However, failure of 3'd nucleosides to bring about an inhibition of mRNA synthesis *in vivo* is at odds with the reports that in cell-free systems both RNA polymerases I and II from calf thymus are equally sensitive to 3'dATP (Blatti *et al.*, 1970) and that RNA polymerase II from yeast is even more sensitive to 3'dATP inhibition than RNA polymerase I (Horowitz *et al.*, 1974). Possibly RNA polymerase II has different catalytic specificities *in vivo* and *in vitro*, or perhaps it draws from a distinct nucleotide triphosphate pool which is not readily accessible to nucleotide triphosphate produced from nucleosides.

Very high levels (substrate levels) of 3'dCyd and 3'dAdo are necessary to inhibit RNA polymerase I and perhaps III in this tissue. This high concentration of 3'dAdo is that required to prevent the appearance during germination of carboxypeptidase C activity (Walbot *et al.*, 1974), which we believe to be an activity resulting from the putative stored mRNA of cotton cotyledons (Ihle and Dure, 1969,1970,1972a,b). This is consistent with the notion that these inhibitors act competitively through their 3'd triphosphates. That is, 3'dATP and 3'dCTP must compete with endogenous levels of ATP and CTP. The endogenous concentration of ATP in this tissue at this developmental stage is about 10^{-3} M. Thus the high inhibitor concentration required may not be unreasonable.

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In Vitro Iodination of DNA. Maximizing Iodination While Minimizing Degradation; Use of Buoyant Density Shifts for DNA–DNA Hybrid Isolation†

Jerry M. Orosz and James G. Wetmur*

ABSTRACT: Conditions have been determined for the two stages of the $TlCl_3$ catalyzed *in vitro* iodination reaction of denatured DNA which maximize iodination while minimizing degradation. Of the cytosine in DNA 50% may be converted to 5-iodocytosine with a melting temperature depression of less than 3°. Chromatography of enzymatic digests of heavily iodinated DNA shows that 5-iodocytosine is the only stable, identifiable DNA base modification. Absorbance at 310 nm, buoyant densities of native and denatured DNA in CsCl and Cs_2SO_4, melting temperatures, and renaturation rates are reported as a function of the degree of iodination. Iodinated λcI 857S7 DNA is used with *Escherichia coli* K12 (λ) DNA to demonstrate DNA–DNA hybrid isolation in a buoyant density gradient.

Commerford (1971) introduced an *in vitro* iodination method for nucleic acids involving the use of $TlCl_3$ and KI. His system is capable of producing a high conversion of cytosine to 5-iodocytosine in denatured DNA and RNA. Furthermore, use of $^{125}I^-$ in the same system leads to DNA or RNA which may be used as a radioactive probe. The documented uses of his method are varied, but have usually dealt with radioisotope labeling. Prensky *et al.* (1973), Scherberg and Refetoff (1973), and Getz *et al.* (1972) have explored the use of ^{125}I labeled RNA in DNA–RNA hybridization experiments. Schmidt *et al.* (1973) have explored the use of iodination as a structural probe in tRNAs. Robertson *et al.* (1973) have used the technique for finger-

† From the School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received May 27, 1974.* Reprint requests may be sent to J. G. Wetmur, Department of Microbiology, Mount Sinai School of Medicine, New York, N. Y. 10029. Supported by Research Grant GM 17215 from the National Institutes of Health. J.M.O. is a Predoctoral Trainee supported by Grant 3T1-GM-00722 from the National Institutes of Health.

printing nucleic acids. Wikman-Coffelt (1972) has used ^{125}I-labeled DNA as a radioactive tracer in immunochemical reactions.

The uses of buoyant density differences for nucleic acid separations involve both natural and induced density differences. Natural differences in DNA buoyant densities are well documented (Mandel *et al.*, 1968). The natural difference in a Cs_2SO_4 gradient between DNA and RNA has been exploited by Spiegelman and coworkers in probing for nuclear DNA complementary to the RNA of various tumor viruses (Axel *et al.*, 1972; Hehlmann *et al.*, 1972a,b). *In vivo* labeling was first introduced by Meselson and Stahl (1958) using stable isotopes. Improved *in vivo* labeling, in terms of buoyant density shifts, has been achieved by substituting halogenated uracil deoxyribonucleotides for thymidine in DNA (Erikson and Szybalski, 1963a,b; Flory and Vinograd, 1973; Buettner and Werchau, 1973).

The advantage of iodination over other methods of DNA labeling occurs when *in vivo* incorporation of heavy nucleotides is difficult or impossible. The buoyant density shifts induced by iodination are as large as those observed with any other modification. Buoyant density labeling of DNA is possible with the Commerford system. Commerford (1971) found, however, that DNA labeled sufficiently to produce a buoyant density shift had a large melting temperature reduction and a broad melting transition, renatured poorly and incompletely, and was degraded to a molecular weight of less than 5×10^5 daltons. We will describe a procedure, necessary for a successful buoyant density labeling method, which minimizes the damage to DNA while maximizing the extent of iodination. We then explore the physical properties and uses of iodinated radioactive buoyant density probes.

