Iodination of Nucleic Acids *in Vitro**

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ABSTRACT: Polynucleotides can be iodinated *in vitro* by heating in an aqueous solution of iodine or thallic trichloride plus iodide at pH 5. Iodine is incorporated into DNA through a stable covalent bond as 5-iodocytosine. There is no reaction with thymidine or adenine residues, and very little, if any, with guanine. Although the reaction markedly favors single-stranded DNA, relatively large amounts of the radioactive isotopes of iodine can be incorporated into native DNA without significantly altering its molecular weight, melting profile, or ability to renature or to transform. Over 95% of the cytosine of single-stranded DNA can be iodinated. This is accompanied by a large increase in buoyant density (in CsCl), a shift toward longer wavelengths in the ultraviolet absorption profile, and loss of ability to renature. Formation of 5-iodocytosine is the only irreversible process observed during this reaction except for a small amount of single-strand breakage.

Iodination of RNA proceeds like that of single-stranded DNA except that a small amount of 5-iodouracil and a large amount of uracil hydrate is also formed.

The *in vitro* iodination of preformed polynucleotides has several potential uses. Since several radioactive isotopes of iodine with suitable half-lives and a variety of decay modes are readily available, it would permit labeling with easily detected γ-emitting isotopes, double labeling, and high-resolution autoradiography; by increasing the density it would enable separation from unlabeled polynucleotides by density-gradient centrifugation; and by modifying the structure of polynucleotides it might help correlate structure and function.

Neither of the two procedures for *in vitro* iodination of polynucleotides are fully satisfactory. Iodination with ICl requires conversion of the nucleic acid into a salt soluble in organic solvents and leads to degradation (Ascoli and Kahan, 1966). Iodination with *N*-iodosuccinimide apparently does not degrade (Brammer, 1963) but only small amounts of iodine are bound and the preparation of labeled reagent is inconvenient. Presented here is a simple method which permits stable incorporation of large amounts of iodine or labeling at high specific activity, and causes little or no degradation of polynucleotides. It also has the provocative feature of being specific for cytosine in DNA with a strong preference for single-stranded cytosine.

Methods

Gel Chromatography. Samples (2 ml) were applied to a 1 × 60 cm gel column and eluted with buffer at 12 ml/hr, collecting 2-ml fractions in a Beckman Model 132 refrigerated fraction collector. Both column and collected fractions were maintained at 5°. Polynucleotides were chromatographed on Sephadex G50 fine. Molecules too large to penetrate the gel eluted at fraction 10 or 11; those small enough to penetrate the gel without hindrance eluted at fraction 26. Nucleosides were chromatographed on Sephadex G10 (Braun, 1967).

Centrifugation. Centrifugation was carried out in a Spinco Model E analytical ultracentrifuge equipped with ultraviolet optics, a photoelectric scanner, and a multiplexer accessory. Equilibrium CsCl density-gradient centrifugation was done in cells containing a double-sector centerpiece, 3 cells at a time in a 4-hole An-F rotor. The sample sector of each cell contained 1–2 μg of *Clostridium perfringens* DNA (the density reference), and 2–5 μg of calf thymus DNA in 0.40 ml of 8.1 *m* CsCl. The solvent sector contained 0.41 ml of 8.1 *m* CsCl. Velocity sedimentation was done in cells containing a type I double-sector, band-forming centerpiece. A 15-μl sample containing 1 μg of DNA in 0.1 M NaOH was placed in the sample well and 0.1 M NaOH–0.9 M NaCl was placed in both solvent and solute sectors; $s_{20,w}$ was calculated from the measured sedimentation coefficient by the solvent and density correction factors given by Studier (1965).

Melting Profile and Renaturation of DNA. The ultraviolet absorption at 260 mμ of a DNA solution was measured as the temperature was increased by 0.5°/min in a Gilford 2400 spectrophotometer. Correction for thermal expansion was made. Renaturation was followed by measuring the ultraviolet absorption of denatured DNA maintained at 58° in 0.4 M NaCl–0.02 M sodium citrate as a function of time.

Ultraviolet Absorption Profile. The ultraviolet absorption of polynucleotides was measured as a function of wavelength in a Cary 15 spectrophotometer. Sulfite was added to all reaction mixtures to eliminate absorption by iodine or thallic ions. The amount of iodide present in the reduced reaction mixture depends on the amount of iodination, and since iodide absorbs strongly below 250 mμ, no weight was given to measurements in this region.

Radioactivity Measurements. ^{125}I, half-life of 60 days, decays to a stable isotope of tellurium by an electron-capture process which results in the emission of a 35-kV γ ray (Myers and Vanderleeden, 1960). Its radioactivity was measured in a well-type NaI crystal scintillator. Counting efficiency was determined by means of a calibrated ^{129}I source (Tracerlab) and was generally ~25%. All measurements are expressed as microCuries or net cpm above background (about 100 cpm).

Assembly of Reaction Mixtures. Iodination in the presence of $TlCl_3$ was always carried out in 0.1 M sodium acetate–0.04 M acetic acid (pH 5.0) buffer unless otherwise indicated. ^{125}I was equilibrated with carrier iodide or iodine before addition of other components. $TlCl_3$ and iodine were added last. The reaction mixture was assembled at 0°, heated in a thermostatted bath for the specified time, and then cooled on ice. Concentration of polynucleotides in the reaction mixture was

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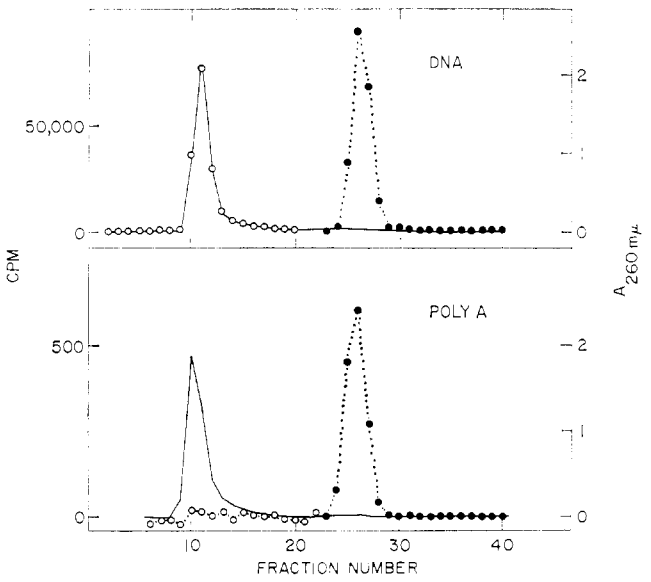

FIGURE 1: Sephadex G50 chromatography of reaction mixtures containing heat-denatured calf thymus DNA or poly(A). The ultraviolet absorption of each fraction is plotted as a solid line (—). The open circles represent net counts above background. The closed circles represent net count above background divided by 3 (DNA) or 200 (poly(A)).

expressed as molarities. This was calculated from the measured ultraviolet absorption of the polynucleotide stock solutions at pH 7 using the values for molar extinction coefficient per nucleotide given by Chamberlin (1965) for homopolynucleotides, by Lawley (1956) for DNA, and by Lindahl *et al.* (1965) for RNA.

