C. There is also present a component

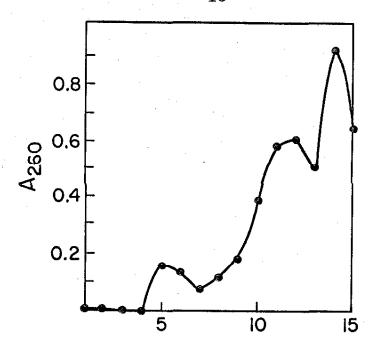


Figure 5. A_{260} pattern resulting from an equilibrium centrifugation of intermediate fractions in Cs_2SO_4 with the mercury to DNA phosphate ratio of 0.1. 15 fractions collected.

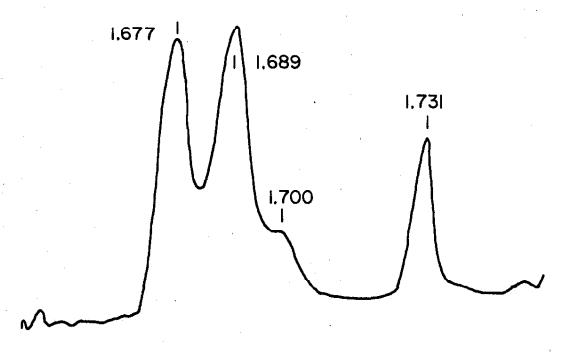


Figure 6. Tracing of a mercury purified intermediate fraction banded in CsCl. Buoyant density values are listed.

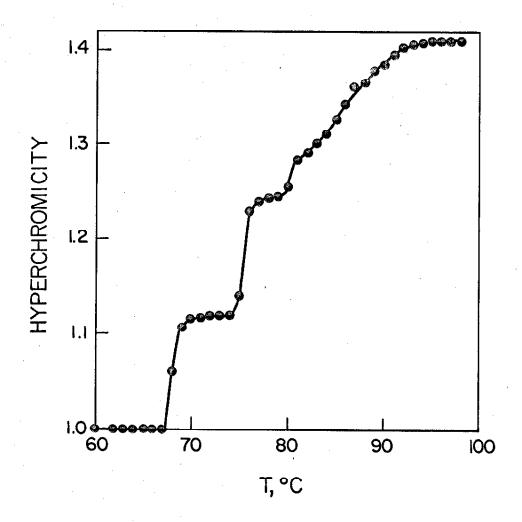


Figure 7. Optical density melting profile of a mercury purified intermediate fraction in SSC.

with a buoyant density of about 1.689 and a Tm of 75.3°C. The melting profile also indicates that there is possibly a fourth component present that melts sharply at 80.2°C.

The DNA melting at 75.3°C was present to the extent of about two per cent in the optical density melting profiles of whole crab DNA (Figure 2). The estimated content in the original DNA based on the melting of the enriched intermediate was about 1.5 per cent. The material melting at 80.2°C was not detectable in the melting profile of whole crab DNA even though it is presumably present to the extent of about one per cent. It is probably undectable because the main component DNA begins to melt at that temperature and a small optical density increase would not be noticed. The material would likewise be undetectable in a cesium chloride density gradient.

The origin of the intermediate DNA species is at present unknown. It is possible, however, that one of them originates in the mitochondria, but experimental evidence is not at hand.

The intermediate material is probably not DNA strands composed of a dAT region joined to a main component DNA region since this structure would have an intermediate buoyant density, but would not melt sharply at an intermediate temperature.

DNA was also isolated from spermatheca by lysing the material directly in cesium chloride solution and banding the DNA and RNA by equilibrium centrifugation.

The composition of the DNA isolated in this way was the same as that of the DNA isolated in the usual manner, but the molecular size of the material as determined by sedimentation was actually smaller rather than larger than the normally isolated DNA. A slight enrichment of one component or the other was noted in the fractions taken from various portions of the DNA band.

2. Melting Characteristics of dAT

The mercury separated dAT (dAT[1]) had a Tm of 67.7°C when first heated, but had a cooling Tm of 62.9°C. The Tm upon remelting was 62.5°C and the melting and cooling profiles were reversible thereafter. The MAK column separated dAT (dAT[2]) melted reversibly at 62.4°C.

The original melting of dAT[1] was very sharp, but the cooling and remelting curves were broader as indicated by an increase of the σ T value from 0.5°C originally to 3.5°C when remelted. The σ T values for all the profiles of dAT[2] were 3.5°C.

Table 1 and Figure 8 also show that the absorbance of $\mathrm{dAT}[1]$ does not return to the original value after heating and cooling. The A_{260} increase remaining after cooling is about 7 per cent of the total hyperchromic increase. A similar value was found when the A_{260} values of melted and unmelted identical $\mathrm{dAT}[1]$ samples were determined with a

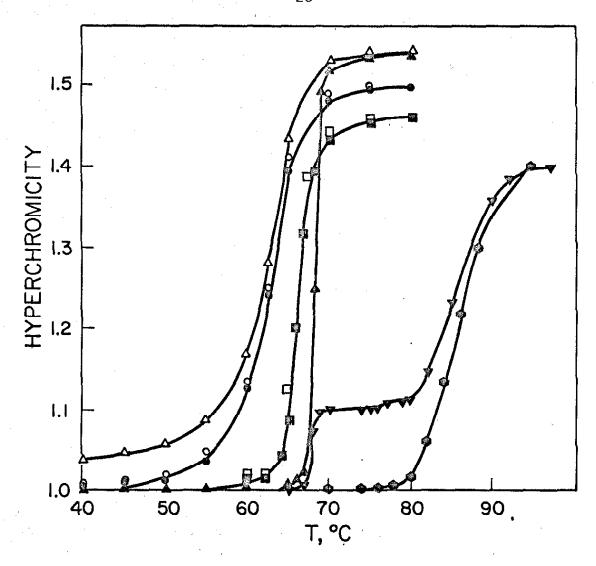


Figure 8. Optical density melting profiles of DNA preparations in SSC. Closed and open points are heating and cooling curves, respectively. ▼, whole crab DNA; □, □, synthetic dAT; △, △, dAT[1]; ○, ○, column separated dAT (dAT[2]); ○, main component DNA[1].

TABLE I

Denaturation Transition Parameters for DNA Samples in SSC

Sample		F1:	rst He	First Heating		:	Seco	Second Heating	ating	
	o G	ц	o C C	Cooling Tm(°C)	% Return	o H	ų	O-T	Cooling Tm	% Return
Whole crab dAT [0]	67.7	· ·								
Main component [0]	85.6	T+-T								
dAT [1]	67.5	1.54	0.0	65.9	93	62.5	62.5 1.49 3.5	w r.	62.2	21-
dAT [2]	4.29	1.49	ω π.	65.9	66	62.5	62.5 1.48 3.5	У	6.19	99.5
Main component [1]	85.4	1.40	0.4							
Main component [2]	85.4	1.39	3.9							. '
Synthetic dAT	65.7	1.46	1.46 1.5	4.99	100	65.7	1.46	ر ب اسا	65.7 1.46 1.5 66.2	001

% Return = 100 x (change in absorbance on cooling)/(change in absorbance on heating). σT is transition width in degrees between 20% below midpoint to 20% above midpoint. Hyperchromic increase, h=A (high temperature)/A (beginning temperature)

spectrophotometer. Slow cooling of the dAT did not result in complete renaturation as evident from the Tm or final optical density.

Thus, the absorbance measurements show that complete renaturation does not occur when heated dAT[1] is cooled. The dAT molecules appear to be changed after strand separation (dAT[2] or dAT[3]) to a form which has a Tm 5° C lower than that of the native form, which melts over a broader temperature range than does the native form, and which shows less hyperchromic increase than the native form, but which returns to the original A_{260} after cooling.

Synthetic dAT shows no change in Tm, σT or absorbance after melting so that evidently complete renaturation occurs with this material.

Apparently, once the crab dAT strands separate they do not return to correct register when renatured. This could presumably be caused by the guanine and cytosine residues that are spaced throughout the alternating AT residues or by a non-perfectly alternating character of the AT bases. Either case would result in regions that do not match up upon strand recombination.

3. Electrophoretic Studies of the dAT Forms*

A study was carried out to determine the electrophoretic

^{*} Very kindly done by B. M. Olivera.

mobilities of the various DNA preparations, since it is known that at 0°C, in 0.01 M NaCl, 0.001 M tris, pH 7.5, the mobilities of native and denatured DNA differ significantly (16). The mobilities of native DNA, denatured DNA and RNA are 2.18×10^{-4} , 1.87×10^{-4} and 1.75×10^{-4} cm² sec⁻¹ volt⁻¹, respectively. The mobilities are independent of molecular weight and base composition.

Two tracings made directly in the electrophoresis apparatus are shown in Figure 9. The results of these experiments show that native whole crab DNA, dAT[1] and melted and unmelted synthetic dAT consist of single bands migrating with a mobility of 2.18 x 10⁻¹⁴ cm² sec⁻¹ volt⁻¹. Mobilities in the range of 1.97 and 1.93 were found for dAT[2] and dAT[3] respectively. Whole crab DNA, which had been heated to a temperature which melted the dAT, but not the main component, had a large peak with a mobility of 2.18 and a smaller one of 1.91.

The mobilities indicate that dAT before melting (dAT[0] or dAT[1]) is native, but after melting and cooling (dAT[2], dAT[3] or melted dAT[0]), the dAT molecules have a mobility closer to that of denatured DNA.

This mobility change indicates that the molecules are not completely native after cooling. The mobility change is not, however, as great as that observed for usual (higher GC containing) DNA after heating. These facts point to a partially denatured structure for once melted dAT.

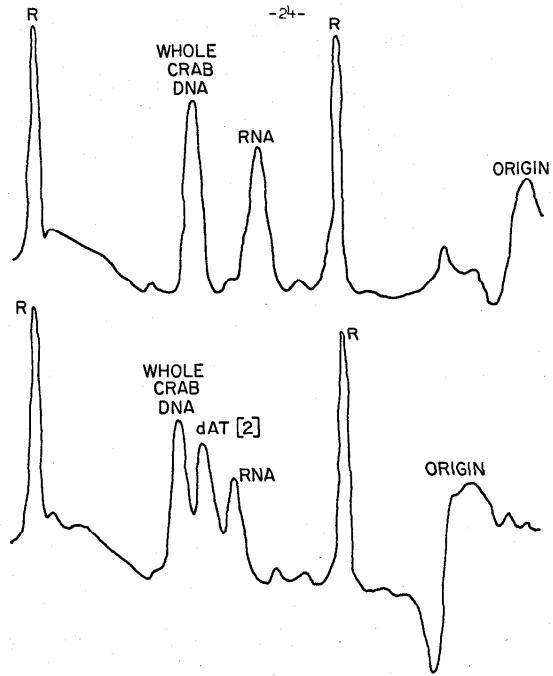


Figure 9. Tracings made during electrophoresis of whole crab DNA and RNA (top) and whole crab DNA, dAT[2] and RNA (bottom). Mobilities of 2.18, 1.99 and 1.75 x 10^{-14} cm² sec⁻¹ volt⁻¹ were calculated for whole crab DNA, dAT[2] and RNA respectively. The very sharp peaks (R) are reference lines and the movement is to the left.

Synthetic dAT is fully hydrogen bonded before and after heating as determined from its mobility.

4. <u>Buoyant Densities of the dAT Forms</u>

The buoyant densities of the various DNA species were determined at least four times in a cesium chloride density gradient at equilibrium and the values are listed in Table 2. The second column lists the values that were obtained after heating and cooling the DNA preparations in SSC.

The <u>C. antennarius</u> dAT species that have never been melted (dAT[0] and dAT[1]) possess a buoyant density of 1.677 g cm⁻³ which increases to 1.680 after heating and cooling. The dAT[2] sample, which was melted before column purification, has a density value of 1.680 both before and after the additional melting. After melting, synthetic dAT exhibits little change while the main component preparations show the usual large increase in density observed with other typical DNA species (14).

Smith (8) observed similar density increases for dAT from <u>C</u>. <u>magister</u> and <u>C</u>. <u>productus</u>, but no increase was noted (1,2) for <u>C</u>. <u>borealis</u> dAT after melting.

Since increased buoyant density in cesium chloride is characteristic of denatured DNA, one can conclude that <u>C</u>.

antennarius dAT after melting is apparently partially denatured. The denaturation is only partial since the increase,

0.003 g cm⁻³, is small compared to the increase of 0.013 g cm⁻³

TABLE 2

DNA Buoyant Densities in Cesium Chloride¹

dAT

				Melted
dAT [O] dAT [1]	1.677 1.677	(dAT	[3])	1.680 1.680
dAT [2] Synthetic dAT	1.680 1.677			1.680 1.678

Main Component DNA

			<u>Melted</u>
In whole crab DNA	[0]	1.701	1.713
Hg isolated	[1]	1.701	1.714
Column isolated	[2]	1.701	1.714

¹Density reference marker, <u>M. lysodeikticus</u> (P = 1.731 g cm⁻³).

noted with typical DNAs in these experiments. The MAK column separated dAT (dAT[2]) possesses this partially denatured character when isolated, since it has been heated and cooled before separation.

5. Electron Microscopic Study of the dAT Forms*

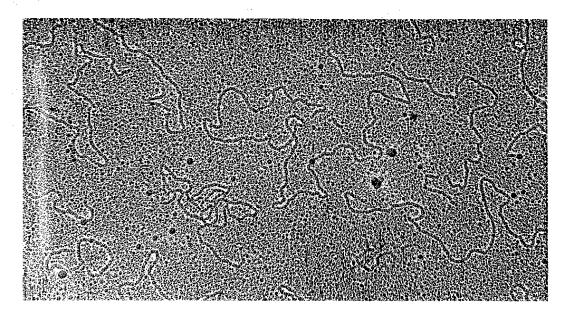
The various DNA species were studied with the electron microscope after preparing the material by the Kleinschmidt technique. Examples of micrographs of the shadowed preparations are shown in Figures 10, 11 and 12.

Whole crab DNA and dAT[1] consist entirely of the usual linear filaments, but the dAT molecules become very highly branched after melting and recooling as shown in Figure 11.

Some branching is also evident for synthetic dAT both before and after melting as shown in Figure 12. Inman and Baldwin (21) showed only linear molecules in their electron micrographs of synthetic dAT, but they now admit that branching is usually present (22).

One can visualize that the branches are formed when the separated strands fold back on themselves during renaturation. Since the crab dAT has essentially the same alternating structure as synthetic dAT, but contains 3.5 per cent GC and also more branching, the GC bases apparently cause the difference in the amount of branching observed. The GC residues

^{*} Very kindly done by Philip Laipis.



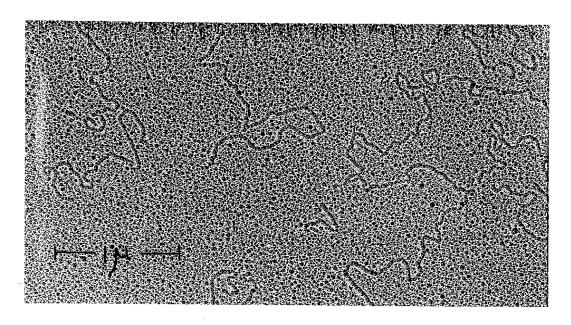
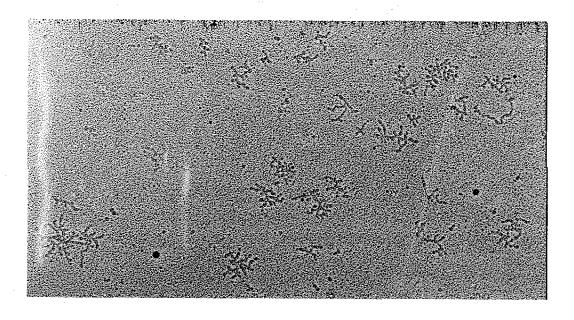


Figure 10. Electron micrographs of whole crab DNA (top) and dAT[1] (bottom) prepared by the Kleinschmidt technique and shadowed. Total magnification, x 32,800.



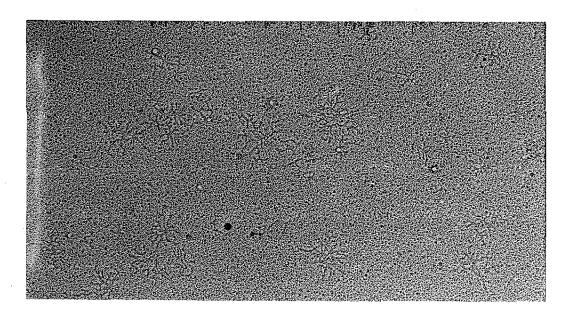
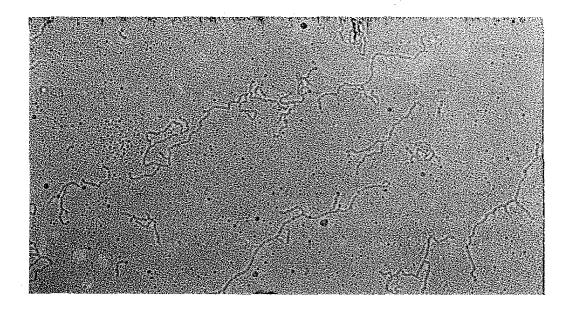


Figure 11. Electron micrographs of dAT[3] (top) and dAT[2] (bottom). Total magnification, x 32,800.



