Iodination of DNA. Studies of the Reaction and Iodination of Papovavirus DNA†

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ABSTRACT: Iodination of DNA by the reaction originally described by S. L. Commerford ((1971), *Biochemistry 10*, 1993) is extremely sensitive to the secondary structure of the DNA. Cytidines in denatured simian virus 40 (SV40) DNA react at a slightly slower rate than free cytidine monophosphate; hydrogen-bonded cytidines in SV40 form I DNA are iodinated considerably more slowly; elimination of the negative supercoils in form I DNA by conversion to form II or form III reduces reactivity even further. The residual reactivity of form II or form III duplex DNA is not due to preferential iodination of unpaired cytidines near phosphodiester bond breaks; rather iodination occurs throughout the molecule. Cytidine monophosphate has been used as a model for DNA, to enable spectral measurements of its reaction with iodine and $TlCl_3$. At temperatures above 42 °C and at pH 5.0, formation of 5-iodocytidine is limited by the rate of formation of an intermediate, probably 5-iodo-6-hydroxydihydrocytidine. At lower temperatures, the conversion of intermediate to product is rate limiting, but can be accelerated by lowering the pH. By appropriate adjustment of pH, or temperature, the formation of intermediate or its conversion to product can be accelerated. Iodination destabilizes the DNA duplex. Iodocytosines in SV40 DNA are preferentially removed by S_1 nuclease. Heavily iodinated DNA does not reassociate normally, but DNA with only 5–10% of its cytosines iodinated appears to reassociate with normal kinetics, if duplex formation is measured by hydroxylapatite chromatography. Conditions are described to permit preparation of DNA, which reassociates normally, having a specific activity of 10^8 cpm/μg.

The iodination reaction originally described by Commerford (1971) has been widely applied to RNAs, but has not often been used to label DNAs. The observed preference of the reaction for unpaired cytosines restricts the extent of iodine incorporation into duplex DNA, but might permit iodination to be used as a probe of DNA secondary structure.

We have further investigated the iodination reaction with respect to its specificity for unpaired regions of DNA, using the well-defined DNAs of Polyoma and SV40[1] viruses. Our studies have been primarily directed toward increasing the utility of the iodination reaction for (a) iodinating DNAs at low temperatures, where iodination might be used as a probe of DNA secondary structure; and (b) for achieving high specific activity DNAs which reassociate normally and can be used as probes in DNA reassociation experiments.

Materials and Methods

Preparation of Viral [^{32}P]DNA. CV-1 monkey cells were grown in Auto-Pow MEM medium (Flow), supplemented with 0.03% glycine, 0.025% L-serine, 5% tryptose phosphate (Difco), and 10% calf serum (Gibco). After adsorption with SV40 at a multiplicity of 0.01 PFU per cell, 30 ml of fresh medium was added, and subsequently changed every 48 h. For the third medium change, 20 ml of labeling medium (Dulbecco's modified Eagle's medium with the NaH_2PO_4 concentration reduced to 12.4 mg/l. containing 50 μCi/ml of $^{32}P_i$, nonessential amino acids, and 10% calf serum) was added. When viral cytopathic effect was extensive (usually 24–48 h later), low molecular weight DNA was extracted by a Hirt procedure (Hirt, 1967) modified by Folk (1973). The SV40 DNA was purified by isopycnic centrifugation in CsCl containing ethidium bromide, and by sucrose gradient band sedimentation as described by Folk and Wang (1974). The yield of [^{32}P]DNA was generally 30–50 μg of DNA/plate (15 cm). SV40 DNA containing 60 ± 5 negative superhelical turns was generously donated by Dr. J. Champoux.

Polyoma [^{32}P]DNA was purified from secondary whole mouse embryo cells or mouse 3T6 cells after infection with low multiplicity, plaque-purified Pasadena large plaque Polyoma virus as described by Folk and Wang (1974). Alternatively, high specific activity [^{32}P]DNA was prepared by nick translation of purified polyoma [^{3}H]DNA with *Escherichia coli* DNA polymerase (the generous gift of A. Kornberg) and [α-^{32}P]deoxynucleoside triphosphates as described by Folk (1973).

Preparation of Enzymes and Proteins. Endonucleases HindII and HindIII were purified from *H. influenzae* as described by Smith and Wilcox (1970). HindII was separated from HindIII by chromatography on DEAE-Sephadex A50 (Pharmacia) equilibrated with 0.01 M $NaPO_4$ (pH 7.1), 0.2 mM mercaptoethanol, and 0.1 mM EDTA. The column was eluted with a linear gradient of 0 to 0.4 M NaCl in the above buffer. Endonuclease HincII was purified as described by Landy et al. (1974).

Endonuclease HpaII was purified from *H. parainfluenzae* essentially as described by Sharp et al. (1973) as modified by Folk and Fishel (1975).

† From the Department of Biological Chemistry, University of Michigan School of Medicine, Ann Arbor, Michigan 48104. *Received July 28, 1975.* The work was supported by Grant No. CA13978-02 from the U.S. Public Health Service and Grant No. NP172 from the American Cancer Society.

‡ National Institutes of Health Trainee, Grant No. GM00187.

[1] Abbreviations used are: SV40, simian virus 40; PFU, plaque-forming units; dCMP, 2′-deoxycytidine 5′-monophosphate; dICMP, 2′-deoxy-5-iodocytidine 5′-monophosphate; Tris, tris(hydroxymethyl)aminomethane; EtBr, ethidium bromide; Cl_3CCOOH, trichloroacetic acid; C_0t_{50}, time required for half-maximal reassociation × initial concentration of DNA; form I DNA, covalently closed circular supercoiled DNA; form II DNA, circular DNA with one or more single-stranded phosphodiester bond breaks; form III DNA, linear DNA.

$EcoR_I$ endonuclease was purified from *E. coli* R_{204} RTF-1 using a modification of the procedure of Yoshimori (1971) described by Folk and Wang (1974). Polyoma $EcoR_I$ linears were prepared as described by Folk and Wang (1974).

S_1 nuclease was purified from *Aspergillus oryzae* Takadiastase powder by the procedure of Vogt (1973).

Iodination of DNA. $TlCl_3$ solutions for iodination were freshly prepared by dilution of a 3.7 M solution of $TlCl_3$ (Thallium Ltd, Norwich, England) into degassed sterile 0.1 M sodium acetate (pH 5.0); $Na^{125}I$ (New England Nuclear) was equilibrated with the desired concentration of KI before the reaction. Reaction mixtures were prepared on ice by mixing 0.1 vol of 10 times the desired final concentration of KI + $Na^{125}I$ and sodium acetate-$TlCl_3$ solution, the DNA, and sufficient H_2O to bring to the desired volume (typically 50 μl). After incubation at the desired temperature reactions were stopped by chilling on ice followed by addition of 0.1 vol of 0.1 M Na_2SO_3. Unstably bound iodine was removed by heating the DNA with 0.2 vol of 1.0 M ammonium acetate (pH 9.0) at 60 °C for 30 min (Commerford, 1971). At this point, samples were analyzed for incorporation of iodine in either of two ways.