Materials and Methods

Materials. All nucleosides, purines and pyrimidines, and *Clostridium perfringens* DNA were obtained from Sigma Chemical Co. Tritiated nucleosides and carrier free $Na^{125}I$ (in NaOH) were obtained from Amersham Searle, Inc. CsCl and Cs_2SO_4 were obtained from Harshaw, Inc. $TlCl_3$ was obtained from Allied Chemical Co. Sephadex G-10 was obtained from Pharmacia Fine Chemicals, Inc. *Escherichia coli* K12 DNA was purchased from General Biochemicals, Inc., and was found to have a single-strand molecular weight of 5×10^5 daltons. *E. coli* K12 $159T^-(\lambda cI857S7)$, hereafter called *E. coli* K12(λ), was a gift of Dr. James Wang. *E. coli* K12(λ) DNA was isolated from a fresh bacterial culture by the procedure of Chow and Davidson (1973) and was found to have a single-stranded molecular weight of 6×10^7 daltons. λ phage was obtained by heat induction of a culture of the lysogen, disruption of the cells, differential centrifugation, and banding in CsCl (density 1.50, 0.0025 M $MgCl_2$, 0.01 M Tris-OH plus HCl, pH 7.8). After dialysis to remove CsCl, phage DNA was isolated by phenol extraction. All DNAs were dialyzed into neutral salt solutions containing EDTA and stored at −20°.

Chromatography. The same procedures were used as those of Braun (1967) except for substitution of deoxyribonucleosides for ribonucleosides. The Sephadex column was 1.5 cm × 100 cm and was eluted at 30 ml/hr. Fractions (5 ml) were collected with a Gibson Medical Fraction Collector.

Melting Temperatures. Melting temperatures were measured as described by Hutton and Wetmur (1973). All DNA samples contained 0.015 M NaH_2PO_4 plus NaOH to pH 6.8. An unmodified DNA sample was included in each set of measurements as a control.

Renaturation Rates. Renaturation rate constants were determined as described by Hutton and Wetmur (1973). *E. coli* DNA with 260-nm absorbance 1.3–1.5 in 0.4 M NaCl–0.01 M phosphate buffer (pH 6.8) was reacted at 60°, which is 33° below the melting temperature of unmodified *E. coli* DNA.

Sedimentation Velocity. Alkaline boundary velocity sedimentation in a Beckman Model E ultracentrifuge was used for DNA with 260-nm absorbance 0.5 in 0.9 M NaCl–0.1 M NaOH. The method and the treatment of the data to give molecular weights are described by Studier (1965).

Buoyant Densities. The buoyant densities of native and denatured iodinated DNA were determined with respect to *E. coli* K12 DNA, which has a density of 1.7035 g/cm^3 in CsCl and 1.4260 g/cm^3 in Cs_2SO_4. Experiments were performed at 40,000 rpm, 20°, in a Beckman Model E ultracentrifuge equipped with a monochromator. Films were traced using a Joyce-Loebl microdensitometer. The separation of *E. coli* K12 DNA and *Clostridium perfringens* DNA in CsCl was measured and found to be in agreement with literature values (Szybalski, 1968). Details of buoyant density calculations are given in the Results section.

Minimal Damage Iodination Conditions. The first stage of iodination is the same as described by Commerford (1971). *E. coli* K12 DNA was denatured at 104° for 5 min and ice quenched. The final reaction mixture contained 1.25×10^{-4} M deoxyribocytidine in DNA, 1.0×10^{-4} M KI, 6.0×10^{-4} M $TlCl_3$, 2 μCi/ml of $Na^{125}I$, 0.1 M sodium acetate, plus 0.04 M acetic acid (pH 5.0). The reaction mixture was heated at 60° for a prescribed length of time and ice quenched. Variations on this procedure are described in the text.

The second stage of iodination is not the same as described by Commerford (1971). Samples were dialyzed exhaustively at 4° against 0.4 M NaCl, 0.015 M NaH_2PO_4 plus NaOH (pH 6.0) and 0.0002 M EDTA. The samples were then renatured while converting any iodohydrate to stable product by heating the DNA in the dialysis tubing at 60° for 5 or more hours. Samples were then dialyzed into the prescribed solvents for subsequent study. Variations on this procedure are described in the text.

Commerford Procedure. The first stage is the same as described above. After reaction, 4-ml samples were treated with 0.2 ml of 0.1 M Na_2SO_3 and 0.8 ml of 1 M NH_4OAc–0.5 M NH_4OH (pH 9.5) and heated at 60° for 20 min. Samples were then exhaustively dialyzed into the desired solvent for subsequent study. Variations on this procedure are described in the text.

$\lambda cI857S7$ Iodination Procedure. λ DNA was dialyzed into a low salt solution, thermally denatured at 100° for 3 min, and ice quenched. Reactants were assembled at 4° to give final concentrations of 0.75×10^{-4} M deoxycytidine in DNA, 0.75×10^{-4} M KI, and 4.5×10^{-4} M $TlCl_3$, and adjusted to pH 4.5 with acetate to produce 5×10^{-3} M acetate. $^{125}I^-$ (10 μCi) was added. The sample was heated to 80° for 10 min and then treated using the conditions for minimal damage described above. The product DNA was 70% labeled and had a buoyant density of 1.810 g/cm^3 and a single-strand molecular weight of 260,000 daltons.