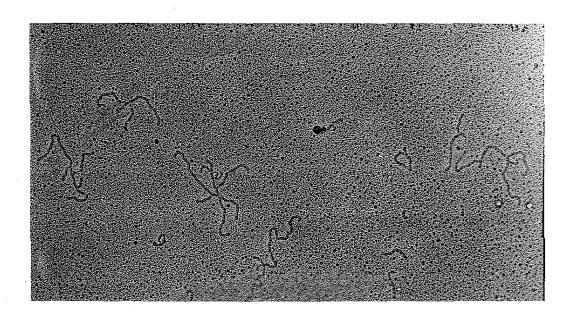


Figure 12. Electron micrographs of synthetic dAT (top) and synthetic dAT after melting (bottom). Total magnification, x 32,800.

probably do not match up and base pair during renaturation.

This conjecture is discussed more thoroughly in a later section.

6. Sedimentation Studies of the dAT Forms

Since sedimentation rates give information concerning the size and shape of macromolecules, a series of experiments were carried out with the crab DNA preparations and synthetic dAT, before and after melting.

Studier (19) has shown that DNA preparations that have been denatured with base and then neutralized possess sedimentation rates in 1 M salt very much higher than when native. He has also devised formulae for molecular weight determination from the sedimentation rates for native DNA (linear and unbranched) $S_{20,W}^{0} = 0.0882 \, M^{0.346}$, neutral denatured DNA (presumably single-stranded after alkaline denaturation) $S_{20,W}^{0} = 0.0105 \, M^{0.549}$, and DNA sedimented as single strands in 0.1 M alkali, $S_{20,W}^{0} = 0.0528 \, M^{0.400}$.

One might expect that since melted dAT apparently does not completely renature and is composed of a branched structure, it would have a sedimentation rate between that expected for native linear structures and that of collapsed denatured single-stranded material.

The analytical band method (18) was used with the sedimentation occurring through 1 M NaCl or 0.9 M NaCl, 0.1 M NaOH. Solvent corrections were applied from Studier (19) and the $S_{20,W}$ values observed were considered as $S_{20,W}^{0}$ values.

Densitometer tracings of a typical experiment are shown in Figures 13 and 14.

Concentrations of dAT[1] from 1-500 µg per ml were melted and fast-cooled and the sedimentation rates were determined using equal amounts of DNA (1.5 µg) in the centrifuge tubes to see if aggregation could be observed. It would seem that if intermolecular hydrogen bonding or entangling occurred at all, it should be more prevalent at high DNA concentrations and nonexistent at very low concentrations.

No systematic differences were observed in the sedimentation rates as is shown in Table 3. Apparently intermolecular aggregation does not occur at these concentrations with \underline{C} . antennarius dAT after heating and cooling.

Table 4 shows the experimental results for the DNA preparations. The molecular weight values listed were calculated from the sedimentation values using Studier's formulae. The results are not of the highest precision, since those for main component DNA[1] and dAT[2] were obtained from only one experiment and the bands are also broad and disappear about half-way down the cell as shown in Figures 13 and 14.

Table 4 shows that mercury separated main component DNA had a native molecular weight of 2.0 x 10 , but apparently

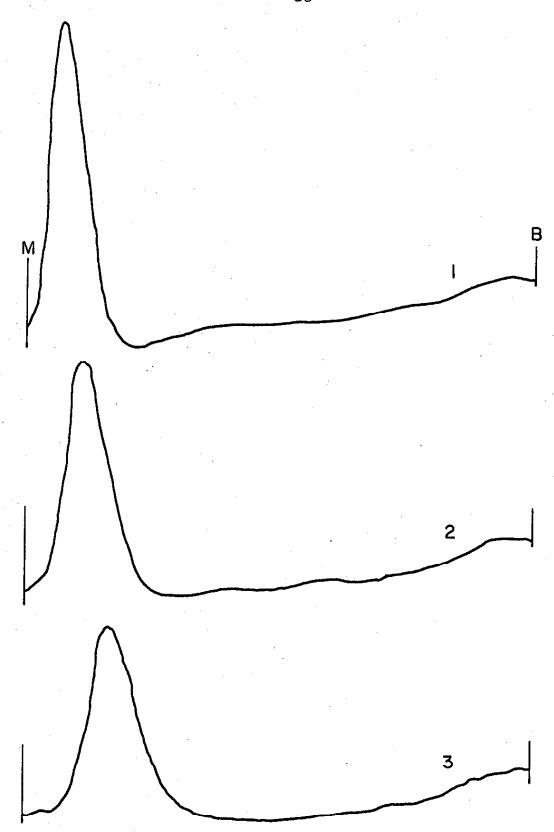


Figure 13. Explanation under Figure 14.

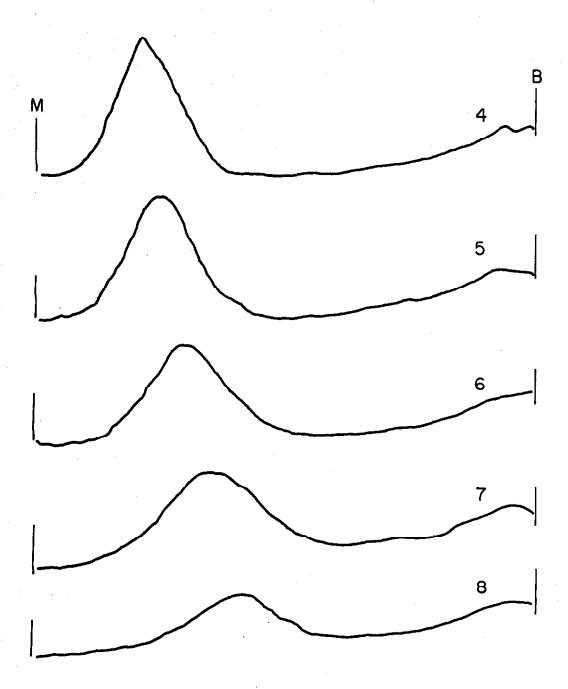


Figure 14. This figure and Figure 13 show densitometer tracings of a typical dAT band sedimentation pattern obtained in 1 M NaCl. About 1.5 µg DNA was sedimented at 29,500 rpm with 8 minutes between each picture, except only 4 minutes between 4 and 5. M=meniscus, B=bottom.

TABLE 3

Sedimentation Rates of dAT Samples Melted and Fast-Cooled in SSC at the Concentrations Listed

Concentration (ид per ml)	^S 20, w
1 25	24.8 26.6
50 100	29.6
100 200	26.2 28.2
500	23.6

contained many single-strand breaks since the alkaline value was 1.3×10^6 . An M of 1.1×10^6 was found for the material after melting, using the formula for denatured DNA, and a value of 1.5×10^6 was determined in alkali. These values after melting are both in agreement with the value obtained before melting, in alkali.

We see that the "true" molecular size is presumably shown by the molecular weight determined from the sedimentation rate of the DNA in 0.1 M NaOH since single-strand breaks and other structural effects are unmasked when the strands are separated and only the primary (covalent) structure of each molecule is maintained.

Synthetic dAT had an original M of 7.9 x 10^6 calculated as native and the value was 4.4 x 10^6 in base. After melting, if the DNA was considered as native, the M was 3.6×10^6 and in base the value was 2.8×10^6 . If the melted synthetic dAT was considered as denatured, the M calculated from the $S_{20,W}^{o}$ would be 6.5×10^5 which is much lower than the alkaline "true" value of 2.8×10^6 .

The M values calculated for this DNA as native both before and after melting are somewhat higher than the alkaline values showing that the material is not exactly like the usual native DNA. This difference is possibly due to the branching which has been observed in electron micrographs of the materials or due to intermolecular aggregation. The original material could possibly have contained single-

strand breaks since the M decrease in alkali is quite profound. Breakage due to melting appears evident since the alkaline M decreases from 4.4 x 10^6 to 2.8 x 10^6 after melting.

The calculated M of dAT[1] which is known to be native was 1.8×10^7 , but decreased to 5.4×10^6 in base, indicating again single-strand breaks. After melting the M value calculated assuming a native configuration was 1.6×10^7 , but the alkaline value was 3.1×10^6 . If, however, the molecular weight is calculated using the formula for denatured DNA, an M of 1.7×10^6 is found for dAT[3] which is much closer to the alkaline value than the value determined assuming a native structure.

The column separated dAT (dAT[2]), if considered as native, had an original M of 1.6 x 10^7 or 1.7 x 10^6 if considered as denatured. The alkaline value of 2.1 x 10^6 is very close to that calculated for denatured DNA in neutral solution. After melting again, M values of 8.6 x 10^6 or 1.1 x 10^6 were calculated assuming native or denatured structures respectively. The alkaline value was 1.9 x 10^6 .

Here as with melted dAT[1] (dAT[3]), the M values calculated, using the conversion factors for denatured DNA instead of native DNA, were much closer to the alkaline values which are assumed to be the actual size values. The alkaline values, to be sure, are between the two other values, but are much closer to the denatured values showing that the melted

TABLE 4

DNA Sedimentation Rates and Molecular Weight Values Before and After Melting

		Ö	Original			Aft	er melti	1g	
Sample	w	nativel M	alkaline alkaline S	alkaline M	melted S	melted denatured ² native ³ alkaline alkaline S S M	native ³ S	alkaline S	alkaline M
Main Component	29.7	29.7 2x10 ⁷	15.0	1.3×10 ⁶	21.6	1.1x106	9.5×10 ⁶	15.7	1.5x10 ⁶
dAT [1]	28.6	28.6 1.8x10 ⁷ 26.	26.1	5.4x10 ⁶	27.6	1.7x10 ⁶	1.6x10 ⁷	20.8	3.1x10 ⁶ .
dAT [2]	27.6	27.6 1.6x10 ^{7*} 17.7	* 17.7	2.1x10 ⁶	22.1	1.1x10 ⁶	8.6x10 ⁶	17.3	1.9×10 ⁶ 0
Synthetic dAT	21.5	21.5 7.9x10 ⁶ 24.	24.1	4.4x10	16.3	6.5x10 ⁵	3.6x10 ⁶	20.1	2,8x10 ⁶

1 Calculated from original S using formula for native DNA.

² Calculated from S after melting, using formula for denatured DNA.

³ Calculated from S after melting, using formula for native DNA.

^{*} If calculated as denatured = 1.7x_{10}^{6}

dAT was neither completely native nor completely denatured, but partially denatured, as has been indicated by numerous other studies presented in this thesis. The electron micrographs particularly show a highly branched structure for dAT[2] and dAT[3].

The data also indicate that most of the DNA preparations contain single-strand breaks which are detected by sedimenting in alkali. With enzymatically synthesized dAT and dAT[1] the alkaline S values are lower after melting, indicating that this treatment caused some breakage.

7. Susceptibility of dAT to Exonuclease I

Our general interpretation of the differences observed between melted and native crab dAT is that most of the alternating AT regions renature, but that the "random" G and C bases do not pair and in this way cause the kinks observed directly by the electron microscope and indirectly by the other methods. If indeed the GC base pairs are not reformed, we should observe a difference in the susceptibility of the two dAT forms to <u>E</u>. <u>coli</u> exonuclease I.

This enzyme attacks DNA from the 3'-hydroxyl end and liberates successively deoxyribonucleotide 5'-monophosphates, but leaves the terminal dinucleotide intact (20). It attacks single-stranded DNA at a very high rate. It attacks synthetic dAT 10 to 200 times more slowly and native typical DNA 40,000 times more slowly than it does heat-denatured DNA (23).

The polymer dGdC is essentially not susceptible to the enzyme (24).

These findings indicate that the enzyme hydrolyzes single-stranded DNA with ease, is slowed somewhat by AT base pairs, but is more effectively slowed by GC base pairs.

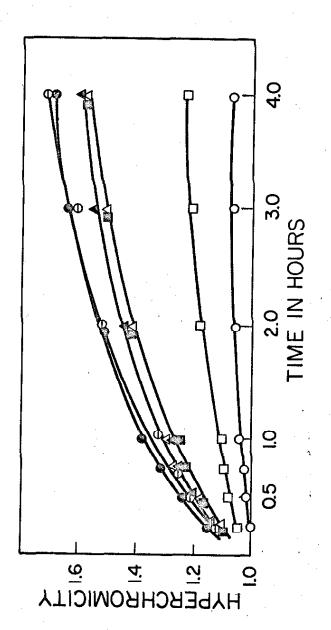
Thus, if the GC regions in dAT are largely unpaired after heating and cooling, the enzyme should hydrolyze this form (dAT[2] and dAT[3]) faster than it does the native form (dAT[1]) and at a rate similar to that at which it attacks synthetic dAT.

Experiments to determine if these expectations are realized were carried out and the results are shown in Figure 15. Complete hydrolysis of synthetic dAT would result in about 80 per cent hyperchromicity (21) and this was attained after about 8 hours of attack by exonuclease I under the conditions employed.

Figure 15 shows that the mercury separated main component DNA was hydrolyzed very slowly and the native crab dAT (dAT[1]) was hydrolyzed at a rate about 40 per cent of that found for the same DNA after melting (dAT[3]). The rate for dAT[3] is similar to that for synthetic dAT and dAT[2].

Clearly the hydrolysis rate increases after melting and becomes comparable with that for synthetic dAT which has no GC base pairs.

The studies were not carried out with rate limiting concentrations of enzyme so that it is possible that melting,



 \blacksquare , dAT[3]; Θ , remelted dAT[2]; \blacksquare , dAT[2]; \triangle , synthetic by the increase in optical density at 260 mm. \square , dAT[1] Each 0.6 ml incubation mixture contained 40 enzyme units dAT; A, melted synthetic dAT; O, main component DNA[1] Figure 15. Hydrolysis of DNA by exonuclease I measured and about 35 mpm DNA.

which can cause some DNA strand breakage, would give the enzyme more strand ends to attack and in this way result in the observed increase in the hydrolysis rate after melting. Indeed, sedimentation studies of the materials in 0.1 M base indicate that breakage does occur during heating, but rate of hydrolysis increases after heating only in the case of dAT[1]. This indicates that the primary cause of the increased hydrolysis rate is the change in secondary structure which results from heating and cooling.

8. Properties of Cancer borealis dAT

A sample of <u>C</u>. <u>borealis</u> DNA was obtained from T. Y. Cheng and the two components were separated by the mercury binding method. The two components separated elegantly and were recovered in pure form.

The melting behavior of <u>C</u>. <u>borealis</u> dAT is similar to that of <u>C</u>. <u>antennarius</u> dAT; the material melts sharply when melted for the first time, and at a temperature higher than the cooling and remelting temperatures. The original Tm in SSC was found to be 65.7°C with the σ T value of 1.3°C. The cooling Tm was found to be 62.8°C and the subsequent Tm value was 62.8°C with a σ T value of 4.0°C. The absorbance returned very nearly to the value before melting.

The buoyant density in cesium chloride of the native isolated dAT was 1.680, but increased to 1.682 after melting. Sueoka and Sueoka and Cheng (1,2) give values of 1.681 both

for the native and the melted material.

Inhibition of RNA synthesis by actinomycin D is a function of the template DNA guanine content (5). Inhibition is less with single-stranded primers also. As is shown later in this thesis, actinomycin D inhibits DNA-dependent RNA synthesis by <u>E. coli</u> RNA polymerase to a lesser extent with primer DNA that has been melted. This phenomenon holds true for <u>C. antennarius</u> dAT as well as for main component DNA.

<u>C. borealis</u> dAT was tested and, indeed, the RNA synthesis with unmelted dAT as primer was inhibited more strongly by actinomycin D than was RNA synthesis with dAT[3] as primer as shown in Figure 23. The differences between the melted and native dAT inhibition are not as large, however, as with <u>C. antennarius</u> dAT.

Incorporation studies using C¹⁴-nucleoside triphosphates, <u>E. coli</u> RNA polymerase and <u>C. borealis</u> dAT as template showed the GC content to be 2.5 per cent. Similar studies indicated that the dAT component from <u>C. antennarius</u> contained 3.5 per cent GC. This GC content difference may explain the quantitative differences noted in the behavior of the dATs obtained from the two species before and after melting. Since the <u>C. antennarius</u> 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 <u>C.</u> borealis dAT.

Indeed, we note that the differences observed are larger for <u>C</u>. <u>antennarius</u> than for <u>C</u>. <u>borealis</u> in all cases: 5°C versus 2.5°C for the differences in Tm 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 studies.

Other facts also indicate that <u>C</u>. <u>antennarius</u> dAT has a higher GC content. Its original Tm is higher, 67.7 versus 66.2°C, and the RNA synthesis primed either by dAT[1] or dAT[3] is inhibited to a greater extent by actinomycin D than is that by the comparable <u>C</u>. <u>borealis</u> dAT forms. The buoyant densities of the two are, however, the reverse and would indicate that <u>C</u>. <u>borealis</u> dAT has the higher GC content.

It is evident that <u>C. borealis</u> dAT, like <u>C. antennarius</u> 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, possibly because this dAT has a higher GC content.

C. Discussion of the Physical Study Results

Before the mercury binding method was developed, the isolation of pure dAT from the DNA of <u>Cancer</u> crabs could be accomplished in quantity only by first melting the DNA and then applying the MAK column separation method. The dAT isolated in this way has been found to be not identical to the same material before melting.

The mercury separation technique, however, has allowed the isolation of dAT that has characteristics identical to those of the dAT present in the whole crab DNA. The work presented here has shown that once the dAT is heated to a temperature which causes strand separation and then cooled, the resulting structure is not identical to that present originally. This alteration in structure is probably caused by the incomplete reformation of the original hydrogen bonds.

When the optical density melting profile is observed the absorbancy increase occurs in a very narrow temperature range when the dAT[1] is heated for the first time. After cooling, the material melts at a lower temperature and over a much broader range; σ T of 0.5°C and Tm 67.7°C originally and subsequently 3.5°C and 62.5°C respectively. The column isolated dAT (dAT[2]) has melting characteristics identical to those of melted dAT[1] (dAT[3]). Synthetic dAT has completely reversible melting characteristics.