(a) Sensitivity to Pancreatic DNase. The sample of DNA was made 0.01 M in $MgCl_2$ and digested with excess pancreatic DNase (after addition of a known amount of [^{32}P]DNA to test the completeness of the DNase digestion). Duplicate samples treated and not treated with DNase were then mixed with carrier calf-thymus DNA (Sigma), precipitated with 5% trichloroacetic acid containing 0.1 M KI, and filtered through Whatman GF/C filters. Following several washes of cold 5% trichloroacetic acid containing 0.1 M KI the filters were dried and counted. DNase-sensitive ^{125}I counts were considered bound to DNA.

(b) Isopycnic Banding in CsCl. Aliquots of the reaction mixtures were passed through columns (0.5 × 12 cm for 50-μl reactions) containing Bio-Gel P-30 or P-100 (Bio-Rad), equilibrated with 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 0.1 mM KI. The excluded [^{125}I]DNA was mixed with either CsCl or CsCl-EtBr and banded in a SW 50.1 rotor at 32 000 rpm for at least 17 h. To reduce losses of DNA, polyallomer tubes were pretreated with a 1% v/v solution of dichlorodimethylsilane (Aldrich) in toluene. Fractions containing the [^{125}I]DNA were collected, and either precipitated with acid and filtered through GF/C filters to determine extent of iodination or ethanol precipitated for further use.

To achieve high specific activity viral DNA, the iodination procedure described by Prensky et al. (1973) for RNA was modified as follows. Carrier-free $Na^{125}I$ (5 μl, containing 1-2 mCi in 0.1 N NaOH) was mixed with 2 μl of 0.3 mM Na_2SO_3 in 0.3 M H_2SO_4 and allowed to stand for 15-30 min in a micropipet. It was then quickly mixed with 4 μl of 4 mM $TlCl_3$ (in 0.2 M sodium acetate and 1.0 M acetic acid) and 4 μl of DNA (2 μg of form I viral DNA), in 100 mM sodium acetate (pH 5.0), sealed in a capillary, and placed in a boiling water bath for 1 min. (The high temperature is required to ensure the DNA is denatured, and remains denatured throughout the reaction.) The capillary was broken open, and the contents expelled into 0.1 ml of 1 M ammonium acetate (pH 9.0) containing 10 mM Na_2SO_3. After incubation at 65 °C for 30 min, an equal volume of 0.4 M sodium phosphate (pH 6.8) containing 0.4% sodium dodecyl sulfate was added, and incubation was continued at 65 °C for an additional hour, to complete reassociation of denatured DNA. The mixture was diluted to 1 ml with 0.4% sodium dodecyl sulfate, and passed over a hydroxylapatite column (Bio-Rad HTP) at 60 °C.

The column was washed extensively with 0.14 M sodium phosphate containing 0.4% sodium dodecyl sulfate, and then the duplex DNA was eluted with 0.4 M sodium phosphate containing 0.4% sodium dodecyl sulfate. To separate nondenaturable DNA from denaturable DNA (see Results section), the fractions of 0.4 M sodium phosphate containing the greatest amount of ^{125}I were pooled, diluted with 2.5 vol of water, and boiled for 2 min. The entire sample was quickly passed over a hydroxylapatite column at 60 °C and washed first with 0.14 M sodium phosphate containing 0.4% sodium dodecyl sulfate and then with 0.4 M sodium phosphate containing 0.4% sodium dodecyl sulfate. Generally, 50-70% of the ^{125}I eluted with the first wash, and was considered "denaturable" DNA. All of the ^{125}I was acid precipitable, and had an average sedimentation coefficient of 4-5 S in alkaline sucrose.

All DNAs to be iodinated were rigorously purified and dialyzed against sodium acetate or against H_2O before iodination. Contaminating proteins as well as Tris or phosphate buffers were found to reduce incorporation of iodine into DNA. DNA mixed with protein (e.g., bovine serum albumin or T4 gene 32 protein (Alberts and Frey, 1970) is poorly iodinated, and iodination of the protein occurs as well. To prevent accidental introduction of proteins all buffers were kept sterile. On several occasions, an unidentified high molecular weight substance has contaminated the $Na^{125}I$ or some component of the reaction mixture. This substance is iodinated during the reaction and is excluded in Bio-Gel P-30 with the DNA. It can be removed from the iodinated DNA by banding the DNA in CsCl or by chromatography through hydroxylapatite at 60 °C.

Polyacrylamide Slab Gel Electrophoresis. Endonuclease fragments of viral DNAs were separated by polyacrylamide slab gel electrophoresis as described by Folk et al. (1975).

Sucrose Gradient Sedimentation. Sucrose gradients (5 to 20%) were prepared by successively layering equal volumes of 20, 15, 10, and 5% sucrose solutions in centrifuge tubes and allowing diffusion to take place overnight at room temperature before use. Neutral sucrose gradients contained 0.1 M Tris-Cl (pH 7.5) and 1 mM EDTA; alkaline gradients contained 0.5 M NaOH and 1 mM EDTA. In cases where both [^{3}H]- and [^{125}I]DNAs were present, the ^{125}I counts were determined separately with a γ counter. The overlap of ^{125}I into the ^3H channel of the liquid scintillation counter was determined and subtracted from the observed ^3H counts.

Identification of Iodinated Product. Iodinated DNA was hydrolyzed to free bases by the method of Wyatt and Cohen (1953). The bases were separated by descending chromatography on Whatman 1 mm paper with 86% butanol v/v as solvent. The paper was dried and scanned with ultraviolet light, and the bases were identified by comigration with standards purchased from Sigma.

DNA-DNA Reassociation. Fragmentation of DNAs and measurement of rates of DNA reassociation were carried out essentially as described by Folk and Wang (1974). In some instances, reassociation mixtures contained 10 mM Tris-Cl (pH 7.5) with 0.1 M NaCl, and duplex DNA was detected by resistance to single-strand specific S_1 nuclease digestion. Then each aliquot was mixed with an equal volume of 60 mM sodium acetate (pH 4.6), 2 mM $ZnSO_4$, and 10% glycerol containing 20 μg/ml of denatured salmon sperm DNA. Sufficient S_1 nuclease was added to degrade

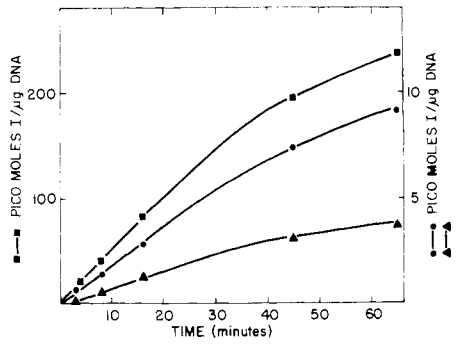


FIGURE 1: Relative rates of iodination of denatured, form II, and form I SV40 DNAs. Reaction mixtures containing 90 mM sodium acetate (pH 5.0), 9.09×10^{-5} M KI, 2.72×10^{-4} M $TlCl_3$, and [^{32}P]DNA at 87.8 μg/ml were incubated at 60 °C. At the indicated time points aliquots were removed and analyzed by banding in CsCl (procedure b under Materials and Methods). The CsCl gradients were collected on filter paper squares and the picomoles of iodine/microgram of DNA was determined from the peak DNA fractions. The symbols represent: (■) denatured DNA, scale on left; (●) form I DNA, scale on right; (▲) form II DNA, scale on right. No correction was made for form I DNA converted to form II during or after the iodination.

