In Vitro Iodination of Low Complexity Nucleic Acids without Chain Scission†

How-Ching Chan, William T. Ruyechan, and James G. Wetmur*

ABSTRACT: In vitro $TlCl_3$ catalyzed iodination of DNA may be performed at 60 °C, pH 4.7, in concentrated $NaClO_4$ solutions where double-stranded DNA is unstable. Other denaturing solvents inhibit iodination. The use of a denaturing solvent prevents DNA renaturation during the iodination procedure. The iodination reaction takes no more than 5 min with 15–20% of input iodide found in iodocytosine. The second heating step, to remove labile iodine, is performed at pH 7 under renaturation conditions to minimize depurination and other damage. A quantitative determination of chain scission accompanying iodination has been made. The single-strand molecular weight of the product DNA is 5×10^6/percent cytosine as iodocytosine. When the iodide to cytosine ratio in the reaction mixture is 0.02 or less, λ DNA may be labeled without significant chain scission as assayed by alkaline sedimentation velocity. A Hind III digest of iodinated, renatured, and endonuclease S1 treated λ DNA has a band pattern identical with unlabeled DNA upon agarose gel electrophoresis. Double-stranded RNA may also be labeled in concentrated $NaClO_4$ solutions.

The many uses of in vitro iodination of nucleic acids have recently been reviewed by Prensky (1976). Orosz and Wetmur (1974) described conditions for using the $TlCl_3$ catalyzed in vitro iodination procedure of Commerford (1971) in a manner which minimized the formation of modified bases which interfere with base pairing. DNA complexity is defined as the number of base pairs present in a nonrepeating sequence. Low complexity DNA could only be iodinated at higher temperatures where renaturation would not occur simultaneous with labeling. Even the use of 80 °C is insufficient to prevent all renaturation. Hence, a heterogeneous product, in terms of iodination, will result. The standard method, and expecially the low complexity method, resulted in significant DNA chain scission with single-strand molecular weights reduced to about 200 000. The reaction which resulted in chain scission was shown to require the formation of iodocytosine or an intermediate in the reaction leading to iodocytosine.

In this work, we have sought solutions to two problems. First, we have looked for and have found a denaturing solvent which allows iodination of low and high complexity DNAs, as well as RNAs, to be performed under the same conditions. Second, we have quantitated chain scission which accompanies iodination. Then, using the known requirements for chain scission, we have obtained iodinated λ DNA with intact single strands. These conditions should allow almost all restriction fragments of all iodinated and renatured DNAs to behave the same way as unlabeled DNA in agarose gel electrophoresis.

Materials and Methods

Materials. λcI857S7 coliphage and DNA were prepared as previously described (Orosz and Wetmur, 1974). *Escherichia coli* DNA was obtained from Sigma Chemical Co. *Penicillium chrysogenum* mycophage double-stranded RNA was a gift of Dr. Richard Douthart, Eli Lilly, Inc. Preparation and assay conditions for endonuclease S1 were those of Hutton and Wetmur (1973). Hind III restriction endonuclease was obtained from Miles Labs, Inc. Carrier-free $Na^{125}I$ (in NaOH) was obtained from Amersham/Searle, Inc. $TlCl_3$ was obtained from Allied Chemical Co. $NaClO_4$ was obtained from G. Frederick Smith Chemical Co. Concentrated $NaClO_4$ solutions were filtered through 0.22-μm Millipore filters before use. Failure to filter the solutions resulted in reduced iodination of DNA if the $NaClO_4$ solution was added to a DNA solution. All other chemicals were reagent grade and were used without further purification.

Sedimentation. Neutral and alkaline band velocity sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge. The method and treatment of data to obtain molecular weights are described by Studier (1965). Preparative CsCl density gradient sedimentation of DNA was performed at density 1.70 in an SW50L rotor.

Electron Microscopy. DNA was visualized in an AEI EM801 electron microscope after mounting by the modified Kleinschmidt technique described in Davis et al. (1971).