Materials

Carrier-free ^{125}I was obtained from New England Nuclear as NaI in 0.1 M NaOH. Calf thymus DNA from Sigma was incubated with 50 μg/ml of RNase at 37°, pH 5 for 1 hr, shaken three times with equal volumes of phenol, precipitated with ethanol until free of phenol, redissolved in pH 5 acetate buffer, and stored frozen at −20°. *E. coli* and *Cl. perfringens* DNA were obtained from Worthington, ribonucleotide homopolymers from Miles, and thallic trichloride from K & K Labs.

Results

Reaction of Polynucleotides with I^- + Tl^{3+}. Significant amounts of iodine are bound to denatured DNA heated with KI in the presence of the oxidizing agent $TlCl_3$. Figure 1 shows the results of heating for 15 min at 60° a mixture containing 6.5×10^{-4} M (as nucleotides) heat-denatured calf thymus DNA, 2.5×10^{-4} M KI in chemical equilibrium with 2 μCi of ^{125}I and 1.5×10^{-3} M $TlCl_3$ in 0.1 M ammonium acetate–0.04 M acetic acid, pH 5.0. The reaction mixture (volume 2.0 ml) was then chilled on ice, mixed with 0.1 ml of 0.1 M Na_2SO_3 to reduce excess $TlCl_3$ and 0.4 ml of 1 M ammonium acetate–0.5 M NH_4OH to raise the pH to ~ 9, heated for 20 min at 60°, and chromatographed on Sephadex G50 fine, eluting with 0.1 M ammonium acetate. The ultraviolet absorption at 260 mμ—a measure of DNA concentration (the other components of the final reaction mixture do not absorb significantly at this wavelength)—and the net radioactivity—a measure of iodine content—were determined for each fraction. The results (Figure 1) show that all the DNA was eluted as a sharp peak at the void volume whereas the iodine was eluted in two sharp, well-separated peaks, the major one (closed circles) at the position where small molecules able to penetrate the gel completely eluted. This component represents inorganic iodine. The other (open circles) appeared at the void volume in nearly perfect coincidence with DNA and thus represents DNA-bound iodine. This iodine was not removed by heating at 100°, pH 5 to 9, and appears to be bound covalently rather than through ionic bonds involving thallium since adding sulfite before, rather than after, the initial heating to 60°, reduces the amount of iodine which elutes with DNA from 16.7% of the total to < 0.02%.

TABLE I: Amount of Iodine Bound to Polynucleotides after Heating with KI + $TlCl_3$.[a]

Polynucleotide Type	Concentration (M × 10^4)	Iodine Bound (%)
Poly(A)	5.0	<0.02
Poly(I)	3.5	<0.02
Poly(G)	2.9	0.06
Poly(U)	6.4	0.80
Poly(C)	5.4	22.1
Native calf thymus DNA	6.9	0.74
Denatured calf thymus DNA	6.5	16.7
E. coli tRNA	3.1	10.1
Wheat germ rRNA	3.3	9.3

[a] Each polynucleotide was heated at 60° for 15 min at the concentration shown above in the presence of 2.5×10^{-4} M KI and 1.5×10^{-3} M $TlCl_3$, pH 5.0. The reaction mixture (volume = 2.0 ml) was then chilled on ice, mixed with 0.10 ml of 0.1 M Na_2SO_3 and 0.40 ml of 1 M ammonium acetate–0.5 M NH_4OH, heated to 60° for 20 min, and then chromatographed on Sephadex G50 fine, eluting with 0.1 M ammonium acetate.

The specificity of the reaction is shown by the fact that not all polynucleotides react with iodide in the presence of thallium. Figure 1 also shows the results of the same reaction conditions applied to 5×10^{-4} M poly(A). As with DNA, all the poly(A) (solid line) elutes as a single sharp peak at the void volume, but all the radioactivity elutes with unreacted iodine. None is seen in coincidence with poly(A) at the void volume although the radioactivity in this region (open circles) is plotted at a scale 200 times that of the other (closed circles). As little as 0.02% could have been detected.

The same reaction conditions have been applied to a variety of polynucleotides; the amount of iodine bound is indicated in Table I. No iodine was bound to poly(I); a very small but reproducible amount (0.06%) reacted with poly(G). Both of the pyrimidine homopolymers tested showed a significant reaction, poly(C) much more so than poly(U) (22.1 and 0.8%, respectively). Wheat germ RNA (primarily ribosomal RNA), *E. coli* tRNA and heat-denatured calf thymus DNA all react with substantial amounts of iodine but the reaction with

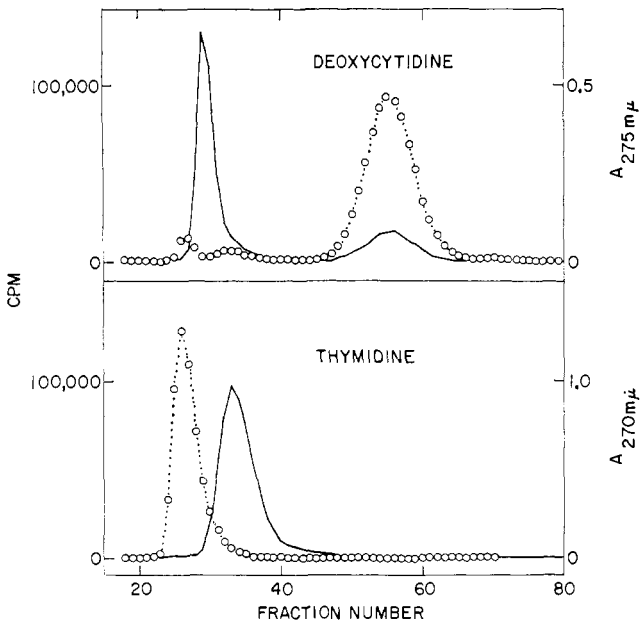

FIGURE 2: Sephadex G10 chromatography of reaction mixtures containing deoxycytidine or thymidine. The heavy line represents the ultraviolet absorption. The open circles represent net cpm above background.