The dAT[1] sample retains a portion of the A₂₆₀ gained during the strand separation even after cooling. This also indicates that the strands do not come back to their original form. This measure shows that the material still possesses 7 per cent of the total hyperchromicity realized during melting which indicates that 7 per cent of the original base pairs have not reformed. Synthetic dAT and dAT[2] do return completely to their original optical density.

Electrophoresis appears to be a very sensitive method for detecting denatured structure since the dAT after melting is only partially denatured, but its mobility is changed from the native value of 2.18 x 10⁻¹ to 1.93-1.97 x 10⁻¹ for dAT[3] and dAT[2]. The mobility of completely denatured DNA is 1.87 x 10⁻¹. Thus melted dAT possesses a mobility closer to that of denatured DNA than to that of native. Clearly, the denatured dAT remains in a partially denatured state. Synthetic dAT, on the other hand both before and after melting, travels at the rate of native DNA.

The quantitative significance of this finding could be important to electrophoretic theory, but is not well understood at present.

Buoyant density determinations show also that dAT[1] is identical to dAT[0] and that dAT[2] is identical to dAT[3]. The former two have buoyant densities of 1.677 and the latter two 1.680. If dAT[2] is remelted the density remains the same. Synthetic dAT shows little change upon melting.

The buoyant density increase observed with main component DNA after heating was 0.013 compared with 0.003 for dAT, again indicating a partially denatured condition for dAT[2] and dAT[3].

Electron micrographs reveal clearly that the linear structure of dAT[0] and dAT[1] is changed drastically by melting and that dAT[2] and dAT[3] consist of highly branched structures. Synthetic dAT is slightly branched both before and after heat treatment.

A highly branched structure is also indicated by sedimentation studies of the dAT forms. A compacted shape would be expected to confer upon the molecule a sedimentation rate higher than that expected for a native DNA molecule of the same size. The measured values are higher than those expected for native molecules and lower than those expected for totally denatured molecules. Again these characteristics are those of a partially denatured structure. The synthetic dAT sedimentation rate is close to that expected for a native form.

The sedimentation studies indicate that intermolecular aggregation does not occur upon cooling of <u>C</u>. <u>antennarius</u> dAT in SSC when the concentrations are 1-500 µg per ml. Sedimentation in 0.1 M alkali revealed that heating does cause some strand breakage.

The experimental results discussed thus far, all indicate that dAT[1] is native linear DNA and that after strand

separation, induced by heating and the subsequent renaturing by cooling, the DNA structure is highly branched and not completely base paired. Synthetic dAT which has a similar base sequence, but without any GC content, appears to base pair almost completely after heating and cooling and is only slightly branched.

It is possible that this dAT is originally in a partially denatured state, since it was formed enzymatically, but the electrophoresis data which indicate a native structure both before and after melting and the sedimentation study which also points to a near native structure for this dAT, rule out this possibility.

It seems probable that the behavioral differences are due to the fact that <u>C</u>. <u>antennarius</u> dAT contains 3.5 per cent GC while synthetic dAT contains none. The guanine and cytidine residues are evidently unable to base pair when the strands fold back on themselves when renaturing. The high proportion of alternating AT structure would fit perfectly until a G or C residue occurs. Since the chances are slight that the G and C residues will match up under these conditions, a non-hydrogen bonding region would occur which would cause a kink and the observed branched structure.

It is possible that the alternating AT sequence is not perfect, but the nearest neighbor studies on <u>C</u>. <u>borealis</u> dAT (4) and studies showing that there are no T-T sequences in <u>C</u>. <u>pagurus</u> dAT (9) indicate that the alternating sequence is

quite perfect.

Two experiments suggest that the GC base pairs are not largely reformed after strand separation.

The first is concerned with the susceptibility of the various DNA species to <u>E. coli</u> exonuclease I, an enzyme whose action is apparently slowed down most effectively by GC base pairs when hydrolyzing DNA. Thus dAT[2] and dAT[3], which hypothetically contain no GC base pairs, should be as susceptible to hydrolysis by the enzyme as synthetic dAT. dAT[1], however, which contains GC base pairs, should be hydrolyzed more slowly than the other DNA species mentioned.

The results fit the hypothesis perfectly; dAT[1] is hydrolyzed more slowly than the other dAT forms utilized in the experiment.

The other line of evidence that supports the aforementioned GC non-pairing concept is reported in the <u>C</u>.

<u>borealis</u> section and also later in this thesis for <u>C</u>.

<u>antennarius</u> dAT. It concerns the actinomycin D inhibition of RNA synthesis primed by native and melted DNA.

It is known that actinomycin D does not bind as strongly to single-stranded DNA guanine residues (5,25) and this weaker binding probably causes the reduced inhibition by actinomycin noted for RNA synthesis with single-stranded primer DNA in <u>in vitro</u> RNA synthesizing systems.

Experimental results reported here show that actinomycin D inhibits RNA synthesis by E. coli RNA polymerase much less

when melted DNA is used as primer than when the native material is used. This is true for <u>C</u>. <u>antennarius</u> main component DNA and dAT as well as <u>C</u>. <u>borealis</u> dAT.

These results indicate that actinomycin D does not bind as well to the melted DNA species, probably because most of the GC base pairs have not reformed upon cooling.

The <u>C. borealis</u> dAT does not exhibit differences, between the native and partially denatured forms measured with buoyant density, optical melting and actinomycin inhibiting techniques, as profound as does the <u>C. antennarius</u> dAT.

This is probably because <u>C. borealis</u> dAT contains 2.5 per cent GC compared to 3.5 per cent for <u>C. antennarius</u> dAT.

Pochon, Massoulie and Michelson (9) using the MAK column separation procedure with unmelted <u>C</u>. <u>pagurus</u> DNA have been able to isolate native dAT about 90 per cent pure. They reached the same conclusion by showing a difference between the native form and the form obtained after strand separation using optical density melting and optical density acid-base titration techniques. "La fraction 'poly-d-AT' du DNA de Crabe comprend plusieurs especes differentes. Ces molecules se presentent sous a forme I, native, et une forme II, obtenue apres dissociation et reformation de la structure secondaire" (9).

Apparently, however, all the previous work carried out by other workers with purified crab dAT (4,5,6) has been done with MAK column separated <u>C. borealis</u> dAT. The partially

denatured character of this material should be considered when evaluating the experimental results.

It is evident, also, that the mercury binding technique is effective for isolating native crab dAT and will probably find many other uses in the future.

II. BIOLOGICAL STUDIES OF CRAB dAT

A. Methods Employed for Biological Studies

DNA was isolated from <u>C. antennarius</u> tissue other than sperm and spermatheca by the method of Smith (8) except that all the steps were done in the cold and the nucleic acids were precipitated with two volumes of cold ethanol after deproteinization. The ethanol precipitate was collected by centrifugation. The material, which was contaminated with RNA, was then analyzed in the analytical ultracentrifuge in cesium chloride.

Purified nuclei were isolated from hepatopancreas and claw muscle by the procedure outlined by Maggio, Siekevitz and Palade (26). However, the material was filtered through both cheesecloth and miracloth after grinding.

Aliquots of each fraction obtained during the preparation of purified claw muscle nuclei were analyzed chemically for DNA, RNA and protein after washing, essentially by the method of Ts'o and Sato (27).

Chemical analyses for DNA, RNA and protein were done by the methods of Burton (28), Dische and Schwarz (29) and Lowry, Rosebrough, Farr and Randall (30), respectively. Calf thymus DNA (Sigma Chemical Co.), yeast RNA and bovine serum albumin (Nutritional Biochemicals Corp.) were used as the respective

standards.

Nuclear fractions were obtained from heart, claw muscle, hepatopancreas and unfertilized eggs either by grinding in the medium used for purified nuclei (0.88 M sucrose, 0.0015 M CaCl₂) or in 0.25 M sucrose, 0.0018 M CaCl₂ (31). Each was centrifuged for the times and at the speeds specified in the text.

DNA was obtained from these nuclear preparations for analysis in cesium chloride density gradients by suspending the nuclear pellets in saline-EDTA containing 1.5 per cent SLS followed by the deproteinization procedure for the DNA isolation from the tissues. Hepatopancreas purified nuclei DNA was banded after direct application of the nuclei to cesium chloride solutions.

Chromatin was prepared from 12 g of freshly dissected hepatopancreas tissue using a modification of the procedures of Raaf (32) and Marushige and Bonner (33). The tissue was homogenized for one minute at 85 volts and two minutes at 45 volts in a blendor in 110 ml of a solution containing 20% sucrose, 0.02 M EDTA and 0.05 M tris, pH 8. This was filtered through 4 layers of cheesecloth and one layer of miracloth and then centrifuged for 2.25 hours at 25,000 rpm in a SW-25 rotor in a Spinco Model L Ultracentrifuge. After pouring off the supernatant, the gelatinous material was scraped from the resulting pellets and homogenized with 7 ml of SSC in a teflonglass homogenizer and stirred for 20 min. with a magnetic stirrer,

all in the cold. A second centrifugation was carried out as before with half of the material after partially mixing it with some fresh sucrose solution. Both preparations were dialyzed against 500 ml of SSC for 5 hours with one change. The chemical composition (DNA, RNA and protein) and priming ability for RNA synthesis were then determined.

The enzyme RNA polymerase was isolated from E. coli by the method of Chamberlin and Berg (34) through fraction 4. RNA synthesis was carried out by essentially their procedure except that the reaction was stopped by pipetting 0.1 ml of the 0.25 ml reaction mixture onto a filter (Bac-T-Flex membrane filter for aqueous solutions type B-6, Carl Schleicher and Schuell and Co., Keene, N. H.) and washed three times with about 6 ml of cold 10% trichloroacetic acid (TCA). Any changes in the reaction conditions are listed in the text.

The base composition of the RNA synthesized with the RNA polymerase system was determined with 4 simultaneous reaction mixtures each containing a different C¹⁴-nucleoside triphosphate with the other three unlabeled.

The specific activities of the nucleoside triphosphates were calculated from radioactivity measurements and the ultraviolet absorption spectra of the stock solutions. The molar extinction coefficients and spectral characteristics were obtained from Pabst Laboratories circular OR-10 (35).

Actinomycin D was a gift of Merck, Sharp and Dohme.
RNA was synthesized for sucrose density gradient

sedimentation analysis as described before, but it was deproteinized after incubation by shaking with an equal volume of chloroform-isoamyl alcohol (24:1). The sample was then centrifuged to separate the phases and the top aqueous layer was recovered and layered on a 29 ml 5-20% sucrose gradient in 0.1 M acetate pH 5.5 and centrifuged for 12 hours at 25,000 rpm in a SW-25 head at 4.C. Fractions of about one ml were collected and an aliquot of each was washed with TCA as described above to remove the sucrose and non-acid precipitable radioactivity.

Each centrifuge tube contained P³² labeled 18 or 28 S HeLa marker RNA (a generous gift of Professor G. Attardi).

The acid precipitated material on each filter was counted with a thin end window gas flow counter and then recounted after placing 3 layers of aluminum foil over the filter. The aluminum foil allows only 1.5 per cent of the C^{14} radiation to be counted, but permits 88.3 per cent of the P^{32} radiation to be detected. The counts obtained during the second counting were then multiplied by 1.133 to correct for the slight absorption to yield the P^{32} marker RNA sedimentation pattern. The P^{32} counts were then substracted from those obtained during the first counting to yield the counts due to the newly synthesized C^{14} RNA.

Reconstituted nucleoprotein was prepared by the method of Huang, Bonner and Murray (36).

Large preparations of RNA were synthesized from dAT[1]

with C^{14} labeled nucleoside triphosphates. The 5 ml reaction mixture contained 140 mp moles dAT[1], 150 units RNA polymerase, 10 μ moles each NTP and the rest of the reaction mixture of Chamberlin and Berg (34). After incubation for 2.5 hours, 0.4 ml of 2.5 per cent SLS, and 0.08 M MgCl, were added. mixture was shaken with an equal volume of water saturated phenol and centrifuged to break the emulsion. The upper aqueous layer was removed and the RNA precipitated from it with 2 volumes of cold ethanol. The precipitate was dissolved in SSC and made up to 3 ml containing cesium chloride with a final density of 1.70. This was centrifuged in an SW-39 head for 72 hours at 25°C at 35,000 rpm. Fractions were collected and the radioactivity and A₂₆₀ patterns determined. fractions containing the Cl4-rAU were dialyzed against frequent changes of 0.5 M sodium citrate, 0.01 M EDTA for 48 hours and then against SSC. This procedure yielded about 650 mµ moles of crab synthetic rAU.

Ribonuclease T_1 (from Calbiochem) treatment was carried out by adding 10 and 50 enzyme units per RNA 0.D. unit to the RNA solution in SSC and incubating for 30 minutes at 37°C. The incubation mixtures were then heated in a boiling water bath for 10 minutes and then cooled. One enzyme unit is defined by Takahashi (37) as the quantity of ribonuclease T_1 that will render 1 0.D. unit of RNA acid soluble in 15 minutes.

The ethanol precipitation was carried out by adding two

volumes of cold ethanol to the ribonuclease T_1 treated RNA and allowing the mixture to sit overnight in ice. The precipitate was pelleted by a 5 minute centrifugation in a clinical centrifuge. The A_{260} or radioactivity values of the supernatant were determined and compared with the respective values originally present.

The isolated rAU was analyzed with a 4.3 ml 5-20 per cent sucrose density gradient in 0.1 M acetate buffer pH 5.5. A sample of 0.2 ml was layered on top of the gradient and 0.5 ml of mineral oil was layered upon this. The tubes were centrifuged in an SW-39 head at 35,000 rpm for 5 or 24 hours at about 4°C. Fractions were collected from a hole in the bottom of the tube and the A₂₆₀ and C¹⁴ content of each determined.

Pea ribosomal RNA was isolated by the method of Scherrer and Darnell (38) with cold phenol from the pea embryo cytoplasmic supernatant resulting from a 15 minute centrifugation of the tissue homogenate at $30,000 \times g$.

C. antennarius hepatopancreas RNA was also isolated by the phenol extraction method or by banding ground material in cesium chloride.

Torula RNA was purchased from Calbiochem.

Incorporation experiments were carried out with \underline{C} . antennarius hepatopancreas tissue (fresh weight 0.5 g) which was incubated in 5 ml of a sterile salt solution (39) contained in 25 ml erlenmeyer flasks on a water-bath shaker at

Addition of actinomycin D was made 1 hour before the C^{14} precursors were added. The solution contained 800 units of penicillin per ml and the incubation time was 3 hours. After the incubation the tissue and incubation solution were ground in a teflon-glass homogenizer with 5 ml cold 20% TCA. The precipitate was sedimented by centrifuging for 5 minutes in a clinical centrifuge. The acid insoluble material was then washed 5 times with cold 10% TCA and once with cold 100% ethanol. The material remaining was taken up in 1 ml 1 M KOH and an aliquot was plated on a glass planchet and counted to determine total incorporation. The remainder was incubated for 16-20 hours at 37°C and then the solution neutralized with concentrated HCl. An equal volume of cold 10% perchloric acid was added and after sitting on ice the precipitate was centrifuged down. An aliquot of the supernatant was counted to determine the incorporation into RNA.

B. Results of Biological Studies

1. Occurrence of dAT in C. anthonyi DNA

DNA has now been studied in 7 <u>Cancer</u> species and the proportion of dAT determined for 6 (1,8,9). The French workers Pochon, Massoulie and Michelson (9) have used <u>C</u>. <u>pagurus</u> partially purified dAT for chemical and physical studies, but they did not report the dAT proportion in the whole crab DNA. Table 5 summarizes the proportion of dAT determined from optical density melting profiles and from buoyant density experiments with the DNA for each of the species.

The species, <u>Cancer anthonyi</u>, which is found in great numbers in the Southern California coastal waters, is described by Light <u>et al</u>. (41) and Schmitt (42).

When the studies reported in this dissertation were first initiated, <u>C. anthonyi</u>, being the most easily obtained <u>Cancer</u> species in this area, was used for the preliminary experiments. As reported in Table 5, however, the DNA from this species contains only 10 to 11 per cent dAT which makes it less valuable as a source of dAT than <u>C. antennarius</u> DNA which contains 23 to 25 per cent dAT.

DNA was isolated from \underline{C} . anthonyi sperm and the material was banded in a cesium chloride density gradient. Two bands

TABLE 5 DNA from <u>Cancer</u> Species

		Tm(°C i	in SSC)	\mathbb{Z}^1	Density ²		2 3
Cancer	antennarius ⁴ 5	65.8 67.7	84.0 85.6	30 25	1.677 1.677	1.700 1.701	26 23
Cancer	anthonyi ⁵	68.5	86.0	11	1.679	1.701	10
Cancer	borealis ⁶	64.2 65.7	85.0 85.5	30 32	1.681 1.680	1.702 1.702	30 34
Cancer	gracilis ⁴	65.6	84.0	12	1.680	1.700	9
Cancer	<u>irroratus</u> 6				1.680	1.700	10
Cancer	magister ¹ +	65.8	84.6	12	1.677	1.701	14
Cancer	productus	66.4	83.2	31	1.679	1.701	32

Percentage dAT based on hyperchromicity of low melting 1. material.

Relative to E. coli, 1.710; P. aeuruginosa-N¹⁵, 1.742; or M. lysodeikticus, 1.731; (14 and 40). These reference densities are all based on the E. coli density of 1.710. Percentage dAT calculated from microdensitometer tracings. 2.

Reported by Smith (8).

Reported in this dissertation.

Reported by Sueoka (1) and Sueoka and Cheng (2).

appeared with densities of 1.679 and 1.701. The band at 1.679, which is apparently dAT, makes up 10 per cent of the total DNA.

The optical density melting profile of the DNA in SSC shows two-step melting with 11 per cent of the DNA melting at 68.5°C. The Tm of the main component DNA was 86.0°C. The total hyperchromicity is 36 per cent.