90% of the total DNA in 1 h were it 100% denatured. Acid precipitable DNA was collected on nitrocellulose filters, dried, and counted.

SV40 DNA Infectivity Assays. The procedure described by Folk and Wang (1974) was used, except that CV-1 cells were substituted for secondary whole mouse embryo cells. For each sample, several different dilutions of DNA were used, each in triplicate. After 10 days the cells were stained with neutral red, and plaques were counted at several day intervals until no additional plaques appeared.

Results

Iodination of Native and Denatured DNAs. Commerford (1971) reported that iodination of denatured calf-thymus DNA occurs at a faster rate than iodination of native DNA. We have repeated that observation using salmon sperm DNA. Iodination of denatured (boiled) DNA occurs at a five- to tenfold faster rate than iodination of "native" DNA, and the extent of DNA iodinated at 60 °C, pH 5.0, is 10- to 20-fold greater when denatured DNA is used.

The increased reactivity of denatured DNA indicates that unpaired cytosines are much more susceptible to iodination than paired cytosines. However, calf-thymus or salmon sperm DNAs, as normally prepared, undoubtedly have a large number of breaks and single-stranded ends, even in the "native" state, and do not provide a stringent test of the specificity of the iodination reaction. For a more critical test of the reaction specificity, we turned to the well-characterized DNAs of SV40 and Polyoma viruses.

The increased rate of iodination of denatured DNA relative to native DNA suggests that supercoiled SV40 form I DNA, which has partial single-stranded character (Bauer and Vinograd, 1968; Vinograd et al., 1968; Dean and Lebowitz, 1971; Beerman and Lebowitz, 1973), should be more reactive than fully duplex form II DNA. The relative rates of iodination of form I, form II, and denatured SV40 DNA are shown in Figure 1. Form I DNA reacts approximately two times faster than form II DNA and denatured DNA reacts approximately 30 times faster than form I. The velocity of the reaction for each DNA varies with the concentration of DNA and iodide, but when measured under the same conditions, denatured DNA is always the most reactive, followed by form I and then form II.

Table I: Iodination of $EcoR_I$ Linear Polyoma [^{32}P]DNA; Iodine Incorporation into Endonuclease HpaII Fragments.

Fragment	% of Genome[b]	Rel Ratio of ^{125}I/^{32}P[a]
HpaII A	27	0.93
$EcoR_I$/HpaII B-a	20	0.96
HpaII C	17	1.08
HpaII D	13	0.86
HpaII E	7.7	0.96
HpaII F	6.7	0.96
HpaII G	5.2	1.15
HpaII H	1.8	1.10
$EcoR_I$/HpaII B-b	1.4	3.13

[a] Relative ratios equal the ratio of ^{125}I/^{32}P for each fragment divided by the average ratio for all the fragments. [b] Griffin et al., 1974; Folk et al., 1975.

During iodination at 60 °C, less than 35% of the form I DNA is converted into form II DNA; however, most of that conversion is caused by the sulfite treatment after iodination is completed, as there is little change in the amount of conversion with increasing iodination produced by longer reaction periods. Acid hydrolyses of several of the iodinated DNAs, followed by chromatography, demonstrated that greater than 97% of the ^{125}I is incorporated as iodocytosine residues.

Nature of the Reactive Site in Duplex DNA. The small, but detectable iodination of fully duplex DNA may be occurring at hydrogen-bonded cytosines, or at cytosines which are transiently unpaired because of "breathing" of the duplex. In an attempt to determine which alternative is correct, we more carefully examined the kinetics of iodination and the nature of the iodinated sites of the duplex DNAs of Polyoma virus and SV40.

To determine if the reactivity of duplex DNA is limited to easily denaturable regions near phosphodiester bond breaks, native Polyoma [^{32}P]DNA with a single double-stranded break at a defined site (that cleaved by the $EcoR_I$ endonuclease) was prepared and iodinated. The iodinated $EcoR_I$ linear DNA was subsequently digested with Hpa_{II} endonuclease, and the eight Hpa_{II} fragments (Griffin et al., 1974) were separated by acrylamide gel electrophoresis (Folk et al., 1975). The ratio of ^{125}I/^{32}P was determined for each fragment and is shown in Table I. If the termini generated by $EcoR_I$ cleavage of the DNA were particularly susceptible to iodination, an elevated ratio of ^{125}I/^{32}P would have been seen in the Hpa_{II} fragments bounding the $EcoR_I$ site. This was not observed: the large $Hpa_{II}B/EcoR_I$ fragment (Folk *et. al.*, 1975) was iodinated to the same extent as several Hpa_{II} fragments removed from the $EcoR_I$ site. The very small $Hpa_{II}B/EcoR_I$ fragment showed a threefold increase in ^{125}I, relative to ^{32}P, but this fragment comigrates with the tracking dye and is likely to be contaminated with traces of free iodide. Thus, there is no evidence that easily denaturable sites near phosphodiester bond breaks are the only reactive sites for iodination.

To determine if iodination of sites in native form II DNA occurs by a different mechanism than iodination of denatured DNA, the initial velocity of iodination of the two forms of SV40 DNA was determined at various temperatures, and an apparent rate constant for iodination of each form of DNA was calculated (Figure 2). The slopes of the Arrhenius plots, which are a function of activation energy, are almost identical, suggesting that iodination of dena-

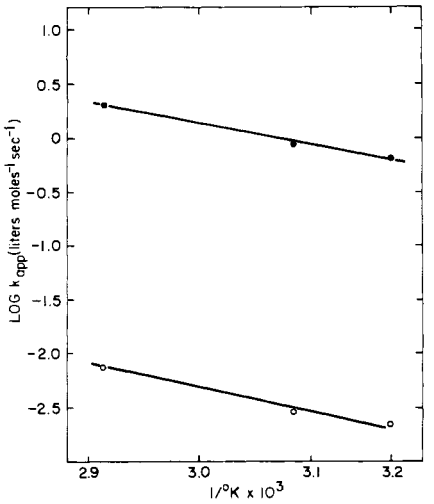


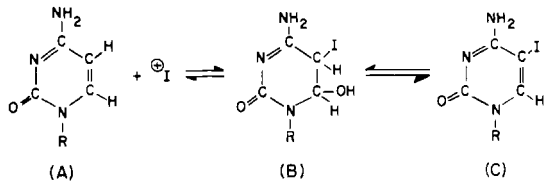
FIGURE 2: Arrhenius plot comparing the rates of denatured and form II SV40 DNA iodination. Denatured and form II SV40 [^{32}P]DNAs (24 μg/ml) were iodinated in a buffer containing 90 mM sodium acetate (pH 5.0), 9.09×10^{-5} M KI, 2.72×10^{-4} M $TlCl_3$. Initial velocities of iodination at 42, 51, and 70 °C were determined by banding in CsCl (procedure b under Materials and Methods). Apparent rate constants were calculated from the initial velocities at each temperature: (●) denatured DNA; (○) form II DNA.

tured and duplex form II DNA occurs by the same reaction mechanism, but that the decreased apparent rate constant for iodination of form II DNA is due to fewer available cytosines.