native calf thymus DNA was <5% that of identically treated heat-denatured DNA. In all cases the reacted polynucleotide was eluted as a single sharp peak at the void volume. The elution profile was identical with that of the untreated polynucleotide. The iodine eluted as a sharp peak at the column volume and, where a reaction took place, iodine was found at the void volume in close coincidence with polynucleotide. Iodine was never found in intermediate positions. The ultraviolet absorption profile of reacted purine homopolymers eluted at the void volume of the chromatographic column was identical to that of the unreacted homopolymers. Reaction of purine homopolymers does not reduce their ultraviolet absorption. Heating 1×10^{-4} M poly(G), poly(A), or poly(I) with 1×10^{-4} M KI and 6×10^{-4} M $TlCl_3$ at pH 5 and 60° for 4 hr decreased their ultraviolet absorption by less than 1%. This was also true of poly(C), poly(U), RNA, and DNA treated in the same way except for the omission of KI. In the presence of KI, the absorbance of these polynucleotides decreased at their wavelength of maximum absorption and increased at longer wavelengths (see below).

The homopolymer of thymidine was unavailable. However, thymidine was treated with iodine; no reaction was observed under conditions where reaction with deoxyuridine was substantial and reaction with deoxycytidine almost complete. Solutions containing 7×10^{-4} M thymidine or deoxycytidine, 2.5×10^{-4} M KI + ^{125}I and 1.5×10^{-3} M $TlCl_3$, pH 5, were heated 90 min at 60° then chromatographed on Sephadex G10. The radioactivity and ultraviolet absorption at 270 mμ (thymidine) or 275 mμ (cytosine) are plotted in Figure 2. With thymidine the bulk of the radioactivity eluted before the thymidine; although the two peaks were not fully resolved, the amount of iodine associated with thymidine must be <1%. With deoxycytidine ~ 90% of the activity was associated with a small ultraviolet-absorbing peak whose absorption profile was similar to that of deoxycytidine but shifted 20 mμ toward longer wavelengths. It was identical with that of 5-iododeoxycytidine. No significant radioactivity was present in fraction

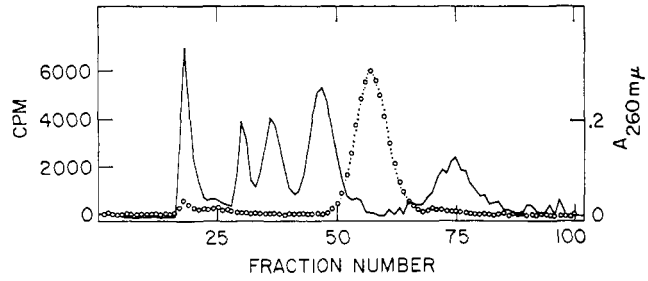

FIGURE 3: Sephadex G10 chromatography of DNase–snake venom digest of iodinated DNA. The ultraviolet absorption at 260 mμ (—) and the net cpm (O. . . O) is plotted for each fraction.

71, indicating absence of deamination of iododeoxycytidine during the reaction since the deamination product, iododeoxyuridine, elutes at this position. Also, the radioactive peak from the thymidine reaction mixture eluting at fraction 26 does not represent iodide, which elutes from this column at fraction 122. It is probably iodate since a sample of KIO_3 eluted at fraction 27 on this column.

The specificity of the reaction with iodine was also checked by chromatography of the nucleosides released by enzymatic digestion of iodinated DNA obtained by heating a mixture containing 8.1×10^{-4} M (as nucleotides) native calf thymus DNA, 2.5×10^{-4} M KI, ^{125}I, and 1.5×10^{-3} M $TlCl_3$ at 60° for 90 min. Unreacted iodine was removed by gel chromatography. The DNA solution was digested in 5 mM $MgSO_4$ and 100 μg/ml of calf pancreas DNase I for 90 min at 37°. The pH was raised to 9 by addition of 1 M NH_4OH, 0.5 mg of snake venom (*Crotalus adamanteus*) was added per ml and the mixture was left overnight at 37°. The resulting nucleoside digest was chromatographed on Sephadex G10. The ultraviolet absorption at 260 mμ and radioactivity of each fraction is plotted in Figure 3. Five ultraviolet-absorbing components are seen. They were identified on the basis of position and ultraviolet-absorption profile as (in order of appearance) DNase and snake venom enzymes, deoxycytidine, thymidine, deoxyadenosine, and deoxyguanosine. The radioactivity eluted as a single component at the same position as iodinated deoxycytidine (Figure 2). No ultraviolet-absorbing material is seen associated with this component since <5% of the cytosine in DNA was iodinated.

Pyrimidines substituted at C5 did not appear to react with iodide in the presence of $TlCl_3$ since there was no significant change in the ultraviolet absorption profiles of IUdR, BrUdR, ICdR, 5-hydroxymethycytosine, or thymidine when these pyrimidines were reacted under conditions causing profound alterations in those of cytosine, cytidine, deoxycytidine, deoxycytidylic acid, poly(C), uridine, deoxyuridine, or poly(U), *e.g.*, the ultraviolet absorption maximum of 1×10^{-4} M solutions of pyrimidines substituted at C5 were the same within ±1% before and after heating with 1×10^{-4} M KI and 1.5×10^{-3} M $TlCl_3$ at 60°, pH 5, for 1 hr, whereas the same treatment of deoxycytidine and deoxycytidylic acid reduced their maximum absorption to 59 and 54%.