Thus, another <u>Cancer</u> species, <u>C</u>. <u>anthonyi</u>, is shown to contain dAT. The dAT proportion, in this case, is 10 to 11 per cent of the total DNA present.

All Cancer species examined so far contain dAT.

2. <u>Distribution of dAT in the Tissues and Localization Within the Cell</u>

The localization of the dAT component within the cell and the distribution in the tissues is important to any interpretation of the biological function of such DNA. The questions to be answered are: a, is this peculiar DNA found in all tissues; and b, in which organelle of the cell is it localized?

DNA was isolated from various tissues, banded in cesium chloride, and the two components quantitated from microdensitometer tracings made from photographs taken of the material at equilibrium. Representative tracings are shown in Figures 16 and 17. The DNA samples were usually contaminated with RNA which moved to the bottom of the centrifuge cell and did

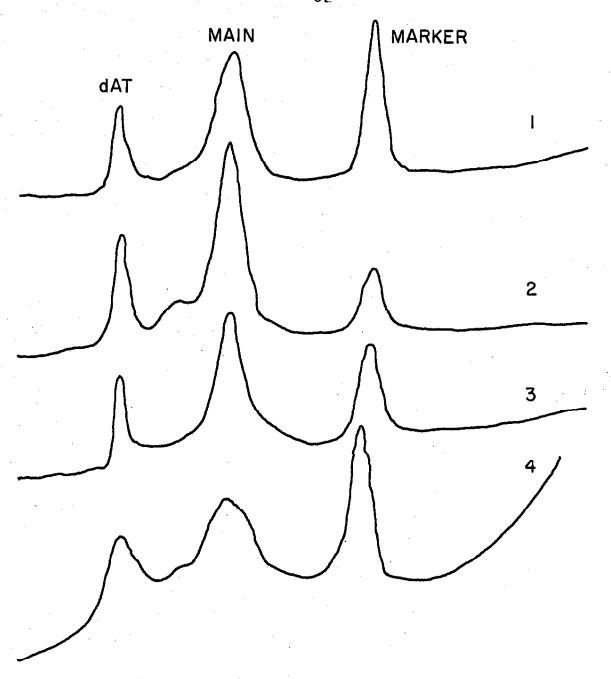


Figure 16. Densitometer tracings of DNA from \underline{C} . antennarius sperm, 1; spermatheca, 2; lysed spermatheca, 3; and hepatopancreas, 4; banded in CsCl. The marker is \underline{M} . lysodeikticus DNA (P = 1.731).

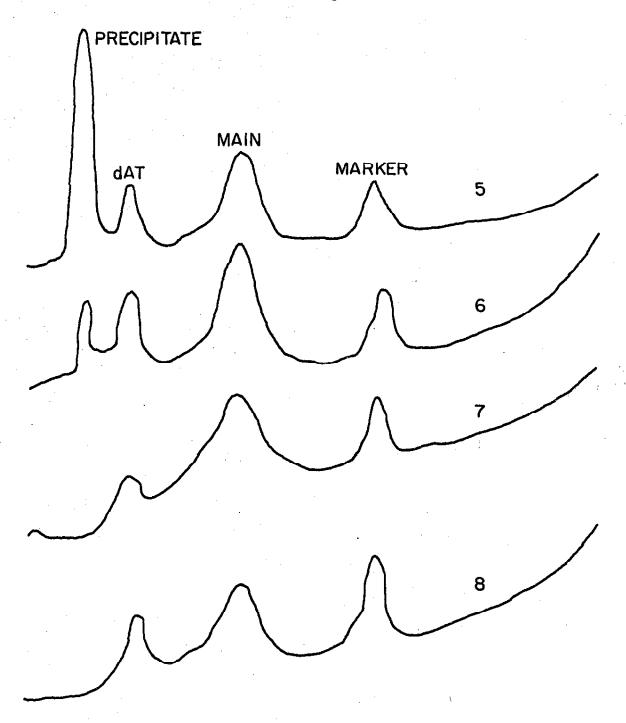


Figure 17. Densitometer tracings of DNA from <u>C</u>. <u>antennarius</u> heart, 5; muscle, 6; embryos, 7; and unfertilized eggs, 8; banded in CsCl. The precipitate bands form within about 4 hours after centrifugation begins.

not interfere with the quantitation. The RNA appears at the cell bottom in the tracings.

We see from Table 6 that all of the seven tissues examined contain about 25 per cent dAT. The variability may be significant since usually two or more individual preparations were analyzed from each tissue.

A precipitate band forms at a density of about 1.6661.675 in most experiments with nucleic acid preparations from heart and claw muscle as well as with hepatopancreas and sometimes with spermatheca tissue when lysed directly in the cesium chloride solution and than banded. This material was determined to be a precipitate because it forms a band within about four hours after the start of centrifugation while the usual DNA bands are visible only after about ten hours.

A sample of heart DNA was found to be free of the precipitate band. This was isolated by the Cheng and Sueoka DNA method (6) which includes phenol extraction and elution from a MAK column. The nature of the precipitate is unknown, although a similar band has been observed recently in directly lysed cells from Aplysia. Apparently completely purified DNA does not contain this material.

Purified nuclei were isolated from the hepatopancreas and claw muscle and enriched nuclear fractions were prepared from these two tissues as well as from heart and unfertilized eggs. The purified nuclei were centrifuged through 2.2 M sucrose which should exclude organelles such as mitochondria.

All of the pellets contained no intact cells and appeared to consist of broken nuclei when examined with a light microscope.

The amounts of DNA, RNA and protein found in the various fractions obtained during the preparation of purified claw muscle nuclei are listed in Table 7. The data show that only 5 per cent of the total DNA in the tissue is recovered in the nuclear pellet. Such DNA is, however, purified markedly: the DNA:RNA:protein ratio goes from 1:6.3:199 in the tissue to 1:3.1:93 in the pellet. It would appear that in this case, at least, we are dealing with purified nuclei or nuclear fragments rather than whole cells.

DNA was prepared from the nuclear pellets and this was analyzed by banding in cesium chloride. Purified hepatopancreas nuclei were placed directly in the cesium chloride solution and analyzed. Table 8 shows the results obtained as well as the specific methods employed. All the tissues and methods give at least 25 per cent dAT in the DNA isolated, indicating that the dAT is contained in the nucleus.

The 40 per cent dAT proportion found for the hepatopancreas nuclei DNA is significantly higher than the usual 25 per cent. No explanation for this high value can be offered, however.

Chromatin was isolated from hepatopancreas tissue and the proportion of dAT was determined by applying the chromatin directly to cesium chloride solution. The once sedimented

TABLE 6

dAT Proportion in DNA Isolated from Crab Tissues

	•	Density ²		
Tissue	dAT(%)	dAT	Main Component	
Heart Unfertilized Eggs Hepatopancreas Claw Muscle Fertilized Eggs Sperm Spermatheca Lysed Spermatheca	26 32 25 27 19 23 23 22	1.677 1.681 1.678 1.677 1.681 1.677 1.677	1.701 1.702 1.701 1.701 1.702 1.701 1.701	

¹Estimated from densitometer tracings of DNA in cesium chloride density gradient.

DNA, RNA and Protein Analyses of Fractions Obtained

During Purified Muscle Nuclei Isolation

	Total µg/fraction1			Ratio				
	DNA	RNA	Protein	DNA	:	RNA	:	Protein
Tissue Homogenate Filtrate Sucrose Supernatant Nuclear Pellet	1037 335 308 49	6510 1029 546 150	206,000 33,000 36,000 4,580	1	:	6.3 3.1 1.8 3.1	:	

¹The values were determined chemically.

²Based on <u>M. lysodeikticus</u> DNA, ρ = 1.731.

dAT Proportion in the DNA from Purified Nuclei and Enriched Nuclear Fractions from Several Crab Tissues

Nuclear Fractions	Centrifugation	Grinding Medium Minutes rpm dAT(%)	0.25 M sucrose 120 16,500 25.5 0.0018 M CaCl ₂	0.25 M sucrose 120 16,500 39 4 0.0018 M CaCl ₂	0.88 M sucrose 20 10,000 26.5 0.0015 M CaCl ₂	0.25 M sucrose 20 2,250 27.5 0.0018 M CaCl ₂
Purified Nuclei		Grinding Medium dAT(%)	0.88 M sucrose 25 0.25 0.0015 M CaCl ₂ 0.0018	0.88 M sucrose 41 0.0015 M CaCl ₂	0.88	ඩිදුලි s
		Tissue	Muscle	Hepatopancreas	Heart	Unfertilized

Each tissue was ground with a teflon-glass homogenizer in the medium listed and filtered through miracloth and cheesecloth. Purified nuclei were centrifuged through 2.2 M sucrose, 0.0005 M CaCl₂, by centrifuging at 44 ,000 x g for 90 minutes. The enriched nuclear fractions were centrifuged in a Servall centrifuge in the grinding medium as described in the table. DNA was isolated as described in the text. chromatin contained 29 per cent dAT banding at a density of 1.678 with the main component at 1.701 and the twice purified chromatin contained 30 per cent dAT banding at a density of 1.679 with the main component at 1.702.

The purified chromatin DNA has been purified markedly as seen from the DNA:RNA:protein ratio of 1:0.9:15 and the yield of DNA based on the amount present originally in the tissue was 35 per cent.

The dAT component appears to be present to the extent of about 25 per cent in every tissue studied in this crab species. Cheng and Sueoka (6) found that the hepatopancreas, unfertilized eggs and claw muscle of <u>C. borealis</u> contained dAT in proportions similar to that found for the testes DNA from the same species.

The dAT component in <u>C</u>. <u>antennarius</u> is associated with the nucleus since all the subcellular purification procedures which should at least select for nuclei give the dAT percentages similar to those found for the tissue itself (or higher). Mitochondrial DNA is ruled out because these organelles do not pellet through 2.2 M sucrose.

The origin of the two intermediate DNA species (with intermediate melting and buoyant density values) is unknown. These DNA components together make up less than 3 per cent of the total DNA. These two species, however, appear to be absent from the DNA of the isolated nuclei.

An attempt to isolate mitochondria and to isolate DNA

from them was unsuccessful so that the hypothesis that these DNA species might be of mitochondrial origin cannot be evaluated.

Further experiments should be carried out with DNA isolated from well fractionated subcellular organelles to determine unequivocally the origin of all of the DNA species found in the <u>C. antennarius</u> tissue.

3. RNA Synthesis from dAT and Inhibitors

Crab dAT should constitute an interesting model system for RNA synthesis studies because of its unique base sequence and low GC content.

C. borealis dAT has been used by other workers as the DNA template for RNA polymerase (5,6) and for DNA polymerase (4). These workers employed dAT[2] which had been melted and then purified on a MAK column. Their material therefore possessed a partially denatured structure and their results must be regarded accordingly.

The effects on the template activity of the structural changes caused by melting are described below and related areas of interest are explored as well.

The RNA polymerase and primer DNA concentrations employed are always in the range where RNA synthesis is proportional to each unless noted. The incubation duration and nucleoside triphosphate levels are as specified in the text, but usually the reaction period is 20 minutes and the reaction mixture

(0.25 ml) contains 50 mm moles of each nucleoside triphosphate.

A corrected guanosine triphosphate specific activity value was utilized due to the presence of an impurity in the sample. The correction is discussed fully in the Appendix at the end of this section.

The base compositions of the RNA synthesized from the dAT and main component DNA from <u>C</u>. <u>antennarius</u> and <u>C</u>. <u>borealis</u> are given in Table 9. The values shown were obtained with pure nucleoside triphosphate stocks so no corrections were necessary. The table shows that the base compositions are similar for the two species, but that the dAT template of <u>C</u>. <u>antennarius</u> must presumably possess the higher GC content (3.5% versus 2.5%). Goldberg, Rabinowitz and Reich (5) reported that <u>C</u>. <u>borealis</u> dAT directed the incorporation of 2.6 per cent GC and the table shows that Swartz, Trautner and Kornberg found 2.7 per cent.

Physical measurements also corroborate the higher GC content of the <u>C. borealis</u> dAT as discussed in the physical studies portion of this dissertation.

The <u>C. antennarius</u> dAT component was found to be an excellent primer for RNA synthesis. Figure 18 shows the incorporation of C^{14} -uridine monophosphate supported by native and melted dAT and main component DNA. It can be calculated from this data that dAT is copied at a rate at saturation of about 2.6 times as great as the main component DNA. The total quantities of RNA synthesized were determined from

TABLE 9

Base Compositions of the RNA Synthesized from

Crab dAT and Main Component DNA Templates

		TAD			-	Main Component				
		A	U	G	С		<u>A</u>	U	G	C
<u>c</u> .	<u>antennarius</u> l	46.8	49.7	1.8	1.7		30.0	29.4	20.9	19.7
<u>c</u> .	<u>borealis</u> 1	48.0	49.5	1.2	1.3		30.5	26.3	23.8	19.4
<u>c</u> .	borealis ²	45.0	52.7	1.5	1.2		31.9	32.5	17.2	18.5

Determined with <u>E. coli</u> RNA polymerase and C^{14} -nucleoside triphosphates.

²Data of Swartz, Trautner and Kornberg (4).

the UMP incorporation data of Figure 18 and from the UMP contents of the RNA products which are given in Table 9.

Figure 18 shows that the concentration necessary for half maximal synthesis rate is less for dAT than for the main component primer.

The lower maximal rate and saturation at lower concentrations noted for the melted DNA is characteristic for denatured primers (43,44) and is apparently due to a greater affinity of the RNA polymerase for single-stranded DNA (43).

The base composition of the RNA synthesized with whole crab DNA as primer is 37.0:49.7:6.8:6.5 (A:U:G:C), instead of 34.2:34.5:16.1:15.2, which is the actual complementary base composition (25 per cent dAT). The reason for the uncomplementary synthesis is unknown. In this case the dAT was copied at a rate which caused synthesis of 2.7 times as much total RNA as did the main component DNA. Since dAT makes up only 25 per cent of the DNA present this means that it is copied at a rate 8.1 times as fast as the main component DNA.

Since, as shown in Figure 18, dAT by itself supports synthesis of RNA at a rate only 2.6 times that of main component DNA under identical conditions, the RNA polymerase must bind preferentially to the dAT molecules in the whole crab DNA, thus magnifying the preferential synthesis of rAU. This agrees with the results of

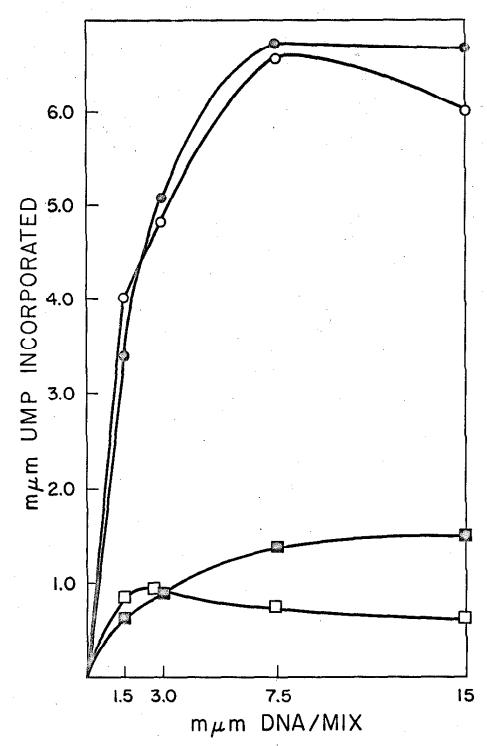


Figure 18. Incorporation of C¹⁴-UMP by <u>E. coli</u> RNA polymerase with the <u>C. antennarius</u> DNA templates; , dAT[1]; O, dAT[3]; , main component DNA[1]; , main component DNA[3]. Incubated for 20 minutes with 3 enzyme units.

Hurwitz et al. (43) who found that synthetic dAT possesses a much greater affinity for RNA polymerase than does ordinary DNA.

The magnification of synthesis noted from the dAT component when whole crab DNA is used as the DNA template is not due to simple degrees of primer saturation. Since in this case the amount of dAT present is always one-third that of the main component, one can see from the curves shown in Figure 18 that in no instance can the RNA synthesis from the dAT be more than 2.6 times that from the main component DNA unless the dAT binds a disproportionate quantity of the enzyme.

In one trial with a longer than usual incubation time, a seven-fold net synthesis of RNA (over the amount of crab dAT primer) was observed. Chamberlin, Baldwin and Berg (12) report that synthetic dAT is a very efficient primer and found up to fifty-fold net increases in RNA over template.

In one experiment with 3 units of enzyme and with 1.5 mpm of synthetic dAT, melted crab dAT and native crab dAT primers, the synthesis of 8.15, 8.35 and 8.34 mpm of RNA resulted in 20 minutes. In this case both enzyme and primer were limiting.

It is apparent that synthetic dAT is no more efficient as template than crab dAT, at least over short times. Hurwitz, Furth, Anders and Evans (43) noted that when synthetic dAT

primes the RNA polymerase reaction, RNA synthesis continues at a linear rate even for 2 hours. Crab dAT, however, follows the usual time course for the RNA synthesis reaction, namely is linear for only about 20 minutes and then slowly levels off with no additional synthesis after about 3 hours.

Only very low rates of RNA synthesis were found with dAT[2] as primer when only ATP and UTP were present in the incubation mixture. The same was true with <u>C</u>. <u>borealis</u> dAT and RNA polymerase (5) and also with DNA polymerase (4). These results show that the G and C residues are a part of the crab dAT molecules and not just part of contaminating DNA molecules.