DNA Restriction and Agarose Gel Electrophoresis. DNA was digested with endonuclease Hind III using conditions specified by the supplier and analyzed by electrophoresis on a 0.7% agarose slab gel (Helling et al., 1974).

Nucleic Acid Iodination and Counting. Nucleic acid concentrations, KI and $Na^{125}I$ concentrations, and denaturing solvent concentrations are given in the text. Unless otherwise specified, a reaction mixture containing DNA, KI, and $Na^{125}I$ as well as 0.1 M acetate buffer, pH 4.7, was heated to 60 °C for 15 min immediately after addition of $TlCl_3$ to a concentration of 9×10^{-4} M. The reaction was stopped by ice quenching. The DNA was dialyzed overnight at 4 °C into 0.4 M NaCl–0.01 M phosphate buffer–2×10^{-4} M EDTA,[1] pH 6.86. After buffer change, the buffer and DNA in the dialysis sac were heated to 60 °C for 2 h. After another buffer change and dialysis, the DNA was recovered from the dialysis sac and stored at −20 °C. Samples were counted using a Beckman Biogamma γ-counting system. The safety precautions for use of ^{125}I described by Prensky (1976) were used throughout this work.

† From the Department of Microbiology, Mount Sinai School of Medicine of the City University of New York, New York 10029. *Received July 2, 1976.* This work was supported by United States Public Health Service Grant No. GM 22029 from the National Institutes of Health.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; IC, iodocytosine; PCM, *Penicillium chrysogenum* mycophage.

TABLE I. Effect of KI and DNA Cytosine Concentrations on Iodination.

[I^-]	[Cytosine]	% ^{125}I as IC	% C as IC
1.5×10^{-4}	8.3×10^{-5}	6.7 (12.1 of possible)	12.1
1.5×10^{-5}	8.3×10^{-5}	17	3.1
	3.5×10^{-5}	18	7.2
	8.3×10^{-7}	0.87 (15.6 of possible)	15.6
1.0×10^{-5}	4.15×10^{-4}	17.7	0.43
	2.07×10^{-4}	18.2	0.88
	2.07×10^{-4}	14.4	0.70
	1.04×10^{-4}	5.5	0.53
	5.2×10^{-5}	12.4	2.4
	2.6×10^{-5}	5.0	1.9
	1.3×10^{-5}	3.0	2.7
1.5×10^{-6}	8.3×10^{-5}	3.0	0.05
	3.5×10^{-5}	1.2	0.05
	8.3×10^{-7}	0.78 (14 of possible)	14

Results and Discussion

Denatured *Escherichia coli* DNA at a cytosine concentration of 1.5×10^{-4} M and KI at 1.5×10^{-4} M plus a trace amount of $Na^{125}I$ were used in an iodination reaction employing the conditions for minimal damage specified by Orosz and Wetmur (1974). Solvents used in the reaction were water or 2.4 M tetraethylammonium chloride (Et_4NCl), 50% dimethyl sulfoxide (Me_2SO), 50% $HCONH_2$, or 5.8 M $NaClO_4$. All of these solvents except water are denaturing solvents. Defining the effectiveness of iodination in water, about 20% conversion of cytosine to iodocytosine, as 100%, the effectiveness of iodination in the denaturing solvents was 3, 6, 6, and 96%, respectively. Only concentrated $NaClO_4$ does not interfere with iodination. The melting temperature (T_m) of a DNA with 50% guanine plus cytosine in 5.8 M $NaClO_4$ is 54 °C. The dependence of T_m on guanine plus cytosine content is 0.52 °C per percent. The effect of $NaClO_4$ concentration on T_m is given in Wetmur and Davidson (1968). Varying $NaClO_4$ concentration in several iodination experiments, always with T_m less than 60 °C, showed no effect of $NaClO_4$ concentration on iodination efficiency. Iodinated DNA was banded in CsCl in a preparative ultracentrifuge. By dripping tube and counting the fractions it was established that the counts banded at a density near 1.7. The efficient iodination of DNA in concentrated $NaClO_4$ solutions means that low complexity DNAs, such as viral or bacteriophage DNAs, may be iodinated at 60 °C without interference by simultaneous renaturation. All further experiments discussed in this work were performed in denaturing $NaClO_4$ solutions.