Reaction Mechanism. Analysis of mixtures of iodide and polynucleotides heated in the presence of $TlCl_3$ show that iodine is bound to pyrimidines in two forms: one stable and associated with the appearance of ultraviolet absorption at 310 mμ; the other relatively unstable and associated with a decrease in ultraviolet absorption at 260 mμ and above. For example, after heating a mixture containing 1.24×10^{-4} M poly(C), 2.5×10^{-4} M KI, and 1.5×10^{-3} M $TlCl_3$ for 15 min

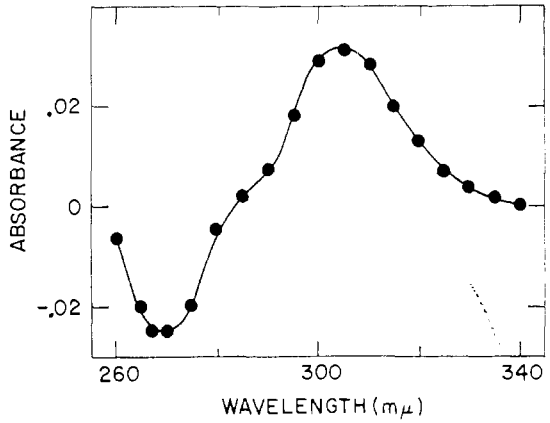

FIGURE 4: The effect of iodination on the ultraviolet absorption profile of poly(C). The difference in the optical density of iodinated poly(C) and unreacted poly(C) is plotted *vs.* wavelength at pH 8.4. Both solutions contained 1×10^{-4} M pyrimidine. The reacted poly(C) contained 11.2% 5-iodocytosine and had been heated to dissociate the unstable iodination component.

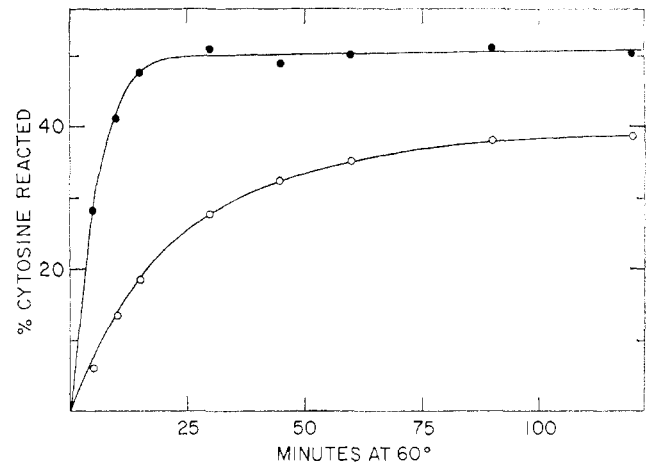

FIGURE 6: The rate of iodination of DNA at 60°, pH 5. Closed circles represent iodine associated with cytosine; open circles represent iodine bound as 5-iodocytosine only.

at 60°, pH 5, and chromatographing on Sephadex G50 with 0.1 M ammonium acetate, 11.8% of the iodine eluted in association with poly(C) at the void volume. If the iodinated poly(C) was immediately rechromatographed, >98% of the iodine eluted at the void volume but if heated to 90° for 10 min or longer, only 52% eluted at the void volume, the rest appearing as a distinct peak at the position where all small molecules eluted. The iodine remaining in the void volume was stable to further heating.

The duration and intensity of heating to completely dissociate the unstable component depended on pH. At pH 9, 10 min at 40° sufficed whereas at pH 7, 1 hr at 60° or 10 min at 90° was necessary. At pH 5, all the unstable form was removed after 15 min at 90°. Evidently some of the unstable form was converted to the stable form by this treatment since the amount of stably bound iodine observed after heating at pH 5 was up to 40% greater than that found after heating at higher pH.

The dissociation of the unstable component by heating was accompanied by changes in the ultraviolet-absorption pattern. Aliquots from the reaction mixture considered above before and after heating at pH 5 were treated with 5 μmoles/ml of Na_2SO_3 and the pH was adjusted to 8.4. They were then heated at 60° for 20 min to completely dissociate the unstable iodination component. This produced an effect on the absorption profile of the iodinated poly(C) equivalent to the reappearance of 10.6% of the original poly(C). The profile of the unreacted poly(C) was unchanged. The ultraviolet absorption of unreacted poly(C) and iodinated poly(C) before and after dissociation was 0.613, 0.524, and 0.588 at 267 mμ and 0.002, 0.032, and 0.031 at 310 mμ, respectively, measured against a blank identical in composition except that buffer was substituted for poly(C). Figure 4 shows the difference spectra between unreacted poly(C) and reacted poly(C) containing 11.2% of 5-iodocytosine at pH 8.4 after heating to remove the unstable component.

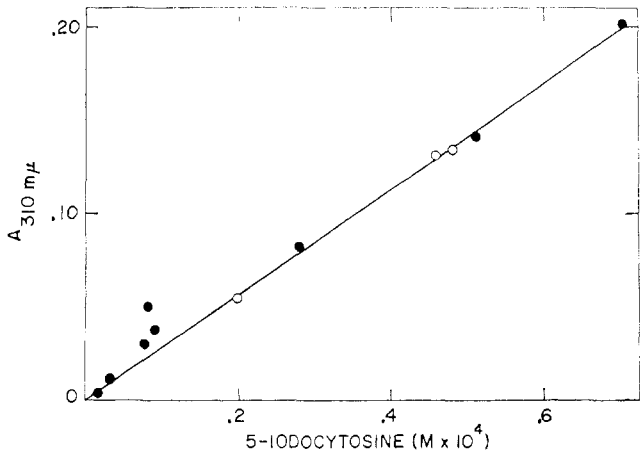

FIGURE 5: The ultraviolet absorption at 310 mμ *vs.* concentration of 5-iodocytosine in solutions of iodinated DNA (●) and poly(C) (○).

At pH 7 and above neither poly(C) nor DNA absorb at 310 mμ. Therefore at this wavelength the absorbance of iodinated polynucleotides is related to the concentration of 5-iodocytosine only. Figure 5 shows that this relationship is linear; extrapolation gives a value of 2820 for the extinction coefficient of a polynucleotide solution 1.0 M in 5-iodocytosine, which is in good agreement with the value found by Michelson and Monny (1967) for polyiodocytosine prepared by polymerizing 5-iodocytidine pyrophosphate. These data were obtained from three samples of iodinated poly(C) and nine samples of iodinated DNA. From 3 to 95% of the cytosine in these samples was iodinated. Concentration of 5-iodocytosine was determined from the concentration of iodine remaining organically bound after heating for 30 min at 60°, pH 9.