These results indicate that iodination of native DNA does not occur preferentially at sites near nicks, or at double-stranded breaks, but occurs throughout the molecule at sites which are limiting because of the structure of the duplex DNA.

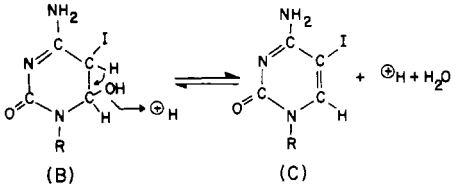
This observed preference of iodination for single-stranded DNAs suggests that the iodination reaction might be used as a probe of DNA secondary structure. To further examine this possibility, we attempted to develop milder conditions for iodination, at which biologically meaningful single-stranded structures might be maintained. To facilitate these studies, 5′-dCMP was used as a reactant in place of DNA.

Iodination of dCMP. The proposed pathway for the $TlCl_3$-mediated iodination of DNA involves the formation of a 5-iodo-6-hydroxydihydrocytidine intermediate (B)



(Commerford, 1971). Using initial reaction velocities, measured at pH 5 at various temperatures, rate constants were calculated for the formation of an intermediate and product, 2′-deoxy-5-iodocytidine 5′-monophosphate (Figure 3). At low temperatures formation of product from intermediate is the rate-limiting step. However, the rate of conversion of intermediate to product appears to have a greater temperature dependence than the rate of formation of intermediate, as seen by the increased slope at low temperature. The rate of formation of product has an altered temperature dependence above 42 °C where the overall rate is then limited by the rate of intermediate formation.

From the proposed pathway, one can predict that conversion of intermediate to product might be acid catalyzed:



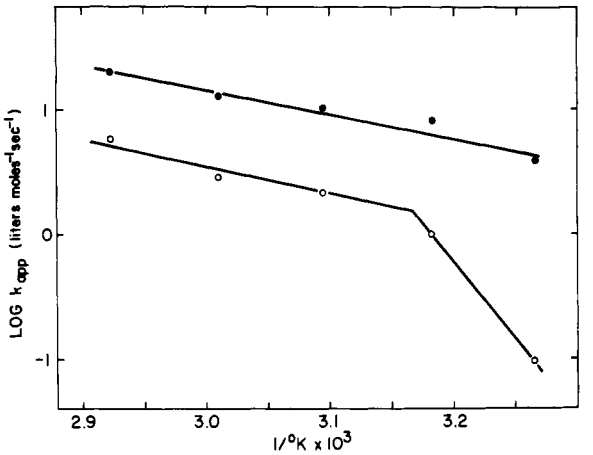


FIGURE 3: Arrhenius plot showing the temperature dependence of the apparent rates of intermediate and product formation in dCMP iodination. Reactions were followed in a Gilford recording spectrophotometer at the indicated temperatures. The disappearance of 5′-dCMP was monitored at 273 nm; the appearance of 5′-dICMP was monitored at 308 nm, a wavelength at which 5′-dCMP does not absorb. It is assumed that the reaction intermediate, probably 5-iodo-6-hydroxydihydrocytidine 5′-monophosphate does not significantly absorb at 273 nm. Using the extinction coefficients of 5′-dCMP and 5′-dICMP under reaction conditions without KI, the absorbancy changes observed during the reaction with KI were converted into molar concentration changes of 5′-dICMP and 5′-dCMP. The concentration of intermediate was calculated by subtracting the observed 5′-dICMP concentration from the difference between the observed 5′-dCMP concentration and the initial 5′-dCMP concentration at any point in the reaction. The rate constants for the formation of product (○) are apparent constants (the composite of at least two reactions is being observed); rate constants for the formation of intermediate (●). Reaction mixtures contained 6×10^{-5} M 5′-dCMP, 5×10^{-5} M KI, 3×10^{-4} M $TlCl_3$, and 0.1 M sodium acetate (pH 5.0).

Therefore, at low temperatures where the conversion of intermediate to product is rate limiting, decreasing the pH should increase the rate of product formation. At pH 5.0 and 37 °C, the intermediate builds up rapidly, but product forms slowly (Figure 4). After 26 h approximately 27% of the 5′-dCMP has been converted to 5′-dICMP and approximately the same amount to intermediate. If the pH is decreased to 4.0 by adding acetic acid, the amount of intermediate decreases and product increases. After 6 h at pH 4.0 approximately 43% 5′-dICMP has been formed. If the pH is decreased to 3.0 by adding acetic acid, intermediate rapidly diminishes and the reaction proceeds to completion (Figure 4).

Stability of Duplex DNA Containing Iodocytosine. Probes of DNA structure should accurately reflect structures that exist prior to reaction and must not in themselves be denaturing agents. Extensive iodination destabilizes the DNA duplex (Commerford, 1971) but the reason for this is not clear; poly(5-iodoC) forms stable complexes with poly(I) (Michelson and Monny, 1967), so the introduction of an iodine atom into the cytidines of DNA should not prevent hydrogen bonding with complementary bases.

Single-strand specific nucleases provide a sensitive method to detect destabilization of the DNA duplex. To test for

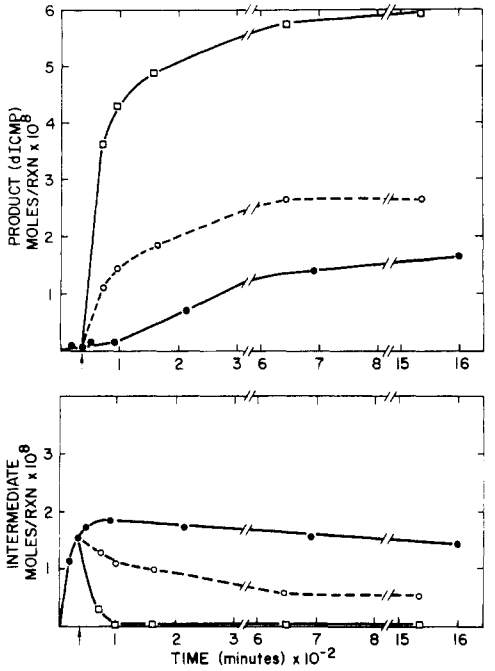


FIGURE 4: Effect of pH on intermediate and product formation in dCMP iodination at 37 °C. Reaction mixtures (1 ml) containing 6×10^5 M 5′-dCMP, 3×10^{-4} M $TlCl_3$, 6×10^{-5} M KI, and 0.1 M sodium acetate (pH 5.0) were incubated at 37 °C for 35 min. Then, to two samples, glacial acetic acid was added to decrease the pH to 4.0 and 3.0, respectively (arrow at bottom of page). Uv spectra were taken at the indicated times. The concentrations of intermediate and product were calculated from the extinction coefficients of 5′-dCMP and 5′-dICMP as described in Figure 1. The results are expressed as moles/reaction to account for the dilution occurring upon the addition of acetic acid: (●) pH 5.0; (○) pH 4.0; (□) pH 3.0.