Aside from the DNA and KI concentrations, which are considered in detail below, potential variables in the in vitro iodination reaction for DNA are the pH and reaction times for the first and second 60 °C heating steps. Because the initial iodination step requires a pH less than or equal to 5 to proceed, no attempt was made to vary the pH 4.7 buffer. Although the rate of depurination is finite at pH 4.7 and 60 °C (Lindahl and Nyberg, 1972), the high salt concentration partially compensates for the low pH. The effect of reaction time was investigated using λ DNA at 1.5×10^{-4} M cytosine and KI at 1.5×10^{-5} M. Using the amount of iodide found in iodocytosine at 15 min as 100% of possible iodination, reactions carried out for 1, 2, 4, and 8 min resulted in 34, 100, 93, and 79%, respectively. Except for the 1-min value, the reaction appears to be completed. A second experiment carried out for 5, 10, and 15 min showed 101, 86, and 100% of possible iodination. These results imply that the iodination reaction is faster in concentrated $NaClO_4$ than it is in H_2O. The data presented below are all the result of 15-min incubations, although there appears to be no reason why shorter reaction times could not be employed.

The solvent conditions for the second heating step, necessary for removing labile iodine, are the same as employed by Orosz and Wetmur (1974) except for pH. Using the pH 6 solvent of Orosz and Wetmur as allowing 100% of maximum labeling, incubations at pH 5, 7, and 8 were found to produce 46, 86, and 46%, respectively. High pH was shown by Orosz and Wetmur (1974) to increase the percent incorrect modification of DNA as measured by T_m dependence of renatured products. However, incubation at pH 6 or pH 7 had the least effect on T_m. In order to minimize possible depurination reactions in the long second heating step, a pH 6.86 buffer was used for all subsequent experiments even though pH 6.0 buffer leads to maximum labeling. The time of the second heating step does not affect the yield. Heating for 2.5, 5, or 7.5 h at 60 °C led to 100, 100, and 94% of maximum labeling. Scherberg and Refetoff (1974) showed that heating beyond 1 h had no effect if the pH was greater than 6. All subsequent second incubations at pH 6.86 were carried out for 2 h.

In order to determine the maximum possible single-strand DNA length achievable using the reaction conditions specified above, λ DNA was incubated at pH 4.7 in 5.8 M $NaClO_4$ with 9×10^{-4} M $TlCl_3$ and 1.5×10^{-4} M KCl (instead of KI) for 15 min at 60 °C. After dialysis to 0.4 M NaCl, 2×10^{-4} M EDTA, 0.01 M phosphate, pH 6.86, the solution was heated for 2 h at 60 °C in the dialysis tubing. These conditions are the same as used for all iodination reactions except for the substitution of KCl for KI. Analysis of the product λ DNA by alkaline band velocity sedimentation showed 80% of the single strand to be intact. Therefore, the reaction time of either the first or second heating step is unimportant in terms of depurination and chain scission as assayed in alkali. Thus, as found before (Orosz and Wetmur, 1974), chain scission due to iodination must be the result of the iodination reaction itself.

The variables remaining are DNA and KI concentrations. These variables affect the yields in terms of both percent I found in iodocytosine (IC) and percent cytosine converted to iodocytosine. The variables also affect the single-strand molecular weight of the product DNA. Yields are shown as a function of the concentration variables in Table I. The results (with one exception) indicate that 12–18% of input iodide is found in iodocytosine if the KI concentration is greater than or equal to 1.0×10^{-5} M and the cytosine concentration is much greater than the iodide concentration. Under these conditions, the iodination reaction in concentrated $NaClO_4$ is quite reproducible. A rather large (up to 16%) conversion of cytosine to iodocytosine is possible. However, significant chain scission follows such a large conversion.