The reaction of iodine with poly(U) is qualitatively the same as with poly(C) but very different quantitatively. After heating a mixture of 1.31×10^{-4} M poly(U), 2.5×10^{-4} M KI, and 1.5×10^{-3} $TlCl_3$ for 15 min at 60°, pH 5, 10.5% of the iodine was associated with poly(U). The ultraviolet absorption at 260 mμ was 79% that of the unreacted control but except for an almost insignificant shoulder at 300 mμ, the absorption profile was identical with poly(U). Heating for 20 min at 40°, pH 9 dissociated 96% of the iodine but had no effect on the ultraviolet absorption. However, after heating 2 hr at 86°, pH 8.3 the ultraviolet absorption had risen to 99% of the control level. The rate of increase was that of a first-order reaction with a rate constant of 0.023 min^{-1}.

Extent of Iodination. Figure 6 shows per cent cytosine con-

TABLE II: Maximum Amount of 5-Iodocytosine Formed in Poly(C).[a]

pH	Iodide (M × 10^4)	$TlCl_3$ (M × 10^4)	% Cytosine as 5-Iodocytosine
5	0.2	6	13
5	0.5	6	27
5	1.0	6	45
5	1.0	0	0
5	1.0	0.7	20
5	1.0	3	34
5	1.0	6	43
5	1.0	15	36
4	1.0	6	96

[a] 10^{-4} M poly(C) was heated with $TlCl_3$ and KI for 1 hr at 80°.

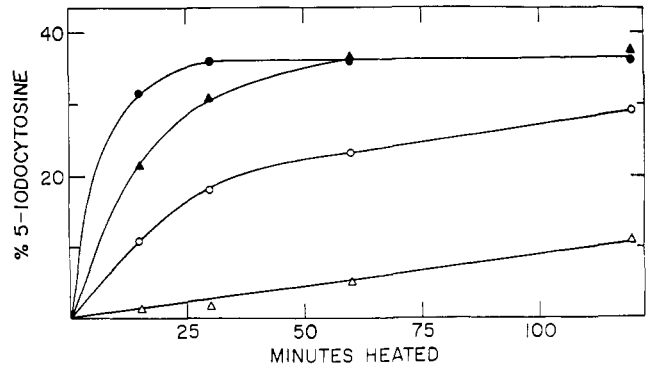

FIGURE 7: The effect of temperature on 5-iodocytosine formation. Solutions of poly(C) were heated at 40° (△), 60° (○), 70° (▲), and 80° (●) at pH 5. The ordinate represents cytosine which had reacted to form 5-iodocytosine.

verted to 5-iodocytosine (open circles) or associated with iodide in either form (closed circles) after heating a solution containing 1.25 × 10^{-4} M DNA cytosine, 1 × 10^{-4} M KI, and 6 × 10^{-4} M $TlCl_3$ at 60°, pH 5, for the times indicated on the abscissa. Similar data appear in Figure 7 where per cent cytosine converted to 5-iodocytosine after heating a solution of 5.27 × 10^{-4} M poly(C), 2.50 × 10^{-4} M KI, and 1.5 × 10^{-3} M $TlCl_3$ at pH 5 and 40, 60, 70, or 80° is plotted. Rate of formation of 5-iodocytosine decreased until a plateau was reached at 40% 5-iodocytosine for DNA and 37% for poly(C). This plateau was independent of temperature, somewhat dependent on $TlCl_3$ concentration, and very dependent on the iodide: cytosine ratio and on pH (Table II). After the plateau was reached, iodination could not be increased unless the polynucleotide was first precipitated or chromatographed on Sephadex, *e.g.*, after heating 1.25 × 10^{-4} M DNA (as cytosine) with 1 × 10^{-4} M KI and 6 × 10^{-4} M $TlCl_3$ for 20 min at 80°, pH 5, 38% of the cytosine was converted to 5-iodocytosine. After precipitating with two volumes of ethanol, redissolving, and again heating under identical conditions, the amount of 5-iodocytosine rose to 58%.

Optimum conditions for trace labeling are generally those which maximize incorporation of the radioactive isotope and minimize that of the stable isotope. Table III indicates that the maximum amount of ^{125}I was incorporated into both native and denatured DNA when the initial iodide concentration was 2.5 × 10^{-5} M. Under these conditions heating 50 μl of a mixture containing 10 μg of native calf thymus DNA and 100 μCi of ^{125}I for 15 min at 60° resulted in incorporation of 2.7 μCi of ^{125}I and iodination of one out of 200 cytosine residues. Higher specific activities could be achieved by decreasing the iodide concentration, but at the cost of diminishing yield. In addition, as the iodide concentration decreases, small impurities may begin to incorporate significant amounts of label. Over 75% of the activity eluting from Sephadex G50 with commercial calf thymus DNA heated at 60° for 15 min with 2.4 × 10^{-6} M KI, 1.5 × 10^{-5} M $TlCl_3$ was bound to an impurity most of which adsorbed irreversibly to the gel after being heated to 90°. All this impurity could be removed either before or after reaction by the standard deproteinization procedure. The native DNA used in these experiments contained no detectable single-stranded material before or after iodination. The ultraviolet absorption of the native DNA in 0.1 M sodium acetate–0.04 M acetic acid at 60° was 1.003 relative to that at 30° in the presence or absence of 1.5 × 10^{-3} M $TlCl_3$, whereas after denaturation it was 1.357.

Iodination with I_2. Polynucleotides can also be iodinated by heating with aqueous iodine. The reaction is the same except that it proceeded more slowly and to a lesser extent than with $TlCl_3$ + iodide. Purines did not react; <0.01% poly(G), poly(A), or poly(I) became associated with iodine when 7 × 10^{-4} M polynucleotide was heated 15 min with 5 × 10^{-5} M I_2 at 60°, pH 5. Table IV shows that denatured DNA was iodinated in preference to native DNA, and cytosine in preference to uracil. The rate of reaction was significantly decreased by adding iodide, raising the pH, or lowering the temperature.

Other Iodinating Agents. Iodination also occurred when DNA was heated with iodide in the presence of auric trichloride. No reaction took place when polynucleotides were heated with iodate.