Per Cent Iodination. The per cent iodination was determined throughout this work by measuring the specific activity of labeled DNA. The scintillation cocktail was 2,5-diphenyloxazole plus naphthalene in dioxane. Samples were

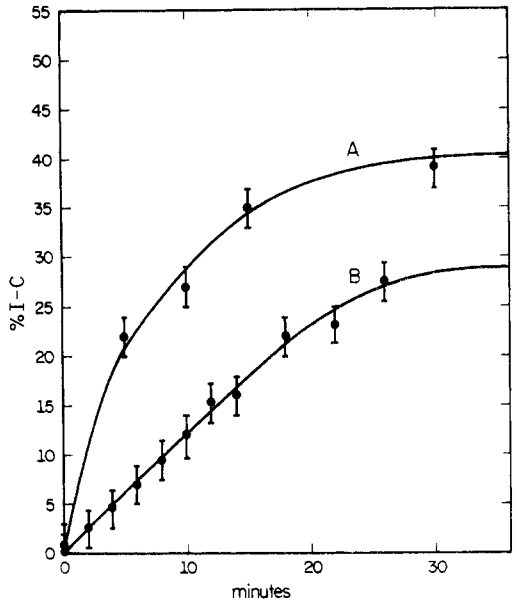

FIGURE 1: The percentage of cytosine in *E. coli* DNA converted to 5-iodocytosine (I-C) is shown as a function of reaction time. Curve A shows the results obtained using the conditions for minimal damage to the DNA which are described under Materials and Methods. Curve B shows the results obtained using the method of Commerford (1971). Error bars in this and most subsequent figures indicate errors inherent in determining per cent iodination by radioactivity measurement.

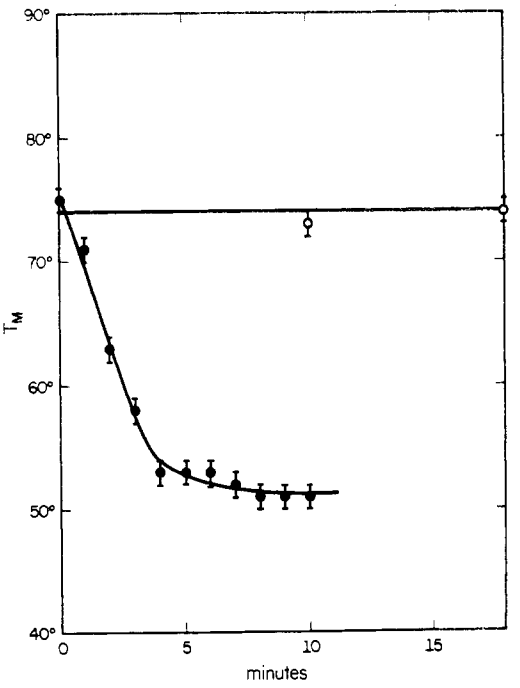

FIGURE 2: (Closed circles) The melting temperature of *E. coli* DNA, measured as described under Materials and Methods, is shown as a function of reaction time using the method of Commerford (1971). (Open circles) Results using the same conditions as for the other curve except KCl is substituted for KI. Error bars show standard deviations of measurements of melting temperatures.

counted on a Nuclear-Chicago Isocap/300 with preset $^{125}I/^{131}I$ channels. The counting efficiency ranged from 60 to 70%.

Results and Discussion

Chemistry of DNA Iodination. In attempting to buoyant density label DNA by iodination, we first used the method of Commerford (1971). Denatured *E. coli* DNA was allowed to react as a function of time and sulfite treated at pH 9. The reaction kinetics are shown in Figure 1B and fit Commerford's data rather well. Figure 2 shows the melting temperature depression due to the reaction. The melting temperature is rapidly reduced to give a depression, ΔT_m, of $-24°$. Control experiments with KCl substituted for KI showed no appreciable ΔT_m. The single-strand molecular weight of the iodinated DNA, measured by alkaline velocity sedimentation, dropped to 1.3×10^5 daltons, and the renaturation rate, measured 33° below the melting temperature of unreacted DNA, was reduced to half that expected for the measured molecular weight (Hutton and Wetmur, 1973). The control DNAs showed no such drop in rates of renaturation compared to the rates expected for the measured molecular weights.

Hutton and Wetmur (1973) have shown that modifications which affect the hydrogen bonding sites of the DNA bases can be quantitatively related to melting temperature depressions and renaturation rates. As we do not know the ΔT_m produced by the specific mismatches caused by iodination side reactions, we take $\Delta T_m = 1.2 \pm 0.3°$ per per cent base pairs modified, as this range appears to fit most mismatches. The iodination results indicate that about 20% of the base pairs are modified in Commerford's procedure. The drop in renaturation rate is consistent with the observed ΔT_m. Having repeated Commerford's observations that highly iodinated DNA has a broad melting transition and renatures poorly, we then attempted to find conditions that would minimize the damage to the DNA caused by iodination. As ΔT_m is a sensitive probe of mismatch, we looked for conditions which minimized this parameter.

In a study of deoxycytidine iodination, we found that sulfite treatment for several hours at pH 5–10 resulted in the loss of all of the 5-iododeoxycytidine generated by the reaction. If the 60° sulfite treatment is carried out at pH 5, only deoxycytidine is recovered. At pH 9.5, however, a new product with a 259-nm absorbance maximum is observed in addition to deoxycytidine. At all pH values studied, the ^{125}I is observed as a $AgNO_3$ precipitable species. The new product at high pH may account for the ΔT_m observed using Commerford's procedure. The loss of 5-iododeoxycytidine is also consistent with the comparison of yields using sulfite treatment (Figure 1B) and the procedure described below (Figure 1A). We chose to discontinue sulfite treatment as this treatment is known to cause deamination of cytosine (Hayatsu *et al.*, 1970) and hydrolysis of the *N*-glycocidic linkage (Kochetkov and Budovskii, 1971). Although we observed no appreciable ΔT_m with control DNAs, the effect of sulfite on 5-iododeoxycytidine and on the intermediate iodohydrate appears to be deleterious. Subsequently, reaction mixtures were treated as described under Materials and Methods under Minimal Damage Iodination Conditions.