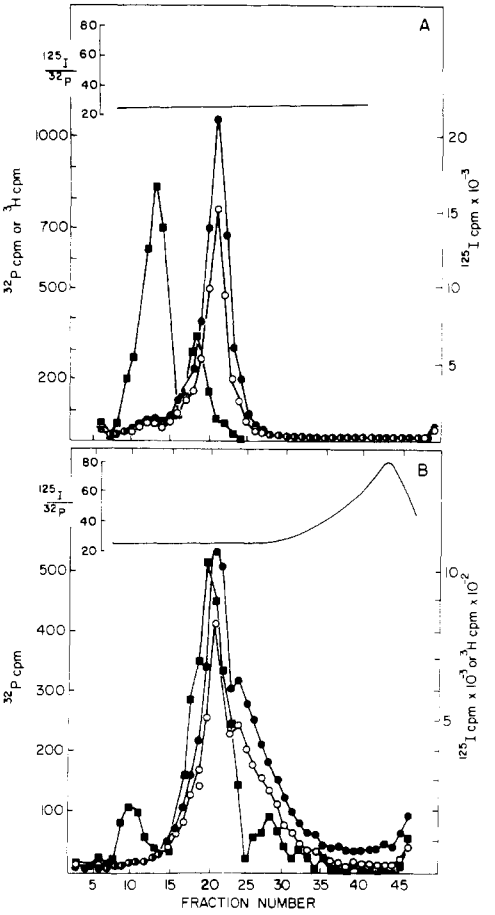


FIGURE 5: Sensitivity of iodinated bases in form II SV40 DNA to S_1 nuclease. Polyoma form I and form II [^{3}H]DNA (4.6 μg) was mixed with 2.4 μg of iodinated SV40 form II [^{32}P]DNA and placed in 30 mM sodium acetate (pH 5.0), 50 mM NaCl, 1 mM zinc acetate, and 5% glycerol containing 10 μg/ml heat-denatured salmon sperm DNA. An aliquot was removed, and the remainder was digested with sufficient S_1 nuclease to degrade 30 μg of denatured DNA to acid-soluble material in 20 min. Both samples were incubated at 37 °C for 30 min, layered on 5–20% neutral sucrose gradients, and centrifuged at 49 000 rpm in a SW50.1 rotor for 210 min at 20 °C. Fractions (8 drop) were collected: (panel A) mixture of DNAs prior to S_1 nuclease digestion; (panel B) mixture of DNAs after S_1 nuclease digestion; (■) a Polyoma [^{3}H]DNA; (○) SV40 DNA (^{32}P, cpm); (●) SV40 DNA (^{125}I, cpm); (—) ratio of ^{125}I/^{32}P.

destabilization of DNA containing only a few iodine atoms, iodinated duplex DNA was incubated with the single-strand specific S_1 nuclease under conditions where normal duplex DNA is not digested but where supercoiled form I DNA is cleaved (Beard et al., 1973). Iodinated SV40 form II [^{32}P]DNA with approximately 1% of the cytidines modified was mixed with polyoma form I and form II [^{3}H]DNA and digested with S_1 nuclease. (The Polyoma DNAs were added to monitor the extent of digestion.) The susceptibility of the DNAs to digestion was determined by sedimentation through neutral sucrose gradients (Figure 5). Panel A shows the profile of the mixture of DNAs prior to digestion; few counts are seen near the top of the gradient, and the polyoma [^{3}H]DNA is mostly form I. After S_1 nuclease digestion, form I polyoma [^{3}H]DNA is largely converted into a sharp peak of form II and linear DNA, and into a few smaller fragments (panel B). Once a molecule is converted to form II or form III, it is resistant to further digestion; however, the iodinated form II SV40 [^{32}P]DNA included in the reaction mixture is slightly degraded by S_1 nuclease, as indicated by the shoulder of lower molecular weight fragments next to the form II DNA peak (the shoulder is absent in the undigested mixture). Radioactivity from the SV40 DNA appears at the top of the gradient; as shown in the insert, the ratio of ^{125}I/^{32}P for the material at the top of the gradient is three times greater than the ratio at the peak of form II DNA, indicating the iodinated DNA is more susceptible to the S_1 nuclease.

As it is unlikely that S_1 nuclease has a preference for iodinated bases, these results indicate that the iodocytosines incorporated into duplex DNA are less stably paired than their uniodinated counterparts. Recently, Shenk et al. (1974) have reported that S_1 nuclease can detect a single base mismatch in heteroduplexed SV40 DNA.

Uniform Iodination of Duplex DNA with a Negative Superhelix Density. Several lines of evidence indicate that form I SV40 and Polyoma DNAs with a negative superhelix density contain specific sites which are more easily denaturable than others (Beard et al., 1973; Morrow and Berg, 1973; Mulder and Delius, 1972; Salzman et al., 1974; Yaniv et al., 1974; Monjardino and James, 1975). However, it is unclear whether some of these sites are single stranded in the absence of denaturing agents. We examined the formation of such single-stranded regions, using the iodination reaction to probe for specific reactive sites. Our first attempts to label form I DNAs were performed at moderate to high temperatures: form I Polyoma and SV40 [^{32}P]DNAs were iodinated at 60 °C, banded in CsCl–EtBr, and digested with site-specific endonucleases (HindII and HindIII for SV40, Hpa_{II} for Polyoma). The fragments were then separated by acrylamide gel electrophoresis and analyzed for ^{125}I incorporation. In several experiments, at levels of iodination ranging from 0.05 to 31 atoms of iodine/

Table II: Iodination of Supercoiled Polyoma DNA; Iodine Incorporation into HpaII Fragments.

HpaII Fragment	% of Genome[b]	Av Rel Ratio of $^{125}I/^{32}P$[a]
A	27	1.04
B	21	0.99
C	17	0.90
D	13	0.97
E	7.7	1.04
F	6.7	0.83
G	5.2	1.03
H	1.8	1.18

[a] The ratios were normalized to the average ratio for each set of fragments as in Table I, producing a relative ratio. The relative ratios from DNAs iodinated to 11.5, 14.2, and 19 molecules of iodine per Polyoma molecule are averaged in the table. [b] Griffin et al., 1974; Folk et al., 1975.