We are interested in finding conditions which affix the 12–18% of input iodide (and ^{125}I) to the DNA without damaging the single strands. Since absence of KI eliminates chain scission, it is logical to conclude that a large cytosine to iodide ratio may achieve the same results. The concentration of KI is kept at 1.0×10^{-5} M because lower KI concentrations lead to lower and less reproducible reaction of input iodide. The high DNA concentrations are easily achieved by dialysis of DNA into 7.2 M $NaClO_4$. This dialysis will concentrate DNA many fold. Furthermore, large impurities in the $NaClO_4$ solution which may have escaped removal by Millipore filtration will not enter dialysis tubing. The results of alkaline sedimentation analyses of λ DNA samples with varying conversion of cytosine

TABLE II. Fraction of Bonds Broken, p, by Iodination.[a]

% C as IC	f	$\langle x_w \rangle$	$10^5 p$	$10^5 p$/% C as IC
0.43	24		3.13	7.3
0.53	24		3.13	5.9
0.70	13		4.47	6.4
1.32		1.13×10^4	8.85	6.7
2.40		6.82×10^3	14.7	6.1

[a] f, $\langle x_w \rangle$, and p are defined in eq 1 and 2.

to iodocytosine are given in Table II. For the cases where whole single strands were detectable, the probability of bond breakage, p, was determined from

$$p = (\ln f)/N \quad (1)$$

where f is the fraction of intact single strands and N is the number of nucleotides in a complete λ DNA single strand. For the cases where many bonds were broken, the value of p was approximated by

$$p = 1/\langle x_w \rangle \quad (2)$$

where $\langle x_w \rangle$ is the weight average degree of polymerization as measured by alkaline sedimentation velocity. p is found to be proportional to percent cytosine converted to iodocytosine. For an infinitely long molecule, the molecular weight would be reduced to about 5×10^6/percent cytosine converted to iodocytosine. This result means that many intact single strands of a viral DNA the size of λ DNA may be obtained if the DNA concentration is above 1.5×10^{-3} M nucleotdes and the KI concentration is 1.0×10^{-5} M. A comparison between these results and those of Orosz and Wetmur (1974) reveals less, though not much less, damage to DNA using the new $NaClO_4$ procedure than previously observed using aqueous solutions and 60 °C.

The actual length of the single strands in the renatured DNA prior to alkaline sedimentation velocity analysis is even longer than given by the formula above. The origin of the chain scission in alkali is probably depyrimidation sites occurring following loss of nonaromatic intermediates in the iodination of cytosine. These sites need not be cleaved prior to exposure to alkali. We looked at the length of the single strands by partially renaturing λ DNA, treating with endonuclease S1, and observing the products in the electron microscope. The majority of the molecules were full length λ DNA even though the weight-average molecular weight from sedimentation analysis in alkali was 3.75×10^6. Alkaline analysis gives a lower limit for single-strand molecular weight.

In order to demonstrate the usefulness of the $NaClO_4$, high cytosine/KI ratio, method of iodination, λ DNA was labeled and subjected to restriction analysis. Figure 1 shows the results of agarose gel electrophoresis of λ DNA cut with Hind III, iodinated (to 1% of cytosines) and renatured λ DNA cut with Hind III, and the same iodinated DNA treated with endonuclease S1 and cut with Hind III. The iodinated and endonuclease S1 treated λ DNA is indistinguishable from control λ DNA except for a band of material which failed to enter the gel. Elimination of S1 treatment led to very little penetration. This is due to the fact that restriction enzymes bind strongly to single-stranded DNA and as a result are no longer available for digestion of double-stranded material (R. W. Davis, personal communication). Thus the material loaded onto the gel remained near the origin as a mixture of uncut double-stranded