The pH dependence for the iodination part of the procedure is shown in Figure 3. Samples reacted at pH 3 or 4 had greatly lowered melting temperatures, probably due to acid-catalyzed depurination (Lindahl and Nyberg, 1972). Samples reacted at pH 6 or above had little labeling. pH 5 reacted samples had only a small ΔT_m for 40% cytosine labeling. Therefore, we chose, as did Commerford (1971), to conduct the first part of the reaction at pH 4.5–5.2. In order to maximize iodine incorporation, we studied the effect of the ratio of concentrations of iodide, $[I^-]$, and deoxycytidine in DNA, [dC]. Results at pH 5, 60°, at pH 5, 80°, and

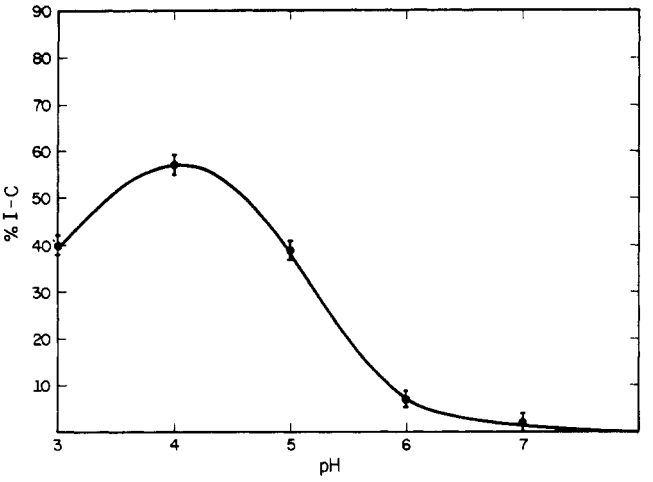

FIGURE 3: The percentage of cytosine in *E. coli* DNA converted to 5-iodocytosine (I-C) is shown as a function of reaction pH. The DNA was subsequently treated using the conditions for minimum damage as described under Materials and Methods. The reaction time was 30 min.

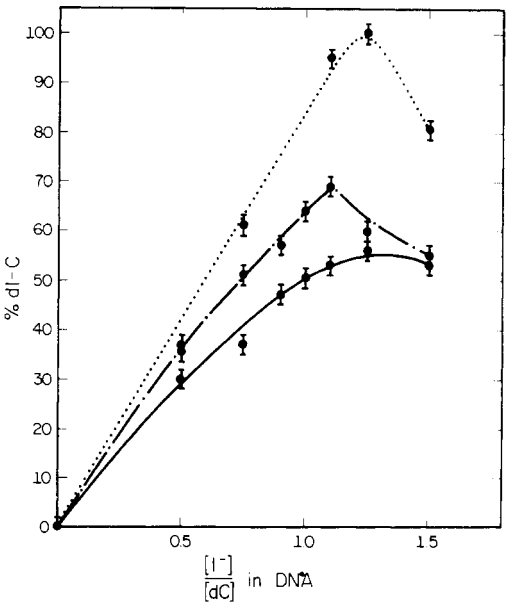

FIGURE 4: (——) The percentage of deoxycytosine (dC) in *E. coli* DNA converted to 5-iododeoxycytidine (dI-C) is shown as a function of the input ratio of iodide (as KI) to deoxycytidine under the conditions of minimal damage described under Materials and Methods. The reaction time was 1 hr. (- - -) The same conditions were employed except for a reaction temperature of 80°. (· · ·) The same conditions were employed except for a reaction temperature of 80° and a reaction pH of 4.0.

at pH 4, 80° are shown in Figure 4. Maximization of iodination occurs at $[I^-]/[dC]$ equal to 1.1–1.25. DNA treated at 80° had lowered melting temperatures. In order to get better than 50% labeling of cytosine at 80°, pH 5, and little damage to the DNA, a reaction time of 10 min may be used. DNA reacted at pH 4 is very badly damaged under all circumstances.

Modified DNA was renatured and dehydrated as a function of pH in order to determine the importance of this parameter in maximizing iodination while minimizing ΔT_m. Samples were allowed to react 15, 30, and 45 min and renatured at pH 5, 6, and 9. The pH 5 and 6 treated samples had a ΔT_m of -1 to $-3°$ while pH 9 treated samples had a ΔT_m of -1 to $-5°$. There was also somewhat less incorporation of iodine at higher pH. We chose to renature and dehydrate all samples at pH 6, where the smallest ΔT_m occurs and where less depurination occurs than at pH 5.

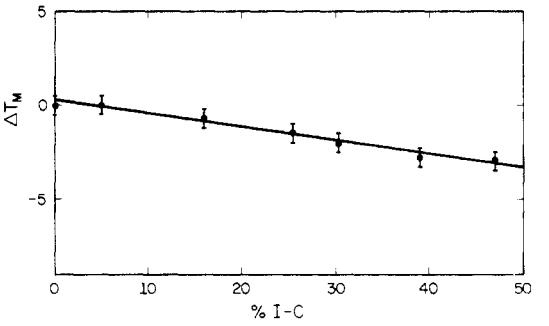

FIGURE 5: The change in the melting temperature of *E. coli* DNA from unreacted *E. coli* DNA, ΔT_m, measured as described under Materials and Methods, is shown as a function of the percentage of cytosine converted into 5-iodocytosine (I-C) under the conditions of minimal damage as described under Materials and Methods.