Studies have been reported with <u>C</u>. <u>borealis</u> dAT (5) and <u>C</u>. <u>productus</u> whole crab DNA (45) related to actinomycin D binding and its relation to RNA synthesis inhibition. These reports with crab DNA and that of Kahan, Kahan and Hurwitz (25) correlated binding of actinomycin D with the deoxyguanosine content of the DNA and have shown that the degree of binding is correlated with the degree of inhibition of RNA synthesis. These workers have also stated that single-stranded DNA does not bind actinomycin as strongly and that its template activity is not as strongly inhibited.

The selectivity of the actinomycin binding is exemplified most clearly when RNA synthesis from whole crab DNA primer is inhibited with actinomycin. The addition of 0.01 µg actinomycin to approximately 0.5 mµm primer DNA and 2 enzyme units causes

the following inhibitions of incorporation: A, 16%; U, 26%, G, 40%; C, 41%. When one µg actinomycin is added, the respective inhibitions are 38, 36, 84 and 89 per cent. This selective GC inhibition is due probably to the selective inhibition of the synthesis from main component DNA rather than selective specific base incorporation inhibition.

Main component <u>C</u>. <u>antennarius</u> DNA was used as primer for the RNA synthesizing system to test the actinomycin D inhibition on native and melted DNA primers. Figure 19 shows the total RNA synthesis from native and melted DNA templates in the presence of actinomycin. Indeed, RNA synthesis from the native DNA primer is inhibited to a greater extent by all actinomycin concentrations than that from the same DNA primer if melted.

The lessened inhibition observed here for the melted DNA has been seen many times before and is due to the fact, according to Goldberg and Reich (45), that actinomycin D does not bind as well to single-stranded DNA. They state also that actinomycin does not compete with the RNA precursors.

Figure 20 shows the base composition of the RNA synthesized at each actinomycin concentration. It is clear that there is a slight inhibition of GC incorporation. For the native and melted DNA without actinomycin, the GC content is 37.5 and 37.7 per cent respectively. These respective values decrease to 27.7 and 27.8 per cent at the concentration of 2 μ g actinomycin per incubation. The base ratio values

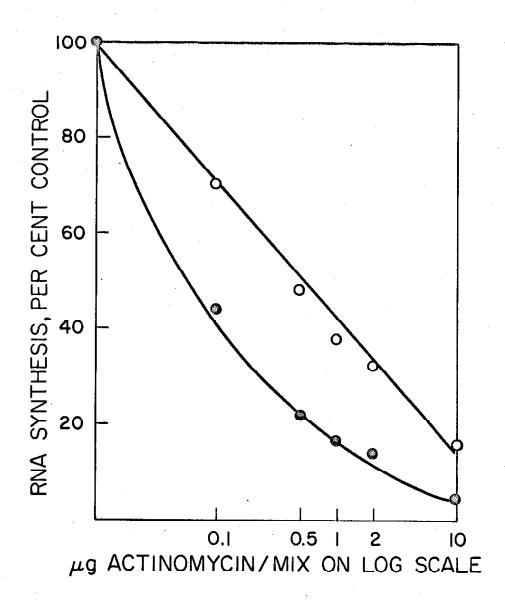


Figure 19. Inhibition of RNA synthesis by actinomycin D using <u>C. antennarius</u> native (①) and melted (①) main component DNA as templates with <u>E. coli</u> RNA polymerase. The 4 C¹⁴-nucleoside triphosphates were incorporated separately and the total RNA synthesis determined. 0.5 mµm DNA was incubated for 20 minutes with 3 enzyme units.

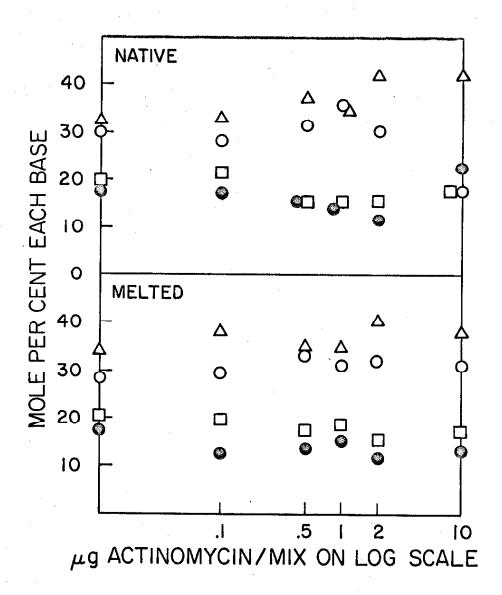


Figure 20. Base compositions of RNA synthesized from native (top) and melted (bottom) main component DNA in the presence of actinomycin.

A, O; U, \(\Delta \); G, \(\Delta \); C, \(\Delta \). Data from the same experiment shown in Figure 19.

found with 10 µg actinomycin are probably in error due to the very low incorporation observed under these conditions.

<u>C. antennarius</u> dAT was also tested and Figure 21 shows that the melted dAT is inhibited much less at all actinomycin concentrations than is the native form, dAT[1]. The inhibition is much less in both cases than that observed for the main component DNA. The inhibition has been shown before to be a function of the guanine content (45).

Figure 22 shows the base composition of the RNA synthesized with each actinomycin D concentration with both dAT forms. The only appreciable trend noted appears to be that the GC content of the RNA synthesized with actinomycin present decreases slightly, from 3.4 to 2.8 per cent in the native case and from 3.4 to 3.0 per cent in the melted dAT case. The C incorporation is apparently inhibited selectively in the native case from 1.7 to 1.2 per cent.

When melted and native \underline{C} . borealis dAT are used as primers the total RNA synthesis is likewise inhibited more with the native material than with the melted form as shown in Figure 23. The inhibition for both forms is less than that for \underline{C} . antennatius dAT. This is probably a result of the lower GC content of \underline{C} . borealis dAT.

Figure 24 shows quite clearly that actinomycin selectively inhibits the incorporation of guanine and cytidine residues.

The GC content of the RNA synthesized from the native dAT decreases from 2.4 per cent in the control to 1.3 per cent

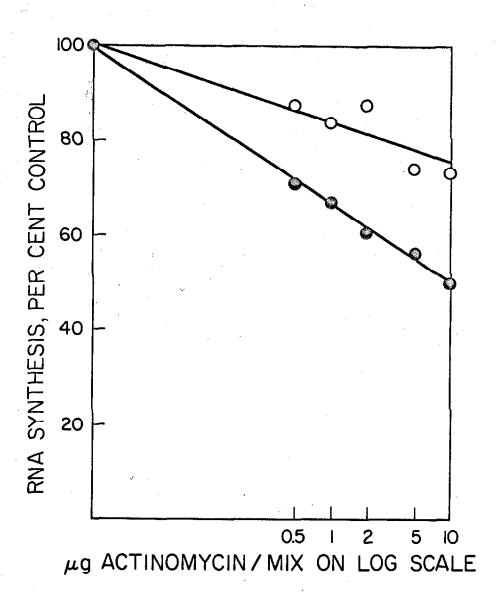


Figure 21. Inhibition of RNA synthesis by actinomycin D using <u>C</u>. antennarius dAT[1] (①) and dAT[3] (①) as templates with <u>E</u>. coli RNA polymerase. The 4 C¹⁴-nucleoside triphosphates were incorporated separately and the total RNA synthesis determined. 1.4 mum DNA was incubated for 20 minutes with 3 enzyme units.

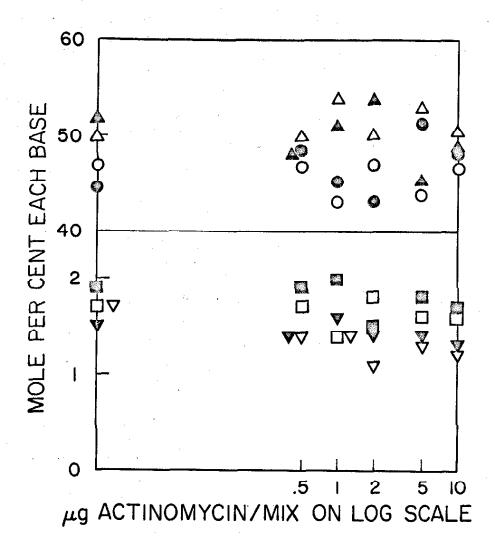


Figure 22. Base compositions of RNA synthesized from C. antennarius native (open symbols) and melted (closed symbols) dAT in the presence of actinomycin. A, O; U, Δ ; C, \Box ; C, ∇ . Data from the same experiment shown in Figure 21.

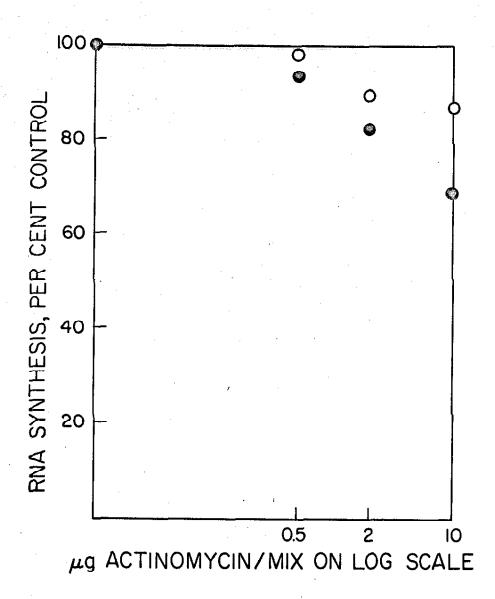


Figure 23. Inhibition of RNA synthesis by actinomycin D using <u>C. borealis</u> dAT[1] (•) and dAT[3] (•) as templates with <u>E. coli</u> RNA polymerase. The + C^{14} -nucleoside triphosphates were incorporated separately and the total RNA synthesis determined. 1.1 mpm DNA was incubated for 20 minutes with 3 enzyme units.

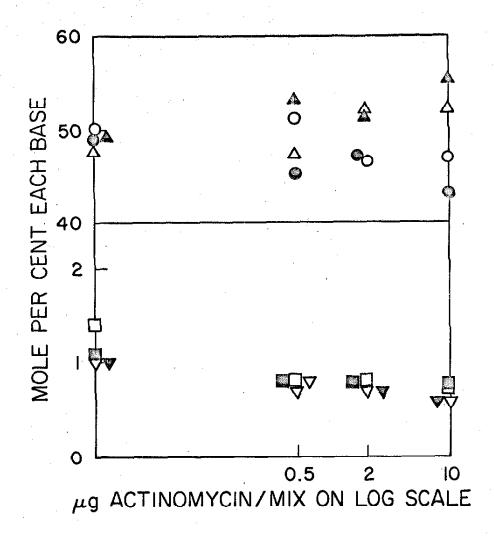


Figure 24. Base compositions of RNA synthesized from C. borealis dAT[1] (open symbols) and dAT[3] (closed symbols) in the presence of actinomycin. A, O; U, Δ ; G, \Box ; C, ∇ . Data from the same experiment shown in Figure 23.

with 10 µg actinomycin and the corresponding values are 2.1 per cent to 1.4 per cent with the melted form. Thus, the selective inhibition by actinomycin of G and C incorporation is more marked for C. borealis dAT than for C. antennarius dAT.

This selective inhibition by actinomycin corresponds to that found by Hurwitz et al. (46) with Dictyostelium discoideum DNA. Nearest neighbor studies showed a decrease of incorporation of P^{32} into GMP and CMP from incorporated α - P^{32} -UTP and α - P^{32} -ATP. Kahan, Kahan and Hurwitz (25) showed with the same template DNA that G and C incorporation was specifically inhibited by actinomycin. They reported inhibitions of 68 per cent for A and U and 80 per cent for C and G. These workers report that this phenomenon is not found with T2, Micrococcus lysodeikticus, calf thymus or Diplococcus pneumoniae DNA.

The <u>Dictyostelium discoideum</u> DNA contains about 20 per cent GC (47), and appears to contain stretches of adenine residues and also stretches of thymine residues which make this DNA unusual as well as different in principle from crab dAT (25).

Goldberg, Rabinowitz and Reich (5) report, however, that "actinomycin lowers the rate of synthesis, but does not alter the base composition of the RNA produced with crab dAT as primer". These workers used <u>C</u>. <u>borealis</u> dAT[2] and <u>E</u>. <u>coli</u> RNA polymerase, but did not display base incorporation

data.

The reasons for the conflicting results are unknown.

The experimental results show clearly that RNA synthesis from the crab dAT components is inhibited less by actinomycin D when melted DNA is used than when native is used. These findings point very strongly to the conclusion that the guanine residues do not appreciably base pair after melting and cooling, thus reinforcing the evidence presented earlier in this dissertation.

The results also indicate that in all cases there is a selective inhibition of G and C incorporation by actinomycin. This has been noted before only by Hurwitz and his collaborators (25,46) and only with an unusual template DNA from Dictyostelium discoideum. Goldberg, Rabinowitz and Reich (5) found no selectivity with C. borealis dAT, but selective inhibition is found with this DNA in the work presented here.

The base composition of the RNA synthesized is not changed by the secondary structure of the DNA primer nor does actinomycin affect the base composition significantly more with native than with melted primers.

Actinomycin does not inhibit RNA synthesis from the synthetic dAT template as has been noted previously (5,48). In this study, with 1.5 mpm of synthetic dAT and 3 enzyme units, 8.15 mpm of RNA were synthesized in the absence of actinomycin and 8.34 mpm were synthesized in the presence of 10 pg actinomycin per incubation after 20 minutes. No guanine

thymus histones Ib, IIb, III and IV (51), listed here in order of decreasing lysine content and in order of increasing arginine content, and with protamine (salmine, from Sigma Chemical Co.). These basic proteins are discussed by Huang, Bonner and Murray (36). The inhibitors are added to the complete incubation mixture before the addition of RNA polymerase. Aggregation of the DNA is probable although the primer concentrations used were so low that no precipitate was visible. This method of addition is the same as that used by Hurwitz et al. (46).

The primers used were native whole crab DNA and the column isolated components. Since the dAT used was column isolated, it was partially denatured. An incubation time of one hour was employed so that low primer and enzyme concentrations could be used. The polymerization rate was not linear for this period, but the extents of synthesis after one hour were proportional to those during the initial linear phase.

Probably the most meaningful experiment, to test the preferential histone binding hypothesis, would be one in which the protein inhibitors are added to whole crab DNA and the base composition of the RNA synthesized determined. This would determine whether the synthesis from the dAT template is preferentially inhibited.

Table 10 displays the results from one such experiment. There is no consistent trend to support the hypothesis of

preferential binding of basic proteins to dAT or to AT rich regions. All the inhibitors employed do inhibit RNA synthesis. The degrees of inhibition shown in Table 10 are greater for histones III and IV because a greater quantity of these proteins were added. Later results show that the lysine rich histones (Ib and IIb) are more potent, on an equal mass basis, than the arginine rich histones (III and IV). Protamine is as effective as the histones Ib and IIb, on a mass basis, as protamine (salmine) is more highly positively charged since it contains 70 per cent arginine (52) while histones Ib and IIb contain less than 30 per cent lysine and arginine together (53).

The results of experiments with the same inhibitors and with the dAT[2] template are shown in Table 11. The data compiled in the table come from three experiments.

There is a general trend indicating a preferential inhibition of AU incorporation for each basic protein inhibitor.

A series of experiments were also carried out with reconstituted nucleoprotein prepared by the method of Huang, Bonner and Murray (36). The reconstituted DNA-basic protein complex is soluble while the complex formed when the basic proteins are added to the DNA in the reaction mixture is largely an insoluble aggregate.

If basic proteins actually prefer to bind to AT rich regions, a gradual placement by gradient dialysis, as accomplished when reconstituted nucleoprotein is made, should

TABLE 10

Inhibition of RNA Synthesis from Whole Crab DNA with Basic Proteins

Protamine 0.7 1.4	24 31 16 24 24	-89-	Protamine	3 + 8 e
.1 0.	32 4 33 6 49 114 61 222			32 26 42 18 18 14 19 13
stone IV	38 13 20 30 30 30	c Proteins	Histone IV	2010
Hi 3.6	11 11 12 12 13 13 13 13 13 13 13 13 13 13 13 13 13	with Basic	111 6.9	66 61 68
Histone III 6	61 87 587 58 58 58 52	11 om dAT	Histone 3.5	1202
Hist 3.6	29 22 32 26 5 22 17 17	TABLE Synthesis fr	IIb 2.1	\$\$\$~2
ne IIb 2.1	29977	of RNA	Histone 1.0	16 37 34 5
Histone 1.1	1182	Inhibition	1b 3.5	827.94 827.94
Histone Ib 1.1^1 3.6	22 34 252 252 252 252 253	II	Histone 1.01	0 88 0 28 0 28 0 28 0 28 0 28 0 28 0 28
Base Hi	A 0 0 - 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0		Ваѕе	4DG0

1 Protein/DNA mass ratio.

Per cent inhibition of each base incorporation relative to the control DNA with no basic protein added. Incubated one hour with 0.5 mym DNA and 2 units E. coli RNA polymerase.

allow the binding process to be more selective.

Histone Ib and protamine were complexed with dAT[2] in the protein/DNA mass ratios of 1.2 and 0.6 respectively. The reconstituted dAT melts at a higher temperature and in a broader temperature range than dAT[2] alone. The respective reconstituted nucleoproteins have Tm values of 47.5 and 49°C while dAT[2] alone melts at 40°C in DSC.

The reconstituted dAT is only slightly less active than the dAT alone as primer for the RNA polymerase reaction. After the enzyme is saturated with template, however, the dAT rate levels off while the reconstituted dAT rates increase slightly and become more active than the dAT alone.

At all concentrations the base ratios of the RNA synthesized are similar to those of the dAT alone, with the exception that the GC content does increase by 0.5 per cent. This indicates a slight tendency for both histone Ib and protamine to complex preferentially with the AT regions of the dAT molecules.

Whole crab DNA was reconstituted with histone IIb at histone to DNA mass ratios of 0.4 and 0.8.