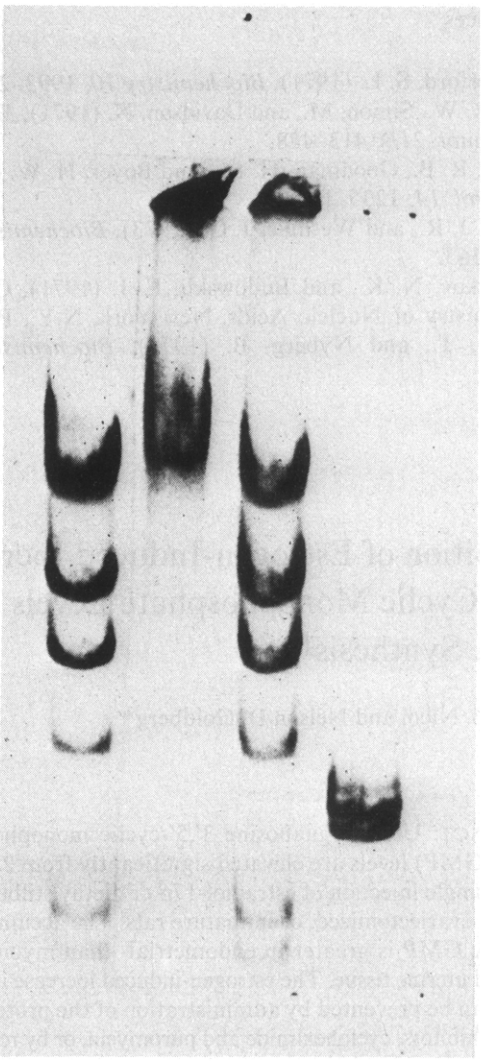

FIGURE 1: Agarose gel electrophoresis of iodinated and noniodinated nucleic acids. Slot 1: λ DNA cut with Hind III. Slot 2: Iodinated λ DNA (1% of cytosines) cut with Hind III. Slot 3: Iodinated λ DNA (1% of cytosines) treated with endonuclease S1 and cut with Hind III. Slot 4: PCM RNA.

DNA and the restriction enzyme–single stranded DNA complex. S1 treatment, therefore, is necessary to eliminate aggregates and/or single-stranded material from the sample before a good gel pattern may be obtained. The gel was sliced and counted. The ^{125}I counts were found in the same positions as the ethidium bromide fluorescence shown in Figure 1.

Finally, we tested iodination of a low complexity RNA. *Penicillium chrysogenum* mycophage (PCM) RNA was found to be denatured at 60 °C in 6.4 M $NaClO_4$. PCM RNA was labeled at 60 °C in this solvent to an extent slightly less than λ DNA. No attempt was made to carefully investigate iodination conditions for this RNA. The RNA contains a segmented double-stranded genome (Nash et al., 1973) as shown in Figure 1. After iodination to about 1% conversion of cytosine to iodocytosine, the RNA ran with the same gel pattern. This result is to be expected considering the increased stability of all bases of RNA, compared with DNA, to acid-catalyzed removal (Kotchetkov and Budowskii, 1971).

Acknowledgment

We thank Dr. Barbara Scher for assistance with agarose gel electrophoresis of nucleic acids.

References

Commerford, S. L. (1971), *Biochemistry 10,* 1993–2000.
Davis, R. W., Simon, M., and Davidson, N. (1971), *Methods Enzymol. 21D,* 413–428.
Helling, R. B., Goodman, H. M., and Boyer, H. W. (1974), *J. Virol. 14,* 1235–1244.
Hutton, J. R., and Wetmur, J. G. (1973), *Biochemistry 12,* 558–563.
Kotchetkov, N. K., and Budowskii, E. I. (1971), Organic Chemistry of Nucleic Acids, New York, N.Y., Plenum.
Lindahl, T., and Nyberg, B. (1972), *Biochemistry 11,* 3610–3618.
Nash, C. H., Douthart, R. J., Ellis, L. F., Van Frank, R. M., Burnett, J. P., and Lemke, P. A. (1973), *Can. J. Microbiol. 19,* 97–103.
Orosz, J. M., and Wetmur, J. G. (1974), *Biochemistry 13,* 5467–5473.
Prensky, W. (1976), *Methods Cell Biol. 13,* 121–152.
Scherberg, N. H., and Refetoff, S. (1974), *J. Biol. Chem. 249,* 2143–2150.
Studier, F. W. (1965), *J. Mol. Biol. 11,* 373–390.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31,* 349–370.