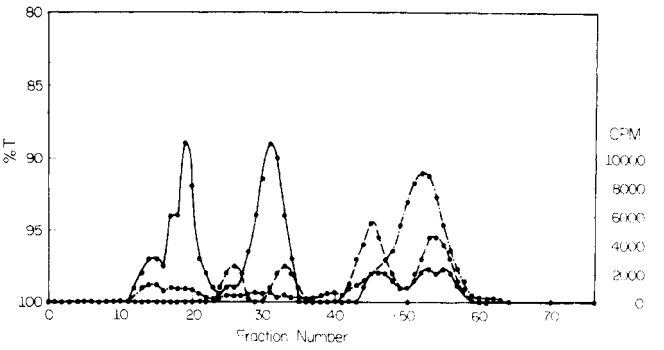

FIGURE 6: (- - -) The elution profile of deoxynucleotides dC, dT, dA, and dG in 0.01 M citrate buffer (pH 5) using a Sephedex G10 column as described under Materials and Methods. Data are given as per cent transmittance (% T). (——) The elution profile, using the same conditions, of an extensively iodinated *E. coli* DNA sample which was enzymatically degraded as described by Commerford (1971). Data are given in % T. (- · -) The radioactivity of the elution fractions for the enzymatically degraded DNA sample.

The kinetics of the labeling reaction using the optimum conditions described above and in Materials and Methods is shown in Figure 1A. The melting temperature depression is shown as a function of per cent iodination in Figure 5. There is an apparent linear relationship between per cent 5-iodocytosine and ΔT_m. This result implies, although it does not prove, that the ΔT_m is the result of either the 5-iododeoxycytidine itself, the formation of a side product *via* a common intermediate, or the formation of a side product from 5-iododeoxycytidine during the second incubation step. To test the latter possibility iodinated DNA was heated at pH 6, 60°, for 72 hr. No additional ^{125}I was lost nor was there any additional ΔT_m. Samples heated at pH 9, 60°, for 72 hr had an additional ΔT_m of $-3°$. The pH 6 result shows that the iodinated DNA product is extremely stable and that the ΔT_m cannot be the result of degradation of the DNA product during the second incubation step. We then looked for the formation of a side product through a common intermediate. Such a mechanism is possible and agrees with the previous result that a control reaction with KCl substituted for KI led to no ΔT_m. Figure 6 shows the results of a chromatographic run of digested 90% iodinated DNA. The solid line shows per cent transmittance. The large peak ending at fraction 23 contains digestive enzymes. The peaks at fractions 26, 31, 46, 53, and 55 are deoxycyti-

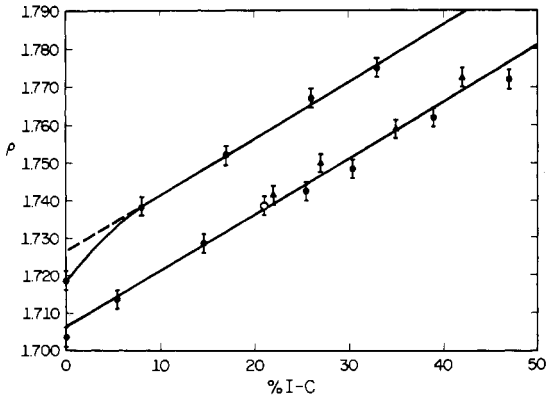

FIGURE 7: The buoyant density of iodinated *E. coli* DNA in a CsCl gradient is shown as a function of the per cent of cytosine in the DNA which is converted to 5-iodocytosine (I-C). The upper curve shows denatured DNA and the lower curve native DNA.

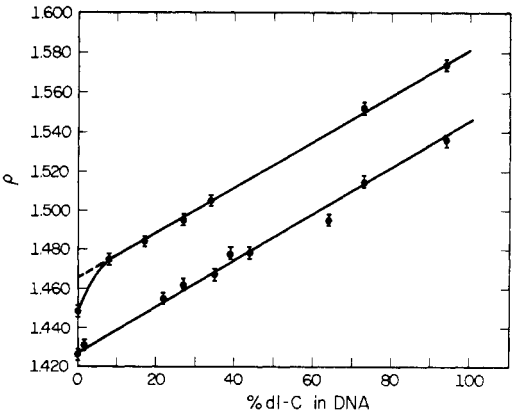

FIGURE 8: The buoyant density of iodinated *E. coli* DNA in a Cs_2SO_4 gradient is shown as a function of the per cent of deoxycytosine in the DNA which is converted to 5-iododeoxycytosine (dI-C). The upper curve shows denatured DNA and the lower curve native DNA.

dine, thymidine, deoxyadenosine, 5-iododeoxycytidine, and deoxyguanosine, respectively. The dashed line shows a mixture of the four DNA nucleosides run separately. Thymidine ran at fraction 33 instead of 31, but otherwise the pattern is about the same as with the digested DNA. The dashed and dotted line shows the radioactivity profile of the digested iodinated DNA. About 5–10% of the radioactivity (2.25–4.5% of the base pairs) eluted at a position other than that of 5-iododeoxycytidine. The existence of these side products could account for a ΔT_m of 2.7–5.4° for this 90% iodinated DNA. We were not able to obtain enough material to identify the structures or stability of any side products.

Beginning with sonicated DNA and using the conditions of minimal damage, the rate of renaturation is not affected by increased iodine incorporation and, for this low molecular weight starting DNA, the single-strand molecular weight is not changed very much. If we begin with larger DNA, the length is rapidly reduced to 150,000–250,000 daltons. For control DNA with KCl substituted for KI, much less length degradation is observed. Lindahl and Nyberg (1972) have determined the rate of depurination of denatured DNA at 60° in 0.1M NaCl in a pH 5 buffer. Using their rate constant, we would expect a DNA about ten times larger than observed following iodination. Sizes observed in the KCl control are in agreement with degradation by depurination. Again, the chain scission with iodination appears to take place through an intermediate in the iodination reaction. Kochetkov and Budovskii (1971) have shown that depyrimidation of nonaromatic DNA base hydrates proceeds faster than depurination. Depyrimidation of the iodohydrate of deoxycytidine, followed by base cleavage of the chain, may account for the observed results. We were not able to find conditions with less chain scission without reducing the degree of iodination.