The melting profiles in DSC again show increased Tm values for both dAT and main component DNA. The dAT[2] Tm increases from 40 to 47 and 51°C for the 0.4 and 0.8 ratio material respectively and the main component DNA Tm values increase only from 65 to 66 and 68°C. Binding selectivity may be shown by these stability increases.

The optical density melting profiles show also that the usual dAT proportion is still present.

Here again, as with the reconstituted dAT, only slight inhibition of RNA synthesis is noted.

The GC content of the RNA synthesized from the reconstituted nucleohistone is increased from 13.5 per cent for the DNA alone to 16.2 and 17.2 per cent for 0.4 and 0.8 protein/DNA mass ratios respectively.

These experiments with RNA synthesis from reconstituted nucleoproteins show a slight selectivity of histones Ib and IIb and protamine for AT rich regions since the RNA synthesized is somewhat enriched in GC content.

Sucrose density gradient sedimentation experiments for the estimation of S values were carried out with C^{14} labeled RNA synthesized by \underline{E} . \underline{coli} RNA polymerase from dAT[2].

After incubation, the reaction mixture was deproteinized by shaking with chloroform-isoamyl alcohol and the resulting RNA solution was sedimented on a 29 ml 5-20 per cent sucrose density gradient. The centrifugation was at 25,000 rpm for 12 hours at about 4°C. One ml fractions were collected and an aliquot of each was filter washed with 10 per cent TCA. The reference RNA used in each tube was 18 or 28 S P³² labeled HeLa RNA. The acid precipitated material was counted and then each filter was covered with 3 layers of aluminum foil and recounted. The foil allows 88.3 per cent of the P³² radiation to be detected, but only 1.5 per cent of the

 C^{14} penetrates. In this way the distribution of P^{32} marker RNA and the C^{14} newly synthesized RNA can be determined.

When dAT is used as primer for the \underline{E} . \underline{coli} RNA polymerase system, the C^{14} labeled RNA made after 10, 30 and 60 minutes gives the sedimentation patterns shown in Figure 25. Clearly some of the material becomes larger as time progresses showing that the RNA molecules increase in length with time and that the synthesis is not of new molecules, but an increase in size of the ones already initiated. This corresponds to the RNA polymerase operational hypothesis of Bremer and Konrad (54).

Figure 26 shows the patterns for RNA synthesized from dAT[2] in the presence of histone Ib, protamine and actinomycin. The inhibitions noted in the three cases were 70, 39 and 24 per cent respectively. The average S value of the histone Ib (9 S) and protamine (10 S) inhibited RNA is less than that of the RNA synthesized from dAT[2] with no inhibitor present (12 S in Figure 27). This indicates that these compounds inhibit by slowing the rate of synthesis by the RNA polymerase molecules present. This would lead to a number of RNA molecules equal to the number of RNA polymerase molecules, but each would be of a smaller size than in the absence of inhibitor.

The RNA made in the presence of actinomycin is no smaller (12 S) than that usually synthesized from dAT

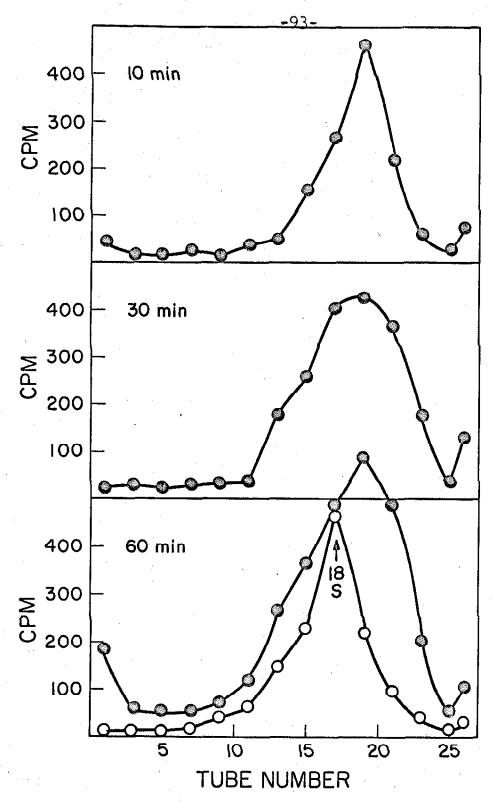


Figure 25. Sucrose density gradient sedimentation patterns of deproteinized Cl4-uridine labeled RNA synthesized from dAT[2] with E. coli RNA polymerase incubated for 10, 30 and 60 minutes. U-Cl4 RNA, (2); P32 HeLa 18 S marker RNA, (2).

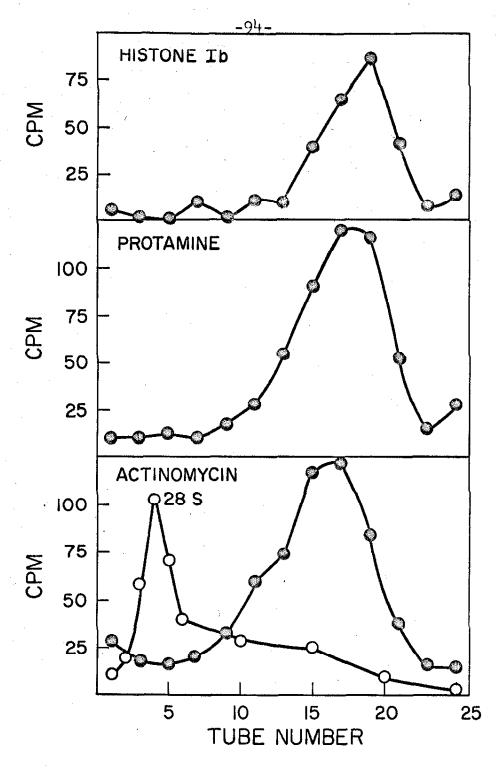


Figure 26. Sucrose density gradient sedimentation patterns obtained with deproteinized Cl4-AMP labeled RNA synthesized from dAT[2] with RNA polymerase incubated for 60 minutes. Top, RNA synthesis inhibited 70% by histone Ib addition; center, inhibited 39% by protamine addition; bottom, inhibited 24% by actinomycin addition. Cl4 RNA (***); P32 HeLa 28 S marker RNA (****). 30 ml gradient centrifuged 12 hrs, 25,000 rpm.

incubated for one hour (12 S). This indicates that actinomycin inhibits not by slowing the synthesis rate by each polymerase molecule, but prevents some of the polymerase molecules from synthesizing RNA. The mechanism of this action could possibly be that of blocking some of the RNA polymerase binding sites of the template.

These proposed inhibition mechanisms are by no means proven, but the evidence presented would fit with such hypotheses.

The RNA synthesized from dAT[2] reconstituted with histone Ib and protamine is as large as the RNA synthesized from dAT[2] alone as shown in Figure 27.

The reconstituted nucleoproteins are only slightly less active as template than dAT[2] alone and no great difference in the RNA size would be expected.

The RNA synthesis studies presented in this section show that \underline{C} . antennarius dAT has a slightly higher GC content than \underline{C} . borealis dAT.

The dAT component is a very efficient template for \underline{E} . $\underline{\text{coli}}$ RNA polymerase and has a very high affinity for the enzyme.

The RNA synthesis from melted dAT or melted main component DNA is inhibited less by actinomycin D than is the synthesis from the respective native templates. In all cases the GC incorporation is inhibited somewhat selectively.

The basic proteins which inhibit RNA synthesis when

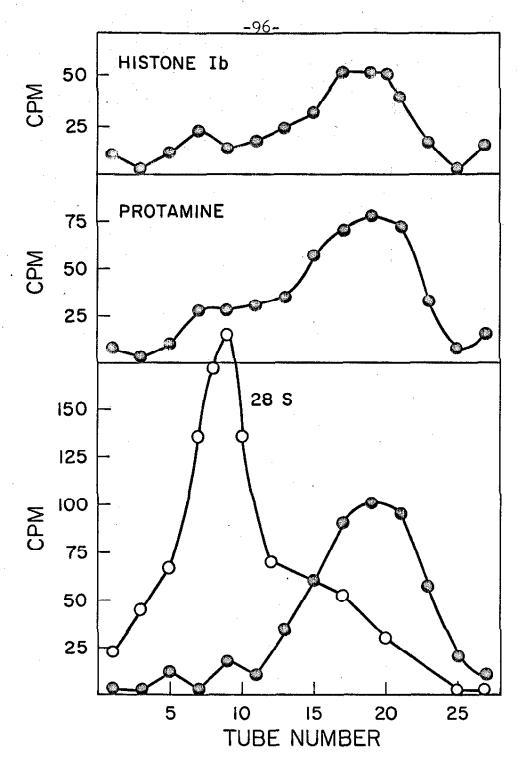


Figure 27. Sucrose density gradient sedimentation pattern of deproteinized C¹⁴-AMP labeled RNA synthesized from dAT[2] with RNA polymerase incubated for 60 minutes. RNA from dAT[2] reconstituted with histone Ib, 1.2:1 ratio (top); from dAT[2] reconstituted with protamine, 0.6:1 ratio (center); from dAT[2] alone (bottom). C¹⁴ RNA (③); P32 HeLa 28 S marker RNA (①). 30 ml gradient centrifuged 12 hours at 25,000 rpm.

complexed to primer DNA appear to bind selectively to AT rich regions of this DNA and to then inhibit the incorporation of A and T bases more than that of G and C bases.

Appendix. GTP Specific Activity Correction

The guanine incorporation was found to be about 1.7 times that which would be expected if C. antennarius DNA were similar to C. borealis DNA in base composition (4) and similar results were obtained by Marushige and Raaf with rat liver, calf thymus and E. coli DNA primers (55,32). Apparently the unlabeled GTP used to dilute the C14-GTP contained an impurity which interfered with measurement of the correct specific activity. The contaminant apparently has a similar ultraviolet absorption spectrum since the spectrum observed appeared to be that of pure GTP. An impurity of this sort would cause the measured specific activity (µc/µm) to be too low as determined from the absorbance, causing the calculated guanine incorporation to be too high. A batch of unlabeled GTP from Schwarz Bioresearch was pure and gave the expected results. All the results reported in this section, except those upon which the base compositions of the C. antennarius and C. borealis dAT and main component DNA are based (Table 9), were determined with the impure GTP stock. A corrected GTP specific activity value was used and the base compositions obtained in this way agreed well with those observed with the pure GTP stock.

This impediment is hopefully not serious since similar GTP conditions are found in all incubations, no matter which nucleoside was labeled.

4. Properties of the dAT RNA Product and Its Absence from Crab Tissue

The RNA made by <u>E. coli</u> RNA polymerase with <u>C. antennarius</u> dAT as template contains on a molar basis, 46.8% A, 49.7% U, 1.8% G and 1.7% C. The template presumably has a structure similar to that of the dAT components of <u>C. borealis</u> (4) and <u>C. pagurus</u> (9) which are both made up almost entirely of an alternating sequence of A and T bases and base compositions similar to that of <u>C. antennarius</u> dAT.

All three of the above DNA polymers are known to renature almost completely after strand separation just as does synthetic dAT which has a perfectly alternating AT structure (7).

Chamberlin, Baldwin and Berg (12) synthesized rAU with \underline{E} . $\underline{\operatorname{coli}}$ RNA polymerase using the synthetic dAT template. The rAU was found to be double-stranded due to its unique alternating base sequence.

RNA preparations were synthesized with \underline{E} . \underline{coli} RNA polymerase from crab dAT and separated from the dAT primer by banding in cesium chloride.

Figure 28 shows an optical density melting profile of the crab dAT primed synthetic rAU in SSC. As with the rAU synthesized from synthetic dAT, the crab synthetic rAU exhibits a reversible strand separation and renaturation, indicative of a double-stranded native character.

The Tm value observed is 67.4°C for the first melting and 66.6°C and 67.0°C for cooling and remelting respectively and the σ T value increases only slightly from 3.1 to 3.6°C from the first to the second melting. Only about 3 per cent of the hyperchromicity is lost and the Tm decreases about 0.4°C after the first heating. These observations indicate that the RNA is changed only slightly by the original strand separation in opposition to the behavior of the primer. This might be expected since the single-stranded RNA polymer must hydrogen bond at random when synthesized.

A comparison of this rAU with that synthesized from synthetic dAT shows that both have Tm values higher than their DNA templates and that the hyperchromicity values are very high (61% for crab synthetic rAU in SSC and 67-70% for synthetic rAU in 0.01 M sodium citrate buffer [12]). These two synthetic rAU polymers appear to have similar structures as deduced from their melting characteristics.

The spacing of the cytidine residues in the crab dAT molecules and thus of the complementary guanine residues in the synthetic rAU has been heretofore unknown. Treatment of the crab synthetic rAU with ribonuclease T_1 , which hydrolyzes the RNA chain specifically adjacent to guanine residues releasing sequences terminated by guanosine 3'-monophosphate,

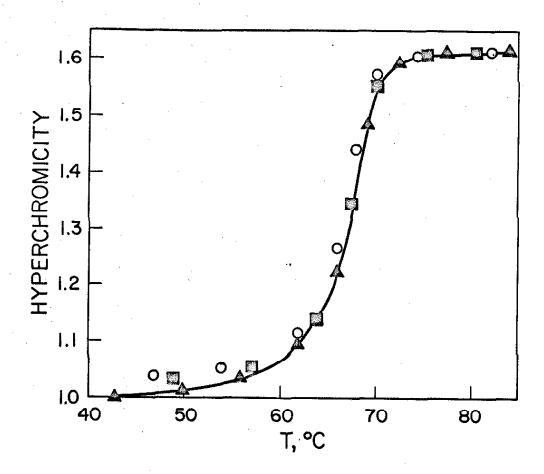


Figure 28. Optical density melting profile of crab synthetic rAU in SSC. A, first melting; O, cooling; I, second melting.

would, however, release polynucleotides containing the sequence of bases between the guanine residues and be terminated by a guanosine 3'-monophosphate. The lengths of the polynucleotides formed would indicate the cytidine spacings in the primer dAT.

Of course, some fragments from the ends of the original rAU molecules will be formed and the copying of the DNA template may not be perfect, but a good indication of the spacings should be obtainable from such experiments.

Samples of rAU labeled with C^{14} were treated with ribonuclease T_1 at levels 10 and 50 times higher than that necessary to hydrolyze the total RNA present (37). The incubation was also carried out for twice the usual time. Each sample was heated to 100°C for 10 minutes to insure strand separation so that no single-strand breaks would be masked. If the samples are not melted the S value of the rAU decreases from 12 to 8 S after ribonuclease T_1 treatment.

Samples of pea microsomal RNA (56), crab hepatopancreas RNA and <u>Torula</u> RNA were rendered largely alcohol soluble by this treatment as shown in Table 12. The rAU, however, which is originally somewhat soluble, is solubilized only partially by treatment with ribonuclease T_1 . This result indicates that rAU even after hydrolysis by the enzyme contains a significant number of polynucleotides large enough to be insoluble in ethanol.

When these samples are sedimented through a sucrose

Sample	Untreated	Treated			
		10 x	50 x		
Pea ²	3%	101%	105%		
Crab Hepatopancreas ²	5%	112%	119%		
Torula ²	3%	90%	106%		
$rAU^3 U-c^{14}$	46%	70%	60%		
$rAU^{3} A-c^{14}$	53%	69%	75%		
$rAU^3 G-C^{14}$	40%	58%	58%		

The percentages shown indicate the proportion of the material soluble in 67% cold ethanol. The concentrations of the rAU samples were approximately 140 mum per ml before ethanol addition and the other RNA samples were at concentrations of approximately 3 mm per ml. lox, treated with 10 times the amount of enzyme usually necessary to hydrolyse the RNA present completely. 50x, treated with 50 times the amount necessary for complete hydrolysis.

²Values determined by measuring A₂₆₀ of supernatant.

 $^{^3}$ Values determined by measuring radioactivity of supernatant.

density gradient, the sizes of the fragments may be determined as shown in Figures 29 and 30.

The sedimentation marker RNA could not be placed in the same tube with the ribonuclease treated RNA because the ribonuclease remained active even after treatment with trypsin, Cu, SLS or pH \pm .5, followed by boiling. A marker was therefore run in each centrifuge run in a separate tube. The sedimentation results were very reproducible from centrifuge tube to centrifuge tube and from experiment to experiment, and the results are considered to be accurate. The A_{260} of the ribonuclease does not interfere since it is very insignificant compared with that of the RNA samples.

Figure 29 shows the pattern resulting after centrifuging the pea RNA marker (closed circles), which is composed of 28, 18 and 4 S components (56) for 5 hours at 35,000 rpm at about 4.00.

The sedimentation pattern is shown for C^{14} -uridine labeled crab synthetic rAU by the open circles in Figure 29. The average sedimentation rate was about 10 S.

The rAU pattern was determined by measuring the C¹⁴ in each fraction. The C¹⁴ cpm values shown for all the sucrose density gradient experiments were corrected, using a table of factors determined empirically, to rectify the error due to absorption by sucrose. When 21 fractions are collected, the factors vary from 1.4 for the first fraction to 1.06 for the last.

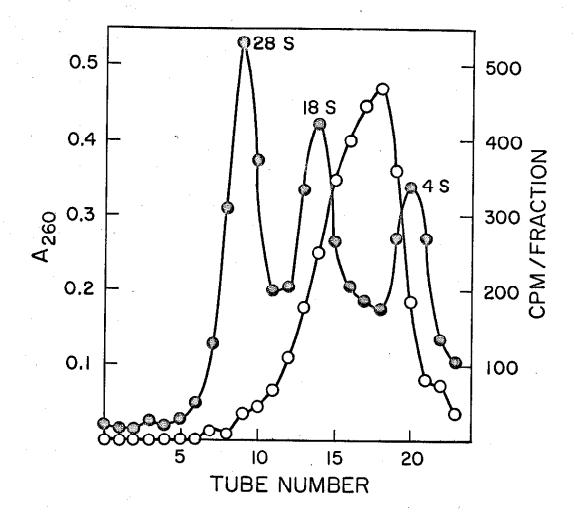


Figure 29. Optical density and C¹⁴ patterns of RNA samples in a sucrose density gradient (5-20% sucrose, 0.1 M acetate pH 5.5). The pea marker RNA (ⓐ, A₂₆₀) and C¹⁴-uridine labeled crab synthetic rAU (O, cpm) in 0.2 ml were layered on a 4.3 ml gradient and centrifuged at 35,000 rpm for 5 hours. Each fraction was diluted with 0.5 ml water and an aliquot was counted and the A₂₆₀ measured.