Table III: Iodination of Supercoiled SV40 DNA; Iodine Incorporation into HindII (HindII) and HindIII Fragments.

Fragment	% of Genome[b]	Av Rel Ratio of $^{125}I/^{32}P$[a]
HincII or HindII		
A	38.5	1.05
B	26	1.11
C	20	0.81
D	7.0	1.09
E	4.5	0.86
HindIII		
A	33	1.08
B	22.5	1.12
C	20.5	0.84
D	10.5	0.86
E	8.5	1.09
F	4.5	1.02

[a] Average relative ratios were calculated as in Table II from DNAs iodinated to levels of 0.05, 0.11, 0.46, 0.90, 1.88, 13.0, and 31 molecules of iodine per SV40 molecule and 68 molecules of iodine per SV40 molecule using SV40 DNA with 60 ± 5 negative superhelical turns (obtained from Dr. J. Champoux). [b] Danna et al., 1973.

DNA molecule, the ratio of $^{125}I/^{32}P$ was uniform throughout both genomes (Tables II and III). Furthermore, SV40 DNA with 60 ± 5 negative superhelical turns was iodinated so that 68 atoms of iodine/DNA molecule were incorporated, but again, with seeming uniformity throughout the DNA molecule.

To lessen the overall melting of DNA which undoubtedly occurs at the elevated temperature used for iodination, similar experiments were repeated at 35 °C either at pH 5 using long reaction times or with a short pH jump to pH 3.0 to achieve adequate iodination for analysis. The results of iodination at the low temperature were the same as at high temperature. Iodine was incorporated throughout the genome with no preferred sites (Table IV). Thus, under both sets of conditions, iodination did not detect any unique single-stranded regions in form I DNAs.

It is quite possible that the conditions employed to achieve detectable iodination of DNA are sufficient to destroy transient single-stranded regions. We have attempted to stabilize such regions in Polyoma and SV40 DNAs by first complexing them with T4 gene 32 protein (Alberts and Frey, 1970). However, when 32 protein is iodinated (not bound to DNA) it loses binding activity. Furthermore, when iodinating the gene 32 protein viral DNA complex, we were unable to demonstrate the protein remained bound during the course of the reaction. The DNAs in such a mixture were iodinated uniformly, as in the absence of protein.

Table IV: Iodination of Supercoiled SV40 DNA at Low Temperature; Iodine Incorporation into HindII Fragments and HindIII Fragments.

Fragment	% of Genome[c]	Av Rel Ratio of $^{125}I/^{32}P$	
		pH 5[a]	pH Jump[b]
HindII			
A	38.5	0.95	0.97
B	26.0	0.91	0.91
C	20.0	0.87	0.87
D	7.0	1.14	1.07
E	4.5	1.12	1.17
HindIII			
A	33	0.84	0.81
B	22.5	0.96	0.99
C	20.5	0.99	1.00
D	10.5	0.94	1.05
E	8.5	1.04	0.91
F	4.5	0.94	1.22

[a] Average relative ratios were calculated as in Table II from DNAs iodinated in 0.1 M NaOAc (pH 5.0) at 37°C with 3×10^{-5} M KI and 3×10^{-4} M $TlCl_3$ for 18 and 30 hr. Iodinated DNAs were purified by hydroxylapatite chromatography before digestion. [b] Average relative ratios were calculated as in Table II from DNAs iodinated in 0.1 M NaOAc (pH 5.0) at 37 °C with 3×10^{-5} M KI and 3×10^{-4} M $TlCl_3$ for 1 hr, followed by 0.2 vol of concentrated acetic acid to decrease the pH to 3.0. At 5, 10, and 20 min aliquots were neutralized, heat treated to remove unstable intermediate passed over P-100 columns, and purified by hydroxylapatite chromatography. [c] Danna et al., 1973.

Preparation of Iodinated DNA Suitable for DNA Reassociation Experiments. The marked preference of iodination for single-stranded DNA undoubtedly limits the amount of iodine which can be incorporated into native DNA. If denatured DNA is iodinated at moderate temperatures, it may reassociate during the course of the reaction, limiting the extent of iodination. This may lead to uneven incorporation of iodine into complex DNAs containing differing degrees of sequence repetition. To overcome these problems, and to achieve DNAs of high activity suitable for reassociation experiments, we increased the temperature at which iodination is carried out. In a series of experiments, we observed a steady increase in extent of iodination of SV40 DNA with increasing temperature. However, not until the reaction was carried out at 95–100 °C could DNA with a specific activity of 10^8 cpm/μg be achieved. Using carrier-free iodine (see Materials and Methods) this represents an incorporation of iodine into approximately 10% of the cytosines.

Reassociation of Iodinated DNA. To determine the effect of iodination upon the capacity of denatured SV40 or Polyoma DNA to reassociate, DNAs were iodinated to varying extents and their rates of reassociation were measured and compared with the rates of reassociation of noniodinated DNAs.

In the course of these experiments an unexpected problem was encountered when, after iodination, attempts were made to fully dissociate the strands of DNA. Often, up to 50% of the iodinated DNA would not denature, as measured by fractionation on hydroxylapatite. This "nonde-

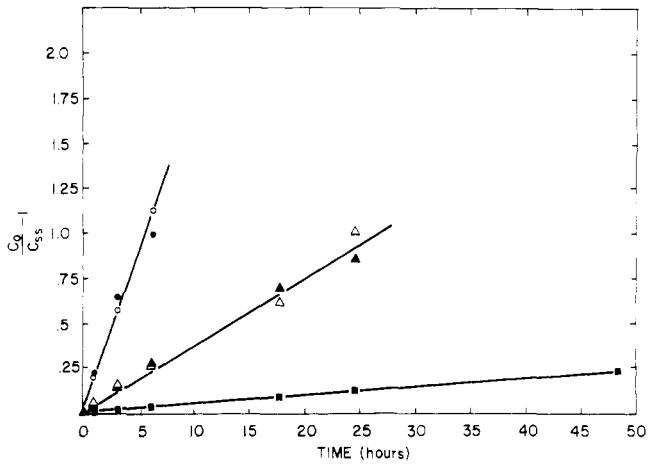


FIGURE 6: Reassociation kinetics of high specific activity [^{125}I]DNA. Polyoma DNA was iodinated to a level of approximately 6×10^7 cpm/μg as described under Materials and Methods. [^{32}P]DNA was prepared by nick translation of Polyoma DNA with *E. coli* DNA polymerase and four α-[^{32}P]deoxyribonucleoside triphosphates (Folk, 1973). The reassociation of polyoma [^{125}I]DNA was examined alone or together with polyoma [^{32}P]DNA. Each DNA had an average sedimentation coefficient in alkaline sucrose of 3-4.5 S. DNAs were denatured in 0.2 N NaOH, neutralized with 0.2 N HCl, and incubated at 68 °C in 1.0 M sodium phosphate (pH 6.8). At the indicated times, 0.1-ml samples were removed and fractionated on hydroxylapatite: (■) [^{125}I]DNA (2.3×10^4 cpm/ml, approximately 3×10^9 cpm/A_{260}); (▲) [^{125}I]DNA (9.3×10^4 cpm/ml, or 3.2×10^{-5} A_{260}/ml) mixed with (△) [^{32}P]DNA (5300 cpm/ml, or 3.2×10^{-5} A_{260}/ml); (●) [^{125}I]DNA (5050 cpm/ml, or 1.7×10^{-6} A_{260}/ml) mixed with (○) [^{32}P]DNA (4.7×10^4 cpm/ml, or 2.8×10^{-4} A_{260}/ml). In all three instances, the calculated C_0t_{50} for the reassociation of the total polyoma sequences in the incubation is 6.5 ($\pm$0.5) $\times 10^{-3}$ mol $\times$ s $\times$ l.$^{-1}$ (corrected to 0.12 M $NaPO_4$).