Iodination of DNA results in a change in the spectrum of DNA (Commerford, 1971). Spectra of renatured iodinated *E. coli* samples in 0.015 M phosphate buffer (pH 6.8) lead to a linear relationship between absorbance at 310 nm, A_{310}, and the molar concentration of 5-iododeoxycytidine. After subtraction of 310-nm absorption of unlabeled DNA, the extinction coefficient obtained for iodinated DNA at 310 nm, in terms of 5-iododeoxycytidine, is 3200 ± 300. The 310-nm absorbance may thus be used to determine the per cent of iodination, although radioisotope incorporation gives more precise results.

Buoyant Densities. Iodinated *E. coli* DNA was prepared as described under Materials and Methods. The maximum melting temperature reduction due to iodination was 3–5° for the most heavily iodinated DNA. Renaturation rate constants were the same as for uniodinated DNA of the same length. Native iodinated DNA was obtained by renaturing each sample to completion at 60° in 0.4 M NaCl–0.0002 M EDTA–0.01 M phosphate buffer (pH 6.0). No denatured DNA band was observed in the iodinated DNA buoyant density runs. Denatured iodinated DNA was obtained by heating the DNA 5–10 min at 100° and ice quenching.

The buoyant density gradient (Vinograd and Hearst, 1962; Schmid and Hearst, 1971) includes the pressure dependence of the buoyant density and is given by

$$\left(\frac{d\rho}{dr}\right)_{\text{buoyancy}} = \left(\frac{1}{\beta^0} + \psi\rho^{0\,2}\right) w^2 r \qquad (1)$$

Our data are expressed in this system. Values of 9.1×10^{-10} gsec^2/cm^5 and 16.1×10^{-10} gsec^2/cm^5 were used for $((1/\beta^0) + \psi\rho^{0\,2})$ for CsCl, initial density 1.730 g/cm^3, and Cs_2SO_4, initial density 1.400 g/cm^3, respectively. w^2 was always 1.75×10^7 sec^{-2}. Thus, for our system

$$\text{CsCl: } \rho = \rho_{\text{marker}} + 0.0159\bar{r}_0\,\Delta r \qquad (2a)$$

$$Cs_2SO_4\text{: } \rho = \rho_{\text{marker}} + 0.0282\bar{r}_0\,\Delta r \qquad (2b)$$

where ρ is the buoyant density of the iodinated DNA, Δr is the band spacing between marker DNA and iodinated DNA, and $\bar{r}_0$ is the average distance of the two bands from the center of rotation. Native *E. coli* DNA was used as a marker

$$\text{CsCl: } \rho_{\text{marker}} = 1.7035 \qquad (3a)$$

$$Cs_2SO_4\text{: } \rho_{\text{marker}} = 1.4260 \qquad (3b)$$

Buoyant density data in CsCl and Cs_2SO_4 are reported in Figures 7 and 8. Empirical equations were obtained for the buoyant densities of iodinated *E. coli* DNA

$$\text{native, CsCl: } \rho = 1.46 \times 10^{-3}(\%\ \text{IC}) + 1.7035 \qquad (4a)$$

$$\text{native, } Cs_2SO_4\text{: } \rho = 1.18 \times 10^{-3}(\%\ \text{IC}) + 1.4270 \qquad (4b)$$

$$\text{denatured, CsCl: } \rho = 1.49 \times 10^{-3}(\%\ \text{IC}) + 1.7265 \qquad (4c)$$

$$\text{denatured, } Cs_2SO_4\text{: } \rho = 1.17 \times 10^{-3}(\%\ \text{IC}) + 1.4635 \qquad (4d)$$

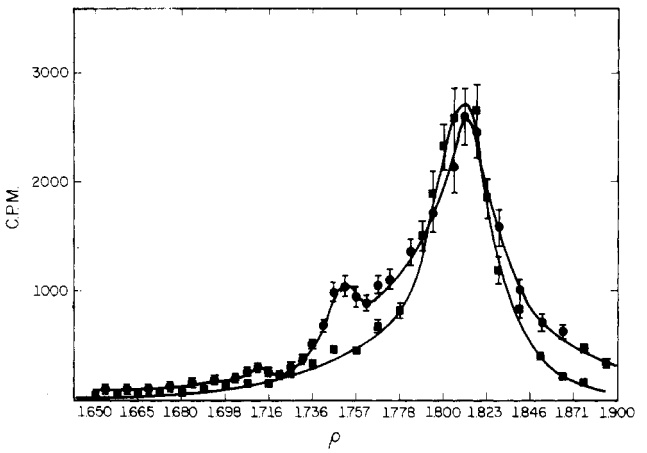

FIGURE 9: The ^{125}I radioactivity is shown as a function of density in a CsCl equilibrium density gradient: (■) native iodinated λDNA; (●) the same λDNA sample after denaturation and renaturation with a ten-fold excess of *E. coli* K12 (λ) DNA.