The C¹⁴-uridine labeled crab synthetic rAU which sediments at a rate of about 10 S has a value of about 8 S after melting as shown by the open circles in Figure 30. This decrease in sedimentation rate apparently indicates strand breakage and/or changes in form during melting.

Figure 30 also displays the pattern obtained with rAU that has been treated with excess ribonuclease T_1 (triangles) in order to cleave the strands adjacent to the guanine residues. The S value of this material is 5-6 indicating that the average chain length is over 100 nucleotides based on a length of 77 nucleotides for the 4 S alanyl-transfer RNA (57). This value for the nucleotide length may be in some error since the secondary structure of the rAU and other factors undoubtedly affect the sedimentation rate.

Figure 31 shows pea RNA treated in the same way. The closed circles show that melted pea RNA sediments largely at a rate greater than the 4 S marker. The marker is represented by the open circles which show the 4 S peak in tube 13. Sedimentation of the melted pea RNA for a shorter time reveals that the bulk of the material is about 7 S compared to the value of 8-10 S reported by Ts'o and Lubell (58).

The open circles represent the pattern made by untreated P^{32} marker pea RNA. The ribonuclease T_1 treated pea RNA, represented by the triangles in Figure 31, has no material sedimenting beyond 4 S.

The small size of the pea RNA treated with ${\bf T}_{1}$ is

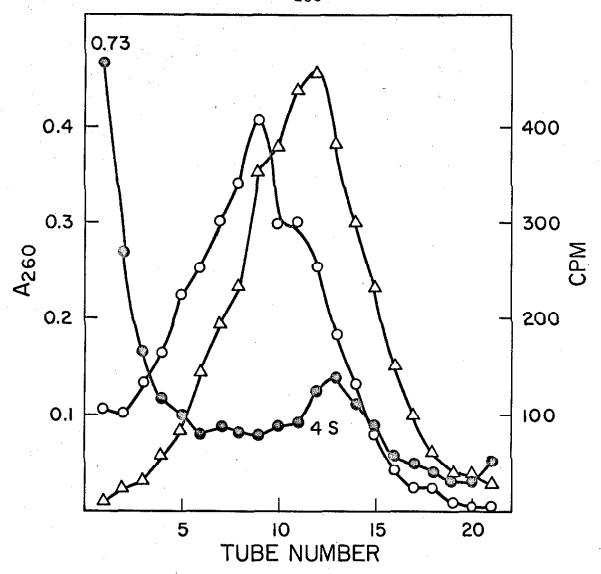


Figure 30. Sedimentation patterns of melted C^{14} uridine labeled crab synthetic rAU (\mathbf{O} , cpm) and
the same material ribonuclease T_1 treated and melted
($\mathbf{\Delta}$, cpm) after 24 hours at 35,000 rpm in a 5-20%
sucrose gradient. The former rAU was contained in
the same centrifuge tube as the pea RNA optical
density marker (\mathbf{O}). The ribonuclease treated rAU
was contained in a separate tube in the same run.

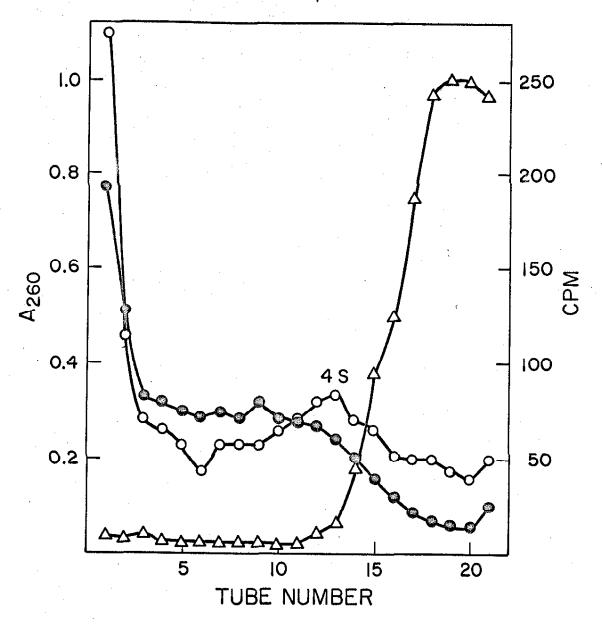


Figure 31. Sedimentation patterns in a sucrose density gradient of melted pea RNA (\bigcirc , A_{260}) and ribonuclease T_1 treated pea RNA (melted after treatment, \triangle , A_{260}). P^{32} labeled pea RNA (\bigcirc , cpm) was used as marker with the melted sample. Centrifuged 35,000 rpm for 24 hours.

expected since pea RNA contains 33 per cent guanine (59) and the spacings between such residues are presumably random. The pieces resulting from hydrolysis of the RNA chain next to each guanine would thus be quite small.

Crab synthetic rAU, on the other hand with only 1.8 per cent guanine, should yield very long pieces after T_1 treatment. The results bear this out, although the size found (greater than 100 nucleotides) is greater than that predicted from the G content (56 nucleotides).

An important biological question to be settled is whether or not RNA is synthesized from the dAT template in The unusual properties of the RNA synthesized the crab. from the dAT with \underline{E} . $\underline{\operatorname{coli}}$ RNA polymerase should be useful for detecting the rAU in RNA isolated from crab tissue. These properties are: 1. The rAU melts sharply at 67°C in SSC, while the usual RNA melts very gradually over a wide temperature range (60); and 2. Since rAU contains a very low guanine content, treatment with ribonuclease T_1 releases polynucleotides which sediment in a sucrose density gradient with an average S value of 5-6 while the usual RNA, after this treatment, consists of very short oligonucleotides due to the higher G content and the lack of long stretches of other bases between the guanine residues. All of the latter oligonucleotides sediment at a rate less than 4 S.

Another property that might be utilized to detect the synthesis of rAU in crab tissue is the fact that actinomycin

D apparently binds to guanine residues in DNA and thus inhibits the synthesis of RNA to an extent greater with primers containing a high G content than with lower G content DNA, at least in in vitro systems.

This is true also with the two components of crab DNA or with whole crab DNA as shown elsewhere in this dissertation; with approximately 0.5 mpm of whole crab DNA per incubation, 0.01 µg actinomycin inhibits the uridine incorporation by 26 per cent and the cytidine incorporation by 40 per cent. If the actinomycin concentration is 1 µg per incubation, the inhibitions are 36 and 89 per cent respectively. Results from experiments with the separated crab DNA components show similar values. These results then are due primarily to selective inhibition of the RNA synthesis from the main component DNA rather than to selective inhibition of cytidine incorporation.

It may be possible that the application of actinomycin D to crab tissue would therefore preferentially inhibit the synthesis of RNA from the main component DNA, yet not affect the synthesis of rAU from the dAT as greatly. If this occurs, the incorporation of labeled cytidine will be inhibited much more than the incorporation of uridine.

Hepatopancreas tissue was incubated in a sterile salt solution similar to sea water, and containing 800 units of penicillin per ml. Tissue was incubated by shaking at 13° C with C^{14} -thymidine for 3 hours and C^{14} -labeled DNA was

isolated from the tissue. The RNA incorporation experiments reported below were carried out with the same system.

The DNA components were separated using the mercury method and the specific activity of the dAT compared with that of the main component DNA indicated that little or no C^{1l_+} was incorporated into DNA other than crab. This finding should rule out microbial contamination and the assumption may be made then that all the C^{1l_+} -precursors incorporated in this system are incorporated within the hepatopancreas tissue itself.

The results of one experiment are shown in Figure 32. The data show generally more inhibition of C incorporation than of U, but the difference is not nearly what would be expected if rAU was being synthesized at a rate corresponding to the proportion of dAT present. The differences noted could be due to selective inhibition of the synthesis of cytidine rich RNA from the main component DNA template and experimental error.

Thymidine and amino acid incorporation are slowed only slightly by the presence of actinomycin D.

The results obtained are not fully conclusive, but do indicate that rAU is not being synthesized at an appreciable rate.

RNA was isolated from fresh crab hepatopancreas tissue by banding directly in cesium chloride or by phenol extraction. Figure 33 shows the sedimentation pattern of

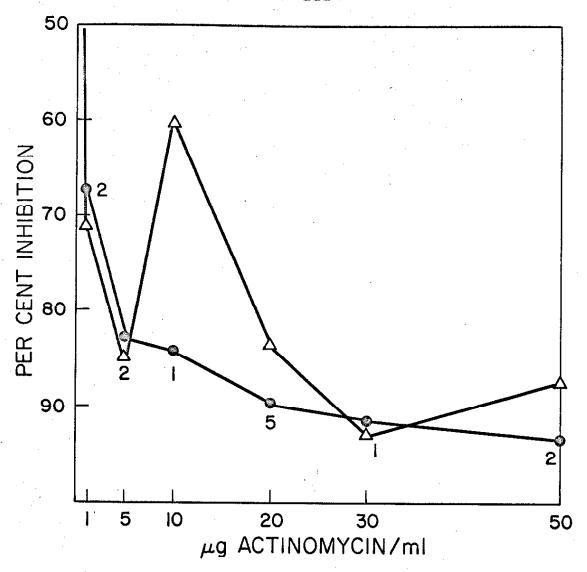


Figure 32. Inhibitory effect of actinomycin D on the incorporation of C^{14} -uridine (Δ) and C^{14} -cytidine (Θ) into RNA in \underline{C} . antennarius hepatopancreas tissue during a 3 hour incubation. The numbers within the figure represent the number of measurements for each point.

phenol extracted hepatopancreas RNA. The peaks, with sedimentation rates of 34, 20 and 3 S, apparently represent ribosomal and transfer RNA. Most of the RNA preparations were partially degraded, however.

The optical density melting profile of the hepatopancreas RNA shown in Figure 34 is very gradual and there is no sharp increase observable in the profile between 60 and 70°C. This melting curve is typical for whole organism RNA (60).

Since crab synthetic rAU gives 61 per cent hyperchromicity upon melting and the crab RNA gives only 22 per cent, the melting profile should detect even a small proportion of rAU.

The optical density melting profiles for pea RNA and Torula RNA are also shown in Figure 34. The pea RNA and crab RNA have very similar melting profiles which may be because both are total extracted RNA made up largely of ribosomal RNA. The Torula RNA was purchased from Calbiochem and was claimed to be in the size range from 20,000 to 30,000 molecular weight. The S value was found to be 3.5.

The optical density melting profile studies indicate that no detectable rAU is present in the crab hepatopancreas RNA.

Another method for detecting rAU is to look for the large polynucleotides characteristic of rAU after ribonuclease \mathbf{T}_1 treatment of the crab RNA.

The hepatopancreas RNA preparations were treated with excess ribonuclease \mathbf{T}_1 and as shown in Table 12, the RNA is

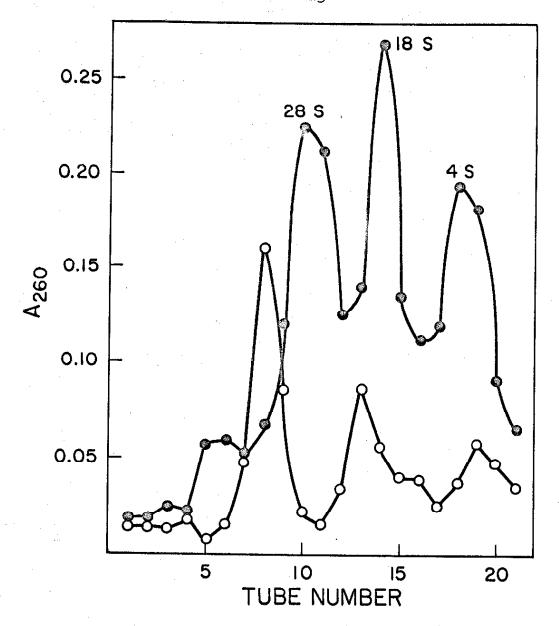
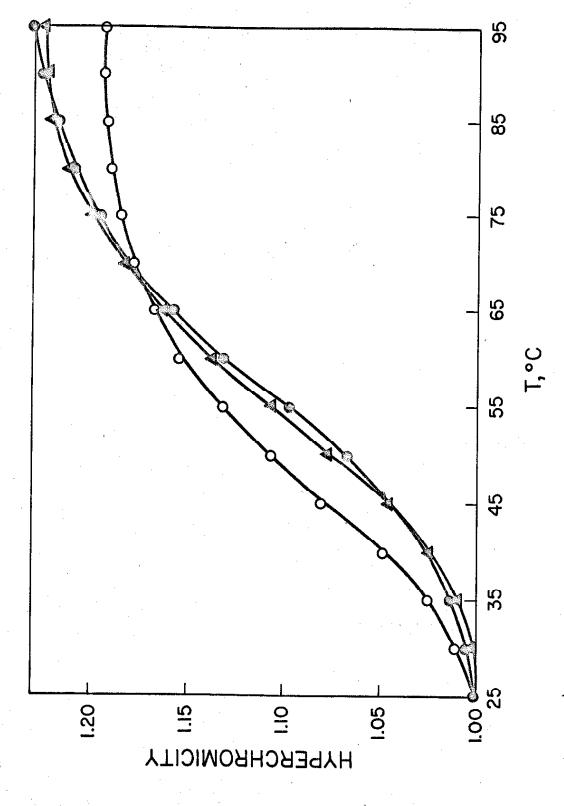


Figure 33. Sedimentation patterns of pea marker RNA (**) and phenol extracted <u>C</u>. <u>antennarius</u> hepatopancreas RNA (**) after centrifuging for 5 hours at 35,000 rpm in a 4.3 ml 5-20% sucrose gradient. The hepatopancreas RNA peaks are approximately 34 S, 20 S and 3 S.



O, pea RNA; ▲, hepatopancreas Figure 34. Melting profiles of RNA samples in SSC. RNA; O, torula RNA.

apparently rendered completely alcohol soluble. When this treated RNA was sedimented in a sucrose density gradient the pattern was obtained as shown in Figure 35. The triangles denote the treated material and the closed circles represent the untreated material. The open circles show the pattern of the P³² marker pea RNA.

It is evident that ribonuclease T_1 treatment reduces the hepatopancreas RNA to oligonucleotides no larger than 4 S. Thus, no rAU is detectable in crab hepatopancreas RNA.

Another innovation is possible with ribonuclease T_1 treated RNA, since the rAU polynucleotides are partially ethanol insoluble. Then an enrichment would be accomplished by treating RNA with T_1 and then precipitating the rAU polynucleotides with ethanol. The precipitate would contain largely the rAU polynucleotides if any are present in the RNA.

This experiment was carried out with five hepatopancreas RNA preparations with the following amounts in each: 10.4, 9.3, 7.7, 4.8 and 2.0 optical density units. After treatment with a 50-fold excess of ribonuclease T_1 and ethanol precipitation, each precipitate was taken up in SSC and centrifuged in a sucrose density gradient.

Approximately 0.2 optical density units of rAU polynucleotides would be easily detected in this centrifuge experiment. As Table 12 shows, only about 35 per cent of the rAU present precipitates in ethanol after ribonuclease T_1

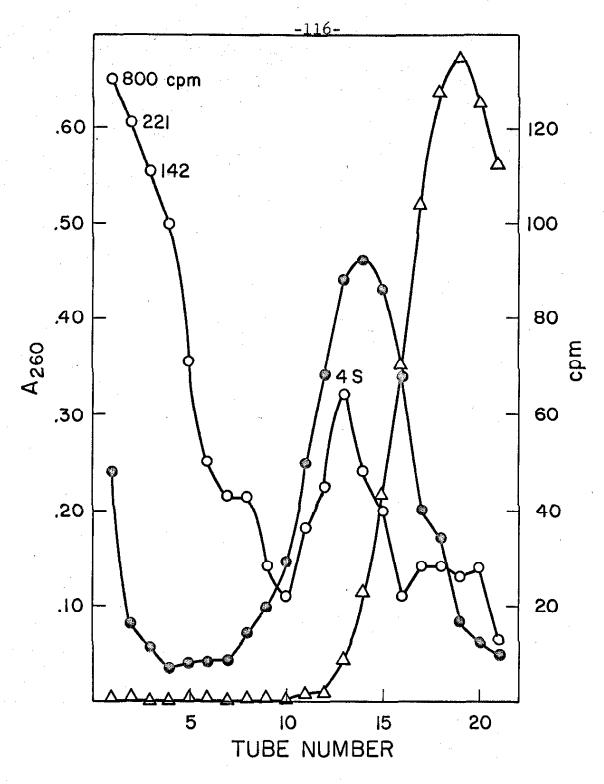


Figure 35. Sedimentation pattern of melted hepatopancreas RNA (\odot), ribonuclease T_1 treated and melted hepatopancreas RNA (Δ) and P³² pea marker RNA (\odot , cpm). Centrifuged in a +.3 ml 5-20% sucrose density gradient for 24 hours at 35,000 rpm.

treatment. Thus, this precipitation and sedimentation method would detect about 0.6 optical density units of rAU in the original RNA preparation. The largest RNA preparation used contained 10.4 optical density units. Then about 6 per cent rAU in the original RNA preparation can be detected by this method.