naturable" material interferes with measurements of the rate of reassociation, and is present in preparations of DNA with differing levels of iodination. In CsCl gradients at neutral pH, the "nondenaturable" material bands as a broad peak with a density ranging from that of single-stranded DNA to that of native DNA. The majority of the material is made acid soluble after digestion with S_1 nuclease, indicating that it is not fully duplex DNA. When SV40 DNA with greater than 60% of the cytosines iodinated was heated to 100 °C, and then cooled to 65 °C to permit reassociation, approximately 40% of the counts appeared as "nondenaturable" DNA at the earliest timepoint (no incubation at 65 °C). This material can be separated from the remainder of the DNA by hydroxylapatite chromatography (see Materials and Methods).

After removal of the nondenaturable DNA, the remainder of the heavily iodinated DNA renatured more slowly than expected. When mixed with an excess of noniodinated DNA, the iodinated DNA still reassociated more slowly than expected, indicating that extensive iodination interferes with reassociation, even when one of the strands contains no iodocytosine. At lower levels of iodination, reassociation between strands appears to occur normally. Polyoma DNA iodinated with carrier-free ^{125}I so that 5% of the cytosines modified (producing a specific activity of approximately 6×10^7 cpm/μg) also contained substantial amounts (50%) of labeled material which fractionated on hydroxylapatite as duplex DNA immediately after heating to 100 °C. After this "nondenaturable" material was separated from "denaturable" iodinated DNA by hydroxylapatite chromatography, the "denaturable" [^{125}I]DNA was allowed to reassociate (Figure 6). Because of the uncertainty

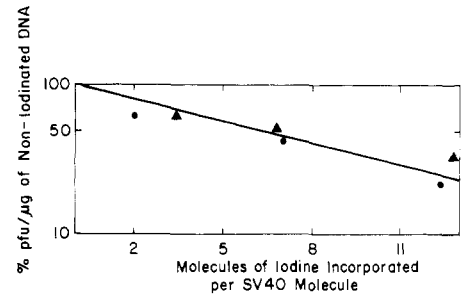


FIGURE 7: Inactivation of SV40 DNA infectivity by iodination. Form I SV40 DNA was iodinated at 60 °C in a buffer containing 90 mM sodium acetate (pH 5.0), 9.09×10^{-5} M KI, 2.72×10^{-4} M $TlCl_3$, and DNA at 60 and 78 μg/ml (two separate experiments) to the indicated levels by varying the length of reaction time. Each iodinated form I DNA was tested for infectivity along with untreated SV40 DNA form I and SV40 DNA form I taken through the iodination procedure either without $TlCl_3$ or without KI. Control DNAs in reactions without $TlCl_3$ or without KI were not iodinated and did not lose infectivity. Circles and triangles represent two independent experiments.

in the specific activity of the iodinated DNA (the quantity of DNA iodinated is too small to permit accurate measurement of the amount recovered) the exact rate of reassociation of the [^{125}I]DNA could not be measured; however, if the iodine was incorporated evenly throughout the "denaturable" and "nondenaturable" material so that radioactivity reflects the amount of DNA, the observed C_0t_{50} (6×10^{-3} mol $\times$ s/l.) for the iodinated DNA is quite close to that expected of Polyoma DNA fragmented to a size of 3-4 S. Furthermore, the correspondence between the rates of reassociation of iodinated DNA and [^{32}P]DNA when the two are mixed together (in equal amounts, or with an excess of [^{32}P]DNA; Figure 6) and allowed to reassociate indicates that the iodinated DNA reassociates normally. Thus, this DNA, at a specific activity of approximately 6×10^7 cpm/μg and with approximately 5% of its cytosines iodinated, cannot be distinguished from normal DNA in its reassociation kinetics. Other SV40 and Polyoma DNAs, with less extensive iodination, also exhibit normal reassociation kinetics. It is likely, however, that rates of reassociation might appear altered if duplex formation is measured by resistance to S_1 nuclease. This is because of the apparent destabilization of the duplex that is caused by the iodocytosine, an effect that is detected by the nuclease but not by hydroxylapatite.

Effect of Iodination on Viral DNA Infectivity. Incorporation of halogenated uridines into DNA induces mutations, possibly by altering base pairing, or base stacking (Freese, 1959; Sternglanz and Bugg, 1975; Kaplan and Ben-Porat, 1966). To determine the effect of iodination of cytosines upon the infectivity of SV40 DNA, the specific infectivity of form I DNAs (repurified by CsCl-EtBr banding after iodination) containing varying amounts of iodine was measured on monolayers of CV-1 cells. Data from two separate iodinations and infectivity assays are shown in Figure 7. In both cases, iodine incorporation diminishes the specific infectivity of form I SV40 DNA. Other DNAs treated under identical conditions, in the absence of iodine, did not lose infectivity.

Discussion

The proposed pathway for the iodination of cytidine involves the formation of an unstable 5-iodo-6-hydroxydihydrocytidine intermediate which is converted by the loss of H_2O to the stable product, 5-iodocytidine. When iodination

of 5′-dCMP is followed spectrophotometrically, the difference between the rate of decrease in absorption of 5′-dCMP and the rate of increase of 5′-dICMP absorption indicates the existence of at least one intermediate in the reaction. If the initial rates of formation of the putative intermediate and of the product are plotted as an Arrhenius plot, the conversion of intermediate to product is seen to be the rate-limiting step at low temperatures. One can predict that the conversion of the proposed intermediate to product would be acid catalyzed. In fact, decreasing the pH accelerates the conversion of intermediate to product when the iodination of 5′-dCMP is conducted at low temperature, where conversion of intermediate to product is rate limiting.

Differences are observed between the iodination of 5′-dCMP and that of DNA. The pH optimum for the reaction with DNA is 4.0 (Orosz and Wetmur, 1974) whereas it is closer to pH 3.0 with 5′-dCMP. Furthermore, denatured DNA reacts more slowly than 5′-dCMP. This may be due in part to steric interference with the electrophilic attack of I^+ by neighboring bases in the polynucleotide chain, or it may be due to changes in the stability of the intermediate formed after electrophilic attack. The stability of uridine hydrates is dependent upon the chain length of polynucleotides (Logan and Whitmore, 1966).