Inhibition of Estrogen-Induced Increases in Uterine Guanosine 3′,5′-Cyclic Monophosphate Levels by Inhibitors of Protein and RNA Synthesis†

Susan E. Nicol and Nelson D. Goldberg*

ABSTRACT: Uterine guanosine 3′,5′-cyclic monophosphate (cyclic GMP) levels are elevated significantly from 2 to 12 h after a single injection of estradiol-17β or diethylstilbestrol to mature, ovariectomized, or immature rats. The accumulation of cyclic GMP is greater in endometrial- than myometrial-enriched uterine tissue. The estrogen-induced increase in cyclic GMP can be prevented by administration of the protein synthesis inhibitors, cycloheximide and puromycin, or by relatively large doses of the RNA synthesis inhibitor, actinomycin D, but not by the muscarinic antagonist, atropine. The requirement for a protein with a relatively rapid rate of turnover is suggested by the demonstration that cycloheximide, when administered after estrogen, can within a 3-h period restore the estrogen-elevated levels of cyclic GMP to those of the non-estrogen-treated tissue.

Uterine cyclic GMP[1] levels have been shown to be elevated in normal estrus-cycling rats at proestrus when plasma estrogen levels are also elevated and in ovariectomized rats following the administration of physiological doses of estradiol-17β or after diethylstilbestrol (Kuehl et al., 1974; Johansson and Andersson, 1975). From these and other related observations, it has been suggested that cyclic GMP may be involved in the expression of estrogen action in uterine tissue.

There are two characteristics of the uterine accumulation of cyclic GMP inducible by estrogen which distinguish it from the increases in the cyclic nucleotide brought about by non-steroidal agents, such as acetylcholine. One is that the increase following estrogen exhibits a lag period of 60–90 min. The second is that the increase is relatively stable, persisting even after the organ is excised and maintained in vitro for at least 30 min. These features of the estrogen-induced increase in uterine cyclic GMP suggest a requirement for RNA and protein synthesis, such as has been demonstrated for a number of other effects of the steroid in estrogen-responsive tissues (Jensen and DeSombre, 1972; O'Malley and Means, 1974). This possibility is examined in the present report along with the alternative that the effect of estrogen to elevate cyclic GMP levels may be brought about indirectly through the release of a neurohumor, such as acetylcholine. The results demonstrate that RNA and protein synthesis appear to be required for estrogen-promoted elevation of uterine cyclic GMP, since the effect can be prevented by cycloheximide, puromycin, or actinomycin D but not by the cholinergic-antagonist atropine.

Materials and Methods

Mature 200–250 g, ovariectomized (1–3 weeks), or immature (21–25 day old) female Sprague-Dawley rats were obtained from Zivic-Miller Laboratories, Allison Park, Pa. Estradiol-17β dissolved in ethanol was diluted with poly(ethylene glycol) 400 and water (1:4.5:4.5, v/v) and administered by ip injection; DES was administered sc in 0.1 ml of sesame oil. Actinomycin D, cycloheximide, and puromycin dissolved in saline and atropine sulfate dissolved in phosphate-buffered saline were administered ip. Studies of uterine contractility were conducted in vitro with uteri suspended in Hanks' balanced salt solution.

Preparation of Tissue and Cyclic Nucleotide Analysis. Tissues were prepared for cyclic nucleotide analysis (Figures

† From the Departments of Pharmacology, Pathology, and Laboratory Medicine, University of Minnesota, Minneapolis, Minnesota 55455. *Received July 7, 1976.* This research was supported by grants from the American Cancer Society (BC-166) and the National Institutes of Health (NS05979).

[1] Abbreviations used are: cyclic GMP, guanosine 3′,5′-cyclic monophosphate; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; DES, diethylstilbestrol; sc, subcutaneously; ip, intraperitoneally; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; IP, estrogen-induced protein.