Biological Properties of Iodinated DNA. Native pneumococcal DNA isolated from a streptomycin-resistant, maltose-utilizing strain was heated 15 min at 60° with 2.5 × 10^{-4} M KI and 1.5 × 10^{-3} M $TlCl_3$ at pH 5. This DNA and unreacted DNA were compared for ability to transform a maltose-negative, streptomycin-sensitive strain of pneumococcus. No sig-

TABLE III: Effect of Iodide Concentration on the Fraction Bound to DNA as 5-Iodocytosine.[a]

KI (M)	% I as 5-Iodocytosine	
	Native DNA	Denatured DNA
0	0.9	2.3
1.0 × 10^{-7}	0.8	6.8
2.5 × 10^{-7}	0.5	5.8
1.0 × 10^{-6}	0.1	5.6
2.5 × 10^{-6}	0.4	5.9
1.0 × 10^{-5}	1.8	17.1
2.5 × 10^{-5}	2.8	31.9
1.0 × 10^{-4}	1.4	21.6
2.5 × 10^{-4}	0.7	9.0

[a] The DNA concentration of each mixture was 1.44 × 10^{-4} M (as cytosine) and the $TlCl_3$ concentration 6 times the iodide concentration, but not less than 6 × 10^{-6} M. The mixtures were heated 15 min at 60°, pH 5.

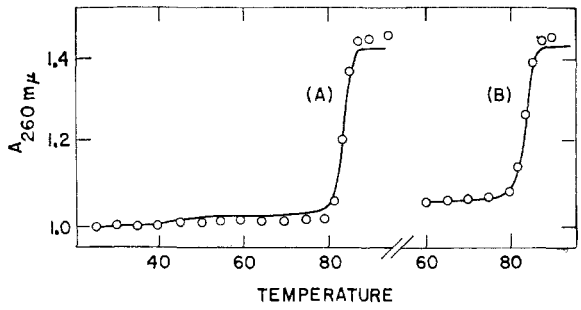

FIGURE 8: Melting profile of native *Cl. perfringens* DNA before (—) and after (O) iodination. The DNA samples were melted (curve A), renatured at 58° for 24 hr, then remelted (curve B).

nificant difference was observed for either marker (S. Lacks, 1970, personal communication).

Physical Properties of DNA. Iodination of native DNA has little or no effect on the melting profile, ability to renature, or sedimentation rate. Iodinated DNA obtained by heating 7×10^{-4} M *Cl. perfringens* DNA with 2.5×10^{-4} M KI and 1.5×10^{-3} M $TlCl_3$ for 32 min at 60°, pH 5, had a sedimentation coefficient of 17.5 S at pH 13 compared to 18.7 S for unreacted *Cl. perfringens* DNA. Figure 8 shows the melting profile of the unreacted DNA (solid line) and iodinated DNA (circles) in 0.4 M NaCl–0.02 M sodium citrate before and after renaturation. Both samples of DNA were heated to 95° (Figure 8, curve A). The temperature of the midpoint of the thermal transition, T_m, was 84.5° for both. Heating the melted DNA solutions at 58° for 24 hr reduced the ultraviolet absorption at 260 mμ of both to 1.05 times that of the unheated solutions. Upon remelting (Figure 8, curve B), the iodinated DNA had a T_m of 83.5° and the unreacted DNA had a T_m of 84°.

Extensive iodination of single-stranded DNA produced significant changes in physical properties. Single-stranded *Cl. perfringens* DNA containing 7% of its cytosine as 5-iodocytosine renatured normally and exhibited a sharp thermal transition when remelted which was identical with that of native *Cl. perfringens* DNA except that it was shifted 1.4° toward lower temperature. Single-stranded *Cl. perfringens* DNA containing 24% 5-iodocytosine no longer fully renatured; when remelted, the thermal transition was relatively broad. Table V shows the results of heating three solutions of 6×10^{-4} M

TABLE IV: Iodination of Polynucleotides with I_2.[a]

Polynucleotide	Departure from Standard Conditions	% 5-Iodocytosine
Denatured DNA	None	4.4
Native DNA	None	0.51
Poly(C)	None	0.65
Poly(U)	None	0.04
Denatured DNA	I_3^-	0.83
Denatured DNA	pH 7	0.40
Denatured DNA	40°	0.44
Denatured DNA	60 min	13.3

[a] Standard conditions were 60°, 15 min, pH 5, 5×10^{-5} M I_2, 1×10^{-4} M polynucleotide cytosine or uracil.

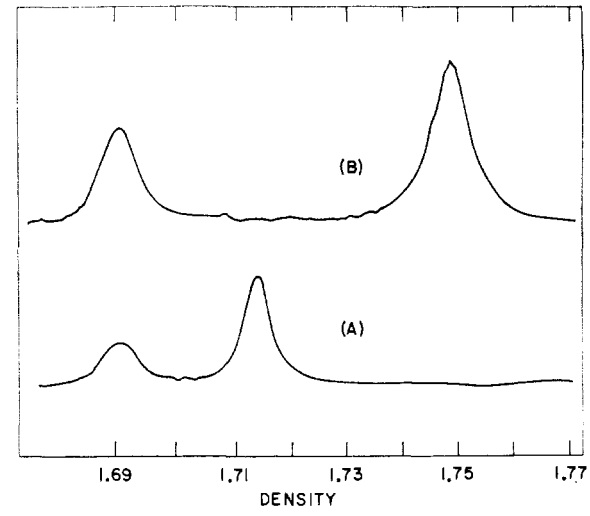

FIGURE 9: Calf thymus DNA in a CsCl density-gradient. The first peak represents *Cl. perfringens* DNA added to provide a density reference. The other peak represents single-stranded calf thymus DNA heated with $TlCl_3$ with (curve B) or without (curve A) iodine.

heat-denatured calf thymus DNA for 15 min at 60°, pH 5. DNA heated in the absence of both $TlCl_3$ and iodide had an $s_{20,w}$ of 15.7 and a buoyant density in CsCl of 1.714. Heating DNA in the presence of $TlCl_3$ did not affect its buoyant density in CsCl but reduced the $s_{20,w}$ to 13.0. Heating DNA in the presence of both $TlCl_3$ and iodide converted 21% of the cytosine to 5-iodocytosine, reduced the $s_{20,w}$ to 9.3, and increased the buoyant density to 1.749. The ultraviolet absorption in CsCl density gradients containing DNA heated with $TlCl_3$ or DNA heated with both iodide and TlC_3 are shown in Figures 9A and 9B, respectively, after centrifuging in 57.6% CsCl for 45 hr at 25°, 44000 rpm. Peak I, at 1.691 g/cm³, represents *Cl. perfringens* DNA which was added as a density marker; peak II represents calf thymus DNA. The pattern for iodinated DNA showed no ultraviolet absorption between the peaks for iodinated calf thymus DNA and *Cl. perfringens* DNA. Evidently all calf thymus DNA was iodinated to about the same extent since otherwise ultraviolet absorption at intermediate densities would have been observed.