Figure 36 shows that no material is found sedimenting past tube 12. If rAU was present, some evidence of RNA would be found in tubes 8 to 11. None of the preparations give any evidence of rAU by this method. Apparently rAU is not found in C. antennarius hepatopancreas RNA.

The evidence presented indicates that rAU is not present in the hepatopancreas RNA and it is probably not synthesized in this tissue. Any conclusions must be qualified in the following ways: 1. The methods employed will not detect rAU in very low concentrations; 2. The RNA isolation procedures may not isolate rAU or the material isolated may be degraded too greatly during the isolation; 3. The rAU component may be synthesized, but be degraded very rapidly so that a very low amount would be present at any time; 4. Actinomycin may not bind selectively in crab tissue.

5. Crab Chromatin and RNA Synthesis

The conclusions, up to this point, imply that the crab dAT component is not utilized as a template for the synthesis of RNA in crab hepatopancreas tissue. If, indeed, this portion

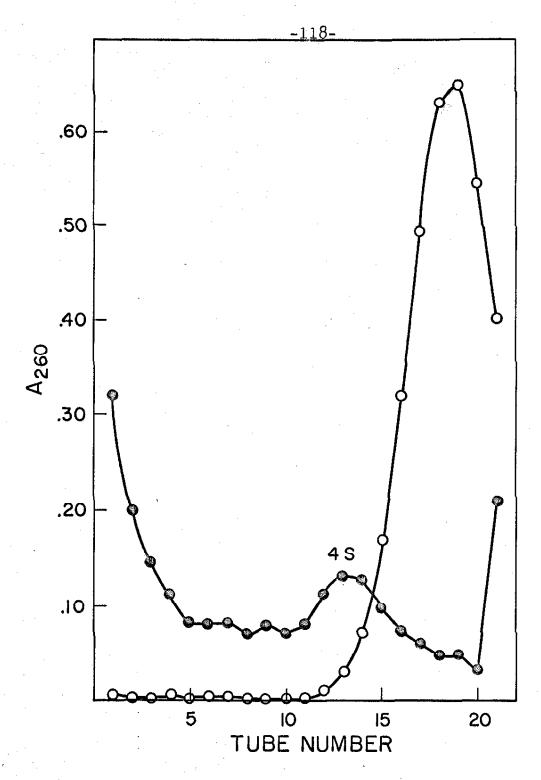


Figure 36. Sucrose density gradient centrifugation patterns obtained after 24 hours at 35,000 rpm with ribonuclease T_1 treated and ethanol precipitated crab hepatopancreas RNA (\bullet) and with pea marker RNA (\bullet) in separate centrifuge tubes.

of the crab "genome" is inactive it should be possible to isolate chromatin containing the dAT in a condition in which it will not function as a template for RNA synthesis. The inactivity of the dAT in chromatin would be presumably due to a histone-dAT complex.

Stedman and Stedman (61) first suggested that histone might regulate genetic activity and Huang and Bonner (50) showed that histone can suppress DNA-dependent RNA synthesis. These workers also presented evidence (62) that the RNA synthesis by the isolated chromatin is at least qualitatively similar to that found in the intact tissue since a gene that is turned off <u>in vivo</u> is also turned off in the chromatin from the tissue and vice versa. In this case, histone appears to regulate the synthesis of RNA from a specific DNA region.

The chromatin material used in this study was isolated from <u>C</u>. <u>antennarius</u> hepatopancreas by sedimenting the homogenized and filtered material through a 20 per cent sucrose solution. A portion of the chromatin was resedimented, but the additional treatment did not purify the material appreciably as shown by chemical analysis.

The crab chromatin DNA contains 29-30 per cent dAT with the remainder main component DNA, as determined by banding the DNA of the chromatin in cesium chloride. The chromatin has a DNA:RNA:protein ratio of 1:0.9:15, which shows that the material is not as pure as pea chromatin for which the corresponding ratio is 1:0.6:1.6 (50) or rat liver chromatin

with the ratio of 1:0.04:1.7 (33). The DNA recovered in the chromatin was about 35 per cent of that originally present in the tissue.

The crab chromatin was active as primer for RNA synthesis with <u>E. coli</u> RNA polymerase. The chromatin alone incorporates no nucleotides into RNA and the material at high concentrations is very inhibitory to the RNA polymerase reaction as shown in Figure 37. Maximal rate of RNA synthesis is attained at a chromatin DNA concentration of about 3 µg per incubation. The twice purified chromatin appears to be somewhat more active than the once purified material.

The base ratio of the RNA synthesized with the crab chromatin template was 33.5:45.7:10.8:10.0 (A:U:G:C), which is intermediate between that complementary to dAT (48.6:49.7:1.8:1.7) and that complementary to main component DNA (30.0:29.4:20.9:19.7). This base composition is not self complementary, but fits most closely to the case where 46.5 per cent of the RNA is synthesized from the main component and 53.5 per cent from the dAT. The actual calculated value for this ratio of RNA synthesis is 39.0:40.2:10.7:10.1 (A:U:G:C).

It has been shown in Section 3 that with whole crab DNA, the dAT is template for 73 per cent of the RNA formed while the main component is complementary to 27 per cent of the RNA synthesized. Normally then, dAT is copied much faster than the other DNA component, in fact, 8.1 times as fast when calculations are made from the 73 per cent total RNA from

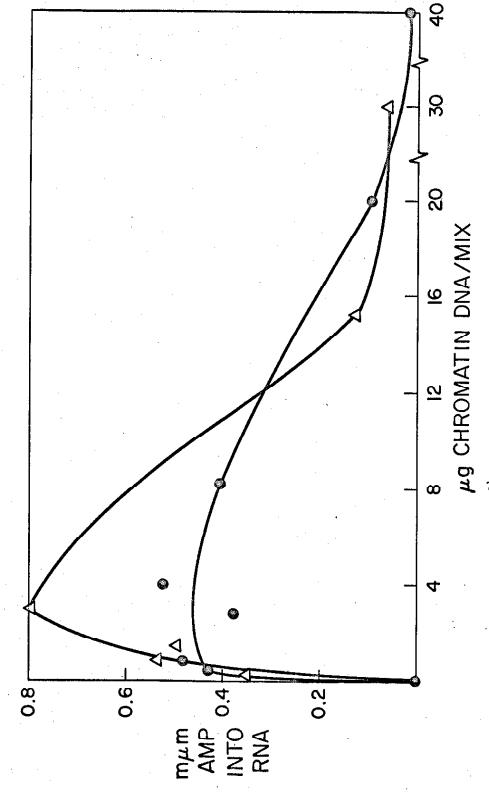


Figure 37. Incorporation of $C^{1\mu}$ -AMP into RNA by E. coli RNA polymerase with crab chromatin as template. Δ , twice purified chromatin; \odot , once purified chromatin. DNA determined by diphenylamine. Incubated for 20 minutes with 8 enzyme units.

the 25 per cent proportion of dAT.

The chromatin DNA, which is enriched in dAT, 29.5 per cent instead of the usual 25 per cent, shows a depressed synthesis of rAU compared to the usual case. Calculations show that the dAT is only 34 per cent as active as expected in comparison to the main component DNA. The calculations are as follows: the dAT template is 8.1 times as active as the main component DNA when whole crab DNA is used as primer, so the DNA in chromatin with 29.5 per cent dAT, should synthesize 8.1 x 0.295 (dAT %) or 2.39 compared to 1 x 0.705 (% main component) or 0.705 for the main component. The ratio of the RNA from dAT to that from the main component would be therefore 2.39:0.705 or 3.4:1.0. The usual DNA, with 25 per cent dAT, gives a value of 2.7:1 for this ratio.

This ratio in the chromatin case would be 0.535:0.465. (% from dAT:% from the main component) or 1.15:1.0. Thus, the template activity of the dAT of chromatin is only 34 per cent (1.15/3.4) of that of the dAT of the whole crab DNA, relative to the main component DNA template activity. The conditions were enzyme and primer limiting in all cases.

The maximal rate of RNA synthesis per unit chromatin concentration in Figure 37 is at about 0.4 μg chromatin DNA per incubation where the incorporation of 0.43 μg of chromatin of 0.43 μg of chromatin DNA synthesis is 1.3 μg with 0.4 μg of chromatin DNA. With the same quantity of RNA polymerase

and deproteinized whole crab DNA, about 8.2 mum of RNA is synthesized. The chromatin then is only about 16 per cent as active as the DNA alone.

In the deproteinized DNA, if the dAT proportion is 29.5 per cent, the proportion of the RNA synthesized from it would be 77 per cent or 6.3 mum with the remainder or 1.9 mum from the main component DNA. In the chromatin case, however, 53.5 per cent or 0.7 mum of the RNA synthesized is complementary to dAT and the remainder or 0.6 mum is made from the main component. By comparing the synthesis from each DNA component of the chromatin with that of the deproteinized DNA, the degree of repression of RNA synthesis for each can be determined.

Thus, the chromatin dAT is 89 per cent repressed and the main component DNA is 69 per cent repressed. These values show that the dAT is more repressed than the main component DNA in crab hepatopancreas chromatin.

Extraction of the chromatin with 0.2 N sulfuric acid results in the removal of about 35 per cent of the protein present. Some of this acid extracted protein moves toward the negative electrode in polyacrylamide gel electrophoresis (63). This apparently basic protein corresponds to the histone which is the usual inhibitor substance in chromatin.

The studies with crab chromatin indicate that indeed the dAT in the chromatin is much less active as template for RNA synthesis than is the main component DNA. About 69 per

cent of the main component DNA is inactive while 89 per cent of the dAT is in the same repressed condition. The available template then would be made up of 13 per cent dAT and 87 per cent main component DNA.

The chromatin from developing pea cotyledons with E. coli RNA polymerase, supports RNA synthesis about 34-42 per cent as well as pure DNA (62) and in the same system, rat liver chromatin has 20-28 per cent of the activity of the DNA alone (33). These workers have found some inhibition at very high chromatin concentrations (55,64), but the crab chromatin must contain a powerful inhibitor of some type possibly ribonuclease, since the inhibition is more severe than usual and occurs at low template concentrations.

The calculations of the rates of RNA synthesis from the two crab DNA components could be in error for several reasons including: the DNA regions copied may not be representative of the base composition of the two DNA components; the inhibitor that is present could affect appreciably the synthesis rate at optimal template levels.

This study indicates, however, that the dAT component in hepatopancreas chromatin is repressed to a greater extent than is the main component DNA of the chromatin when tested with the \underline{E} . \underline{coli} RNA polymerase system.

C. Discussion of the Biological Study Results

An additional <u>Cancer</u> species, <u>C. anthonyi</u>, has been found to contain 10-11 per cent dAT in its DNA. So far then, DNA from every one of the 8 <u>Cancer</u> species examined contains dAT.

The <u>C</u>. <u>antennarius</u> dAT component is found in all tissues and to the same extent of about 25 per cent. Cheng and Sueoka (6) found that in <u>C</u>. <u>borealis</u> the 3 tissues examined contained about 32 per cent dAT. Evidently then, the dAT is universal to all tissues of the crab.

The <u>C. antennarius</u> dAT is localized in the nucleus. Its presence in all tissues and localization in the nucleus suggests that it is part of the hereditary material of the crab.

The localization of the two DNA species with intermediate melting and buoyant density characteristics has not been accomplished, however, and their significance is unknown. A mitochondrial origin seems most probable.

The dAT is very active as template for \underline{E} . \underline{coli} polymerase so apparently could function as a template in the crab for RNA synthesis.

The RNA product is an alternating rAU polymer which forms a double-stranded structure with a Tm value of 67°C in SSC.

This RNA would be expected to be inactive in stimulating

amino acid incorporation in <u>in vitro</u> ribosomal systems because of its hydrogen bonded secondary structure. Take nami and Okamoto (65) have reported that the presence of secondary structure makes ribosome-polynucleotide association impossible and Singer, Jones and Nirenberg (66) have shown that secondary structure abolishes the ability of a polyribonucleotide to cause amino acid incorporation in the ribosome system.

Preliminary in vitro experiments appear to show no evidence of any amino acid incorporation and Sueoka (67) was unable to make any protein using the RNA synthesized from the C. borealis dAT[2] template. It may be that the partially denatured structure of the dAT[2] template causes the resulting RNA to be inactive as primer since Wood and Berg (68) have shown that RNA synthesized from denatured DNA is inactive.

It is possible, however, that <u>in vivo</u> the single RNA strands when synthesized are protected in some way so that the single strands do not hydrogen bond and in this way remain active as messenger RNA.

If the rAU had caused amino acid incorporation by ribosomes, the code for the two alternating amino acids involved would have been discovered. The amino acids would have the code letters AUA and UAU. The latest coding information (69) shows that these amino acids are methionine and tyrosine, respectively.

Since the amino acid code words are now known, the best approach to find out if dAT is an active genetic determinant would be to look for this unusual protein (methionine-tyrosine alternating copolymer).

Actinomycin inhibition studies with <u>C. antennarius</u> hepatopancreas tissue show only a very slight selective inhibition of cytidine incorporation into RNA as compared with that of uridine. If dAT is acting as primer for RNA synthesis to an appreciable extent, the actinomycin D should selectively inhibit the synthesis from the higher GC main component DNA. This would thus selectively inhibit the C incorporation compared with the U incorporation.

Since such selectivity is not found, either little or no rAU is synthesized <u>in vivo</u> or actinomycin does not act selectively in living tissues.

Optical density melting profiles of the RNA isolated from the hepatopancreas gives no indication of any material melting sharply at 67°C and ribonuclease T_1 treatment cuts all of this RNA into oligonucleotides smaller than 4 S. These facts indicate that rAU is not present.

Crab synthetic rAU treated with ribonuclease T_1 is cut into polynucleotides which are partially ethanol precipitable and which have an average S value of 5-6 in a sucrose density gradient. The specific hydrolysis adjacent to the G residues by ribonuclease T_1 produces 5-6 S polynucleotides since the G content of the rAU is only 1.8 per cent. Since no RNA

with S values greater than 4 is found in ribonuclease \mathbf{T}_1 treated hepatopancreas RNA, there is little or no rAU present in this RNA.

The dAT of chromatin isolated from <u>C</u>. <u>antennarius</u> hepatopancreas tissue is 11 per cent as active a template as is the deproteinized dAT in whole crab DNA. The template activity was measured by RNA synthesis catalyzed by <u>E</u>. <u>coli</u> RNA polymerase. The main component DNA in chromatin is 31 per cent as active as when deproteinized. These values are determined from the total RNA incorporation and base composition of the RNA made from the chromatin and from whole crab DNA templates.

In chromatin, then, we find the dAT selectively repressed indicating that since the isolated chromatin probably represents qualitatively, at least, the pattern of RNA synthesis of the living organism (62), the dAT is inactive or much less active than the main component DNA in RNA synthesis <u>in</u> vivo.

It appears then that although <u>C</u>. <u>antennarius</u> dAT is an excellent template for RNA polymerase, it is nonetheless not an active template in the hepatopancreas tissue of the crab. If, however, rAU is being synthesized as a messenger RNA it would probably not ever be present to a very great extent since messenger RNA always constitutes a small proportion of the total cellular RNA. The detection methods employed are then not sensitive enough to determine unequivocally whether or

not rAU is synthesized.

The dAT may function as a nucleotide storage material or it may have some other function. It is also certainly possible that it serves no function, although this is unlikely.

The RNA synthesis from the dAT template with <u>E. coli</u>
RNA polymerase progresses at a rate 2.6 times as fast as from the main component DNA template. If whole crab DNA is used as template, 2.7 times as much RNA is synthesized from the 25 per cent dAT proportion than from the main component DNA. This means that the dAT is being copied at a rate 8.1 times as fast as the main component DNA. The dAT must bind more enzyme than does the main component DNA in the mixture since usually the dAT under noncompetitive conditions is copied at a rate only 2.6 times as fast as main component DNA. Hurwitz et al. (43) reported that synthetic dAT has a higher affinity for the enzyme than does usual DNA.

Actinomycin D inhibits RNA synthesis more strongly from native dAT of both <u>C</u>. <u>antennarius</u> and <u>C</u>. <u>borealis</u> than from melted dAT. The same is true of <u>C</u>. <u>antennarius</u> main component DNA. The inhibition differences would be expected if most of the guanine residues have not base paired after melting.

Actinomycin, in all cases, selectively inhibits the incorporation of guanine and cytidine. Goldberg, Rabinowitz and Reich (5) did not find this with <u>C. borealis</u> dAT[2] and

Hurwitz and collaborators (45,46) found this only with Dictyostelium discoideum DNA primer and not with T2, Micrococcus lysodeikticus, calf thymus and Diplococcus pneumoniae DNA. The reason for this discrepancy is not known.

The basic proteins, histones Ib, IIb, III, IV and protamine, are potent inhibitors of RNA synthesis when added to the DNA template before the addition of RNA polymerase. The lysine rich histones Ib and IIb are more potent than the arginine rich histones, III and IV. Protamine is as effective as histones Ib and IIb apparently because it is more highly positively charged on a mass basis. Protamine contains about 70 per cent arginine and histones Ib and IIb contain less than 30 per cent arginine plus lysine.

The very dilute primer and inhibitor conditions used may explain the high inhibitor to DNA ratios necessary for appreciable inhibition of RNA synthesis.

These basic proteins exhibit a slight binding selectivity as deduced from a somewhat stronger inhibition of AT incorporation compared to that of GC. Also the Tm of dAT in whole crab DNA reconstituted with histone IIb is raised substantially, while that of the main component is elevated only slightly. These findings corroborate the reports of Mahler and Methrotra (49) who show a greater stabilizing of AT rich DNA by diamines and Hurwitz, Evans, Babinet and Skalka (46) who found a selective inhibition of AT incorporation by added histone.

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