The observation that iodination proceeds faster with denatured DNA than with native DNA suggests that either base stacking, or base pairing, interferes with the reaction, or that the iodination of the two forms of DNA proceeds by different mechanisms. Kinetic analysis of the iodination of native and denatured DNA indicates that the reaction with each has the same activation energy, which is consistent with a similar mechanism for both forms of DNA. The slower apparent rate of reaction of native DNA may simply be due to a decreased concentration of reactive bases. Since there does not appear to be preferential iodination of DNA surrounding the termini of linear molecules of Polyoma DNA, it seems likely that "breathing" of native DNA (von Hippel and Wong, 1971; Utiyama and Doty, 1971) provides the unpaired bases which can react with the iodine.

Form I SV40 DNA, because of its superhelical character, is in a higher energy state than form II SV40 DNA (Bauer and Vinograd, 1968), and reacts more readily with iodine. The positive free energy contributed by the superhelical turns destabilizes the duplex, generating a greater amount of single-stranded DNA than is found in form II DNA (Dean and Lebowitz, 1971; Beerman and Lebowitz, 1973). Increasing the temperature accelerates the rate of iodination of form I DNA relative to denatured DNA, supporting the notion that melting of the DNA is prerequisite for iodination. It seems likely, then, that the iodination reaction could be used to examine the dynamic aspects of DNA structure in a fashion similar to that used with formaldehyde.

We reacted SV40 form I DNA with iodine to determine if specific regions of the duplex were opened more frequently than other regions. This was not observed; iodination occurred uniformly throughout the DNA. This is in contrast to the observation that a water-soluble carbodiimide, *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide, with a specificity for unpaired bases, reacts preferentially with only three regions of SV40 form I DNA (Salzman et al., 1974). There are several possible explanations for these divergent results.

(1) The water-soluble carbodiimide reacts with guanine and thymine residues (Ho and Gilham, 1967); the latter occur frequently in regions having a low T_m. On the other hand, iodination proceeds only with cytosine residues, which occur more frequently in regions having a high T_m. Thus, the specificity of iodination makes it a less suitable probe for regions of DNA which are only transiently single stranded.

(2) The water-soluble carbodiimide reacts with ring nitrogens involved in base pairing, thereby disrupting the duplex. Reaction with DNA may be cooperative, and lead to "unzippering" of regions of DNA that are not normally completely denatured. This might magnify the extent of single-stranded character at any one site in the DNA molecule. On the other hand, iodination is less likely to be cooperative, as the reactive site in the pyrimidine ring is not involved in base pairing; thus, reactivity with iodine may more accurately reflect the dynamic breathing of the form I DNA.

It is presently not possible to rule out the existence in negatively supercoiled DNAs of specific regions with stable single-stranded character such as the hairpin structures proposed by Gierer (1966). Reaction of form I DNA at specific sites with single-strand specific nucleases (Beard et al., 1973; Kato et al., 1973), T4 gene 32 protein (Morrow and Berg, 1973; Yaniv et al., 1974; Monjardino and James, 1975), and a water-soluble carbodiimide (Salzman et al., 1974) supports the notion that there are regions which can be easily denatured. However, as mentioned before, the potential of these reagents to react cooperatively, to denature DNA, makes it likely that they do not accurately reflect the extent and duration of transient single-stranded character in form I DNA. Evidence against the existence of such stable single-stranded regions in other DNAs can be drawn from several different experimental approaches (Maniatis and Ptashne, 1973; Wang et al., 1974; Chan and Wells, 1974; Wang, 1974). More extensive studies of the changes in physical properties of supercoiled DNA upon reaction with such reagents, as well as of the dynamics of breathing of supercoiled DNA, are required before a judgment can be made.

Iodination, even to a low level, does affect the stability of the duplex, as shown by the increased susceptibility of iodinated bases to S_1 nuclease excision. Recently Shenk et al. (1974) have observed that S_1 nuclease may detect regions of DNA with single mispaired bases. The increased susceptibility of iodinated DNA to S_1 nuclease warrants extra caution when studying rates of DNA reassociation using single-strand specific nucleases.

At low or moderate levels of iodination, there is no effect upon the apparent rate of reassociation of DNA when hydroxylapatite is used to separate duplex from single-stranded DNA. The majority of the bases in such duplexes, however, are not iodinated. Heavily iodinated DNA does reassociate more slowly than noniodinated DNA. Other observations, consistent with the destabilizing effect of iodination, have shown that heavily iodinated DNA has a decreased T_m and a broader thermal transition than noniodinated DNA (Commerford, 1971; Orosz and Wetmur, 1974).

After iodination a variable amount of DNA fractionates on hydroxylapatite as if it were "nondenaturable" by boiling or alkali treatment. The nondenaturable character of this material decreases with time when the DNA is stored. We considered the possibility that a fraction of the DNA is cross-linked during the reaction, but this could not conclusively be proven. This "nondenaturable" material has a

buoyant density in CsCl that is intermediate between native and denatured DNA, and it can be separated from single-stranded DNA by fractionation on hydroxylapatite. Thus, before iodinated DNA can be used as a probe for DNA reassociation experiments, this material should be removed or else it will yield a high background of seemingly duplex DNA. Extrinsic iodination should prove to be an easy, useful method to make probes for DNA–DNA reassociation studies. We have obtained specific activities as high as 1×10^8 cpm/μg without an apparent effect upon the reassociation kinetics (measured by hydroxylapatite).

Iodination inactivates the infectivity of SV40 DNA. This is in contrast to the observation that bacterial transforming DNA is not affected by low levels of iodination (Commerford, 1971) but agrees with the interpretation of Brammer (1963) on the effect of iodination on TMV-RNA. Buettner and Werchau (1973) have shown that incorporation of iododeoxyuridine inactivates SV40 DNA. Experiments in which iodinated DNA was exposed to strong fluorescent light for over an hour (unpublished) suggest that chain breakage from visible light absorption is insufficient to account for the observed inactivation. Brammer (1963) suggested the large size of the iodine atom might cause it to act primarily as an inactivating agent. However, iodinated nucleotides can be utilized by DNA polymerase (Kamen et al., 1974; Shaw et al., 1975; Scherberg and Refetoff, 1974) and Kaplan and Ben Porat (1966) concluded that iododeoxyuridine caused the synthesis of nonfunctional proteins involved in pseudorabies virus assembly. X-ray crystallographic evidence (Sternglanz and Bugg, 1975) suggests that altered stacking interactions account for mispairing between 5-halogenated uracil residues and guanine residues in DNA. Presently, we are continuing studies of the effect of iodination upon the biological activity of DNAs.

Acknowledgment

We thank Dr. Jules Shafer for helpful discussions, Dr. Prasanta Datta for critical evaluation of the manuscript, and Barbara Fishel for assistance with several of the enzyme purifications.

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