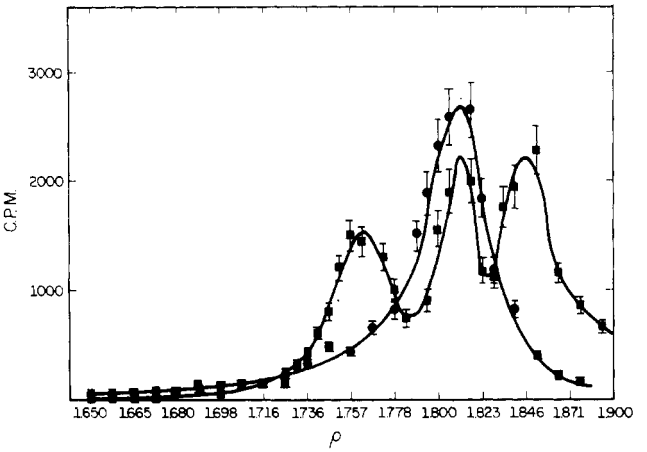

FIGURE 10: The ^{125}I radioactivity is shown as a function of density in a CsCl equilibrium density gradient: (●) native, iodinated λDNA; (■) the same λDNA sample after denaturation and renaturation with a 100-fold excess of *E. coli* K12 (λ) DNA.

(% IC) is the percentage of deoxycytidine converted to 5-iododeoxycytidine in the DNA. The relationships hold for native DNA over the entire ranges reported, 0–50% IC for CsCl and 0–100% IC for Cs_2SO_4. The relationships hold above 8% IC for denatured DNA. The data point of Commerford (1971) is shown in Figure 7 and agrees with our results. Iodination (100%) changes ρ for *E. coli* DNA about 0.146 g/cm^3 in CsCl and 0.118 g/cm^3 in Cs_2SO_4. Within experimental error, the slopes of the empirical equations are the same for native and denatured DNA.

Simple isotopic substitution in DNA should produce a density difference given by

$$\text{CsCl: } \rho - \rho_{\text{marker}} = 0.0159\left\{\frac{\Delta m}{m}\frac{\rho_{s,0}}{w^2}\frac{\beta_{\text{eff}}}{1+\Gamma'}\right\} \quad (5a)$$

$$Cs_2SO_4\text{: } \rho - \rho_{\text{marker}} = 0.0282\left\{\frac{\Delta m}{m}\frac{\rho_{s,0}}{w^2}\frac{\beta_{\text{eff}}}{1+\Gamma'}\right\} \quad (5b)$$

where $\rho_{s,0}$ is the solvent density, $\Delta m/m$ is the relative change in mass due to isotopic substitution, and $\beta_{\text{eff}}/(1+\Gamma')$ is given for CsCl and Cs_2SO_4 by Schmid and Hearst (1971). Erikson and Szybalski (1963b) grew *E. coli* 15 T$^-$ in 5-iododeoxyuridine and found a shift of 0.076 g/cm^3 in Cs_2SO_4 and 0.119 g/cm^3 in CsCl. Buettner and Werchau (1973) have recently studied the same effect in SV40 DNA as a function of the degree of 5-iododeoxyuridine substitution. They find a linear relationship between degree of substitution and buoyant density. Their results agree with the results of Erikson and Szybalski for 100% substitution. Predicted values using a substitution of I for CH_3 would be 0.0755 g/cm^3 in Cs_2SO_4 and 0.122 g/cm^3 in CsCl, in good agreement with simple isotopic substitution with no changes in hydration. When the same overly simple theory is applied to substitution of I for H in *in vitro* iodination of DNA, the calculated value in CsCl is 0.137 g/cm^3 for 100% substitution, about 6% less than the experimental value of 0.146 g/cm^3. In Cs_2SO_4, the calculated value of 0.085 g/cm^3 is 28% less than the experimental value of 0.118 g/cm^3. An increase in DNA volume upon iodination would decrease, rather than increase, the theoretical shift in buoyant density. As a decrease in volume upon substitution of I for H is unreasonable, we conclude that iodination of cytosine must change the preferential hydration of DNA and affect the $\beta_{\text{eff}}/(1+\Gamma')$ term in eq 5a and 5b.

Erikson and Szybalski (1963a) noted that heat denatured 5-iododeoxyuridine substituted DNA banded in Cs_2SO_4 at a density 0.046 ± 0.004 g/cm^3 higher than native DNA instead of a shift of 0.022 g/cm^3 observed with unsubstituted DNA. We have observed the same effect in both CsCl and Cs_2SO_4 for *in vitro* iodinated DNA after 8% of the cytosines are iodinated. We have no explanation of this phenomenon. We note, however, that the good agreement between our results *in vitro* and an analogous *in vivo* modification system demonstrates that there is nothing different or unusual about the *in vitro* system.

DNA–DNA Hybrid Isolation. The *E. coli* K12 (λ) system is a well-characterized virus integration system. The molecular weight of *E. coli* DNA is 2.5 ± 0.5 × 10^9 daltons, and the molecular weight of λcI857S7 DNA is 30.8 ± 1.0 × 10^6 daltons. One λDNA inserts per *E. coli* K12 genome. The physical properties of λDNA are described in detail by Davidson and Szybalski (1971).