Discussion

Pyrimidine iodination probably proceeds according to a mechanism similar to that proposed for pyrimidine bromination (Brammer, 1963; Yu and Zamecnik, 1963): addition of IOH across the 5,6 double bond of the pyrimidine to form the moderately stable 5-iodo-6-hydroxydihydropyrimidine. These compounds should have a weakly bound iodine (since it is

TABLE V: Physical Properties of Iodinated Calf Thymus DNA.

	$TlCl_3$	KI	% 5-Iodocytosine	$s_{20,w}$ (S)	CsCl
1	0	0	0	15.7	1.714
2	1.5×10^{-3}	0	0	13.0	1.714
3	4.5×10^{-4}	7.5×10^{-5}	21	9.3	1.749

attached to a saturated carbon), and would be expected to share some of the properties of the structurally similar uracil and cytosine hydrates, *e.g.*, a tendency to reestablish the 5,6 double bond by splitting out water, which is much more pronounced for cytosine hydrate than for uracil hydrate (Johns *et al.*, 1965; Logan and Whitmore, 1966), and a loss of ultraviolet absorbance above 260 mμ. Because iodine is more electronegative than hydrogen, the pK of 5-iodo-6-hydroxydihydrocytidylic acid should be <5.56, the pK of the hydrate of cytidylic acid (Johns *et al.*, 1965).

On the basis of these assumptions the experimental observations can be explained. Iodination is enhanced by $TlCl_3$, inhibited by excess iodide, and is absent in the presence of iodide or iodate alone because the rate of reaction depends on IOH concentration. The reaction does not go to completion even in the presence of excess iodine because the iodide formed as a result of the reaction inhibits further reaction. No reaction takes place with the 5-substituted pyrimidines thymidine or 5-hydroxymethylcytosine because of steric hindrance. The unstable reaction product of iodine and pyrimidines is the 5-iodo-6-hydroxydihydropyrimidine. At or below pH 5 a significant amount of 5-iodocytosine is formed because dehydration of the protonated cytosine intermediate to form the stable 5-iodocytosine is very rapid. At higher pH, less 5-iodocytosine is formed because the neutral cytosine intermediate dehydrates more slowly and the predominant reaction is deiodination to form cytosine hydrate followed by rapid dehydration to cytosine. Very little 5-iodouracil is formed at any pH for the same reason. The loss of ultraviolet absorption in poly(U) solutions heated with iodine or $TlCl_3$ + iodide is due to the formation of 5-iodo-6-hydroxydihydrouracil. The slow reappearance of ultraviolet absorption during heating at 86°, pH 8.3, denotes dehydration of uracil hydrate to uracil (Logan and Whitmore, 1966).

The effect of DNA conformation on iodination is the same as its effect on photohydration (Grossman and Rodgers, 1968; Setlow and Carrier, 1970) suggesting that the 5,6 double bond of cytosine in native DNA is more resistant to the addition of IOH or H_2O. Conceivably, cytosine which participates in complementary pair formation with guanine of another strand is completely resistant to iodination, and the iodination observed is with single-stranded cytosine already present as an impurity or temporarily present in regions of local melting. This seems unlikely since the DNA used in these experiments contained <1% single-stranded material and no melting was observed below 70°.

Brammer (1963) and Hsu (1964) did not detect binding of iodine after heating RNA or DNA with aqueous iodine. This is not surprising since their reactions were carried out at relatively low temperature (37°), relatively high pH (7 to 10), and in the presence of iodide. Optimum conditions for iodination of nucleic acids fall in a fairly narrow range. Despite this it is possible to incorporate large amounts of radioactive iodine isotopes into native DNA without significantly altering its biological or physical properties. After iodination of native DNA the value of $s_{20,w}$ for the single strands was 17.5 compared to 18.7 before treatment. This corresponds to a single strand molecular weight of 2.4×10^6 before iodination and 2.0×10^6 after. Therefore iodination produced less than one single-strand break per molecule. No other irreversible changes such as deamination were observed. No significant changes were seen in the melting profile or ability to renature or transform.

Extensive iodination of single-stranded DNA was accompanied by significantly changed physical properties. Some of these, *e.g.*, alteration of the ultraviolet absorption profile and increase in density can be attributed to formation of 5-iodocytosine. The decrease in sedimentation rate of single strands at pH 13, which takes place when 21% of the cytosine is iodinated, could denote change in conformation brought about by 5-iodocytosine formation. However a very few single-strand breaks could give the same result since the values of $s_{20,w}$ correspond to a single-strand molecular weight of 1.5×10^6 before iodination and 0.4×10^6 after. Loss of ability to renature when 24% of the cytosine of *Cl. perfringens* DNA was iodinated is difficult to explain. Iodine occupies a position on cytosine equivalent to that of the methyl group on thymidine or of the iodine on iodouracil, hence it should present no steric hindrance to double-strand formation. Moreover, Michelson and Monny (1967) showed that the complex formed between polyiodocytosine and poly(I) is more stable than that formed between poly(C) and poly(I). There are two possibilities: either the structure formed between polyiodocytosine and poly(I) is not compatible with the Watson–Crick structure assumed by the rest of the DNA or an as yet unrecognized irreversible reaction, *e.g.*, formation of diiodocytosine, underlies the disturbance in renaturation. Iodination of guanine is not the cause since although iodine was found to elute at the void volume of a Sephadex G50 column after reaction with poly(G), the amount of iodine was very small, and it may have been bound to an impurity in the poly(G) sample. Degradation of nucleic acid bases is not a factor since heating purine homopolymers in the presence of $TlCl_3$ and KI and heating pyrimidine homopolymers in the presence of $TlCl_3$ does not reduce their ultraviolet absorption. The decrease in the ultraviolet absorption of polyuracil produced by heating with $TlCl_3$ and KI is due to uracil hydrate formation, not degradation, since heating at pH 8.6 restores 99% of the original absorbance. The decrease in the ultraviolet absorption of polycytosine heated with $TlCl_3$ and KI must be due primarily to formation of 5-iodocytosine and cytosine hydrate since at least 96% of the cytosine can be converted to 5-iodocytosine.

Iodination of RNA is complicated by the reaction with uracil, producing small amounts of stable 5-iodouracil and large amounts of moderately stable uracil hydrate which reverts to uracil only after extensive heating (Logan and Whitmore, 1966). However this can be done at nearly neutral pH where depurination and single-strand breakage are minimal.

References

Ascoli, F., and Kahan, F. M. (1966), *J. Biol. Chem. 241*, 428.

Brammer, K. W. (1963), *Biochim. Biophys. Acta 72*, 217.

Braun, R. (1967), *Biochim. Biophys. Acta 149*, 601.

Chamberlin, M. J. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 1446.

Grossman, L., and Rodgers, E. (1968), *Biochem. Biophys. Res. Commun. 33*, 975.

Hsu, Y. (1964), *Nature (London) 203*, 152.

Johns, H. E., Le Blanc, J. C., and Freeman, K. B. (1965), *J. Mol. Biol. 13*, 849.

Lawley, P. D. (1956), *Biochim. Biophys. Acta 21*, 481.

Lindahl, T., Henley, D. D., and Fresco, J. (1965), *J. Amer. Chem. Soc. 87*, 4961.

Logan, D. M., and Whitmore, G. F. (1966), *Photochem. Photobiol. 5*, 143.

Michelson, A. M., and Monny, C. (1967), *Biochim. Biophys. Acta 149*, 88.

Myers, W. G., and Vanderleeden, J. C. (1960), *J. Nucl. Med. 1*, 149.

Setlow, R. B., and Carrier, W. L. (1970), *Biophys. J. 10*, 255a.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Yu, C., and Zamecnik, P. C. (1963), *Biochim. Biophys. Acta 76*, 209.

Studies on Acid Deoxyribonuclease. IX. 5′-Hydroxy-Terminal and Penultimate Nucleotides of Oligonucleotides Obtained from Calf Thymus Deoxyribonucleic Acid

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ABSTRACT: A new procedure has been developed in order to determine the 5′-hydroxy-terminal and penultimate nucleotides of oligonucleotides derived from calf thymus DNA by digestion with hog spleen acid DNase. The essential point in this procedure is the isolation in high yield of the 5′-hydroxy-terminal dinucleoside monophosphates produced by the action of snake venom exonuclease. The dinucleoside monophosphates are then split with spleen exonuclease; the resulting nucleosides and nucleotides, derived from the penultimate and terminal positions of oligonucleotides, respectively, are separated and analyzed. The results obtained on oligonucleotides having an average chain length close to 10 show that purine nucleotides largely predominate at the 5′-hydroxy-penultimate positions (A forming 52% of the nucleotides, G 23%, T 16%, and C 9%), whereas the 5′-hydroxy-terminals show a predominance of G and C (G 34%, C 31%, A 22%, and T 13%). Since the 3′-phosphate-terminal nucleotides of the fragments are formed by G 39%, A 31%, T 21%, and C 9%, respectively, it appears that acid DNase can recognize sequences of at least three nucleotides in native DNA. A novel purification procedure for snake venom exonuclease is described.

It is widely believed that the specificity of DNases is too low to be of any use in the study of nucleotide sequences in DNAs. This opinion is rather due to the difficulty of demonstrating a specificity in DNases than to a well-demonstrated lack of specificity. As a matter of fact, DNases having the evident specificity of pancreatic RNase or T1 RNase, for instance, have not been found yet. Since all four nucleotides are present in the terminals formed by DNases, precise quantitative determinations of the terminal nucleotides are required in order to obtain information on the enzyme specificity. This means that one needs very accurate methods for separation and analysis, besides extremely pure DNases and contaminant-free ancillary enzymes (exonucleases and phosphatases). An effort has been made in recent years in our laboratory to set up an improved technology in this area. This has been used, so far, to investigate the specificity of acid DNase from hog spleen.

Some results concerning the 3′-phosphate-terminal nucleotides of the oligonucleotides obtained from calf thymus DNA have already been published (Carrara and Bernardi, 1968). We wish to present here a new procedure developed in order to determine the 5′-hydroxy-terminal and penultimate nucleotides of oligonucleotides derived from calf thymus DNA by acid DNase digestion. Initially, we tried the classical approach of degrading the dephosphorylated fragments with snake venom exonuclease in order to release the 5′-hydroxy-terminal nucleotides as nucleosides. We rapidly realized, however, that 5′-hydroxy-terminal dinucleoside monophosphates were accumulating in the digestion mixture as a result of enzyme action. The possibility of isolating them in a very high yield encouraged us to set up a procedure for the analysis of the 5′-hydroxy-terminal and penultimate nucleotides. Very briefly, the procedure, summarized in Figure 1, is the following. Oligonucleotide 3′-phosphates, released from calf thymus DNA by spleen acid DNase digestion, are dephosphorylated (step 1), treated with pancreatic DNase in order to decrease their average size (step 2), and digested with venom exonuclease (step 3). The 5′-hydroxy-terminal dinucleoside monophosphates thus liberated are isolated and split with spleen exonuclease (step 4) to release the 5′-hydroxy-terminal nucleotide as a nucleoside 3′-phosphate and the 5′-hydroxy-penultimate nucleotide as a nucleoside. These are subsequently separated and analyzed. This procedure, involving several new techniques to be described below, has led to the quantitative determination of 5′-hydroxy-terminal and penultimate nucleotides of oligonucleotides released by acid DNase.

The results obtained on oligonucleotides having an average chain length close to 10 show that purine nucleotides largely predominate at the 5′-hydroxy-penultimate positions (A forming 52% of the nucleotides, G 23%, T 16%, and C 9%), whereas the 5′-hydroxy-terminals show a predominance of G and C (G 34%, C 31%, A 22%, and T 13%). On the other hand, the 3′-phosphate-terminal nucleotides of the

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