E. coli K12 (λ) DNA was prepared as described under Materials and Methods. The single-strand molecular weight was 6 × 10^7 daltons. λDNA was iodinated as described under Materials and Methods to give a buoyant density for native λDNA of 1.810. The single-strand molecular weight was 260,000 daltons. A 2-ml reaction mixture was assembled containing 200 μg/ml of *E. coli* K12 (λ) DNA, 20 μg/ml of iodinated λDNA, and 0.0002 M EDTA–0.015 M phosphate buffer (pH 6.8). The reaction mixture was heated at 105° for 3 min to denature the DNA, ice quenched, and renatured 5 *E. coli* K12 (λ) half-times at 60°. The reaction mixture was then diluted and CsCl added to give 5 ml of 1 g/ml CsCl. The solution was then banded at 40,000 rpm in an SW50 rotor for 60 hr. The tube was dripped into scintillation vials and counted as described under Materials and Methods. Figure 9 shows the results of this experiment. A density gradient run of native iodinated λDNA is shown for comparison. Three distinct peaks are seen in the gradient with *E. coli* and λDNA, one at 1.81 ± 0.005 g/cm^3, which is native λ iodinated λDNA. A second peak at density 1.750 ± 0.005 is an *E. coli* K12 (λ)–λDNA hybrid. A small peak appears at 1.710 ± 0.005, which is label drawn into the native *E. coli* band. The ratio of counts under the heavy band to counts under the hybrid band is 7.2 ± 0.72 for this reaction mixture made up with ten times as much λDNA as λ prophage in *E. coli* K12(λ).

A one-to-one ratio of λDNA to λ prophage in *E. coli* K12(λ) was treated as previously described except that 2 μg/ml of λDNA was used. The DNA was renatured 2 *E. coli* K12 (λ) half-times. The results are shown in Figure 10,

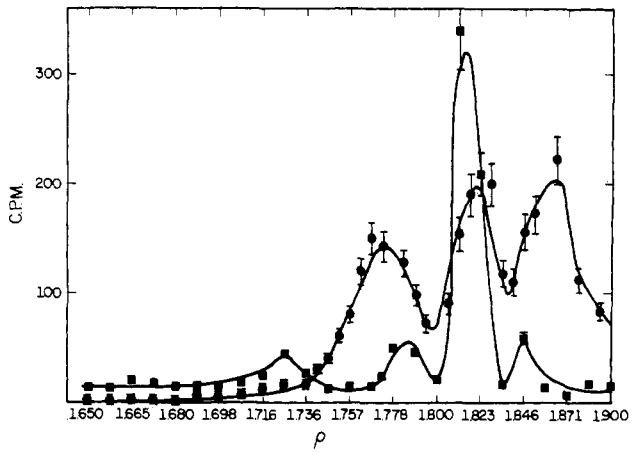

FIGURE 11: The ^{125}I radioactivity is shown as a function of density in a CsCl equilibrium density gradient: (●) iodinated λDNA denatured and renatured with a 100-fold excess of *E. coli* K12 (λ) DNA; (■) iodinated λDNA denatured and renatured with a 100-fold excess of *E. coli* K12 DNA.

again with a native iodinated λ gradient displayed for comparison. Three bands are seen at 1.846 ± 0.005, 1.81 ± 0.005, and 1.762 ± 0.005 g/cm^3. The first and second bands are denatured and native iodinated λDNA. The third band is hybrid DNA, which appears at a higher density than in the previous run, probably because the shorter reaction time did not allow as many of the excess *E. coli* strand segments in the hybrid to react with other *E. coli* strands. The ratio of counts under the native heavy band to counts under the hybrid band is 1.0 ± 0.1 for this mixture of 1:1 λDNA to λ prophage DNA.

In order to test for hybridization between λDNA and *E. coli* K12 DNA other than the prophage of *E. coli* K12 (λ), *E. coli* K12 DNA was treated the same way as in the previous experiment except for a renaturation of 4 *E. coli* K12 half-times. Figure 11 shows the results of this experiment, with the 1:1 λDNA to λ prophage DNA experiment displayed for comparison. Native and denatured λDNA are seen again at 1.810 ± 0.005 and 1.846 ± 0.005 g/cm^3, respectively. Two other bands are observed at 1.783 and 1.726 g/cm^3. These bands apparently represent hybrid formation with an excess of either *E. coli* K12 or λDNA strands. The ratio of counts in the native heavy band to the counts in the two hybrid bands is 3.4 ± 0.34. This result indicates that some homology may exist between *E. coli* K12 DNA and λDNA, although a quantitative conclusion of 30 ± 3%, based on the ratio, is not very convincing because of the placement of the bands in the density gradient. Cowie and Szafranski (1966) have demonstrated a 33% homology between λ and *E. coli* DNA using kinetics methods. Our results agree at least qualitatively with their conclusion.

If we accept 30% homology in the absence of prophage, the results in the 10:1 λDNA to prophage λDNA experiment are in exact agreement. For the case of the 1:1 λDNA to prophage DNA experiment, the observed ratio of 1.0 ± 0.1 is lower than might be expected from a stoichiometric argument. However, the *E. coli* K12 (λ) DNA was much larger than the λDNA and, even if somewhat degraded during the denaturation and renaturation steps, would be expected to renature with itself faster than the prophage DNA would be expected to renature with the λDNA.

Finally, when a 1:1 ratio of λDNA to prophage DNA in *E. coli* K12 (λ) DNA was renatured 15 half-times, much of the iodinated DNA appeared in a band close to native iodinated *E. coli* DNA, which suggests that the iodinated DNA strands represented only a small fraction of a larger polymeric structure containing mostly uniodinated DNA. The ratio of heavy DNA to hybrid DNA was 0.9 ± 0.1, just as in the less renatured case. For the purpose of achieving hybrid separation, renaturation of 2-3 half-times is to be preferred.

In order to demonstrate the existence of a provirus, renaturation kinetics is the best available method. However, the procedure described above may be used as an adjunct and, in addition, could lead to the isolation of the DNA.

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