Iodination of Nucleic Acids in Organic Solvents with Iodine Monochloride*

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SUMMARY

Iodine monochloride, labeled with $^{125}$I, iodinated ribonucleic acid and deoxyribonucleic acid dissolved as hexadecyltrimethylammonium salts in anhydrous dimethylformamide or $N$-ethyl acetamide. In the case of RNA (polyuridylate, polycytidylate, and soluble RNA) 5-iodouridine 5'-phosphate, 8-iodoguanosine 5'-phosphate, and 5-iodocytidine 5'-phosphate are found. After 24 hours of reaction, 32% of UMP, 8% of GMP, and 9% of CMP residues in soluble RNA were found to be iodinated. In the case of DNA, after 24 hours of reaction, 8-iodo-dGMP and 5-iodo-dCMP represent 1.4 and 14%, respectively, of the parent nucleotides present. The use of ion exchange column chromatography and ion exchange paper in the analysis of the iodinated nucleotides present in enzymatic digests of the nucleic acids is described. These techniques provide a means of introducing radioactivity into preformed polynucleotides and of studying the influence of iodinated bases on the biological activity of nucleic acids.

Extensive, direct halogenation of nucleic acids has until now been accomplished only by the use of brominating agents (1-3). Neither nucleic acids nor their constituent bases have been found to react with iodine in water (1, 4); however, the formation of small amounts of an iodinated product has been demonstrated when viral ribonucleic acid was exposed to N-iodosuccinimide (5). Recently, iodine monochloride was shown by Lipkin et al. (5) to iodinate, by a substitution reaction, solutions of nucleosides, nucleotides, and nucleoside polyphosphates in anhydrous solvents. In this paper we show that iodine monochloride may be used to introduce substantial amounts of iodine into the constituent nucleotides of polyuridylate, polycytidylate, soluble ribonucleic acid, and deoxyribonucleic acid when these polymers are dissolved in anhydrous solvents. This method provides a practical means of introducing radioactivity (in this case $^{125}$I) into preformed polynucleotides, and also permits studies of the influence of iodinated bases on the biological properties of nucleic acids.

EXPERIMENTAL PROCEDURE

Polyribonucleotides—Soluble RNA from Escherichia coli strain B was purchased from General Biochemicals. Salmon sperm DNA was obtained from Calbiochem. Polyuridylate and polycytidylate were gifts of Dr. R. E. Thach.

Solvents—Dimethylformamide was distilled at reduced pressure in the presence of phthalic anhydride; $N$-ethylacetamide was stored over magnesium sulfate and distilled before use. Carbon tetrachloride was dried over Drierite and distilled.

Iodine Monochloride—Solutions in carbon tetrachloride of $^{125}$ICl having a specific activity of 40 to 60 $\mu$C per $\mu$ mole were purchased from Volk. Before the reactions were begun, $^{125}$ICl was diluted to the desired specific activity by adding nonradioactive ICl (Eastman).

Hydrolitic Enzymes—Crystalline pancreatic RNAse, pancreatic DNase I, purified Crotalus adamanteus venom phosphodiesterase, and Escherichia coli alkaline phosphatase were purchased from Worthington.

Measurement of Radioactivity—Up to 3 ml of solutions containing $^{125}$I were counted in a Nuclear Chicago well counter, model DS5-5; a counting efficiency of 50% was observed. Paper strips bearing radioactivity were either counted in the well counter or immersed in a toluene-based scintillation fluid within a Packard Tri-Carb liquid scintillation spectrometer. The latter conditions provided a counting efficiency of 25%.

Iodonucleosides and Iodonucleotides—Samples of nonradioactive 5-iodouridine and 5-iodocytidine were purchased from Calbiochem. Radioactivity was introduced into cytidine, guanosine, deoxguanosine, uridine, dGMP, dCMP, GMP, CMP, and UMP by allowing solutions of these compounds in $N$-ethylacetamide to react with 2-fold molar excesses of $^{125}$ICl for 18 hours at $25^\circ$ (6). The radioactive products were separated from unreacted material in the case of the nucleosides by paper chromatography in 1-butanol-formic acid-water (70:14:10) as described by Lipkin et al. (5). The radioactive nucleotides were purified.
with the use of DEAE-formate paper (7) under conditions described in the legend to Fig. 1. Each of the labeled nucleotide preparations yielded, after treatment with bacterial alkaline phosphatase, a radioactive compound with a mobility, under the aforementioned conditions of paper chromatography, equal to that of the corresponding labeled nucleoside preparation.

RESULTS

Iodination of Soluble RNA—Since ICl is rapidly hydrolyzed in aqueous solutions to hypoiiodous acid, which has been shown incapable of iodinating nucleic acids (4), it was necessary to conduct the following experiments in nonaqueous solvents. Nucleic acids were rendered readily soluble in these solvents by converting them to hexadecyltrimethylammonium salts according to the procedures of Aubel-Sadron, Beeck, and Ebel (8) and Weil and Ebel (9). These water-insoluble salts were collected by centrifugation, washed with water, and lyophilized for 18 hours at a pressure of 50 μ of Hg. To iodinate sRNA,1 solutions of the hexadecyltrimethylammonium salt at a concentration of 10 μ moles (nucleotide phosphate per ml) in dimethylformamide were combined with 0.1 volume of 0.2 M 125IICl in CCl₄. After 18 hours, the reactions were terminated by the addition of 0.4 ml of 3 M NaCl containing 0.11 M sodium thiosulfate to each milliliter of the reaction mixture. The sodium salt of sRNA was thereby regenerated and precipitated. The precipitate was dissolved in 0.01 M potassium acetate, pH 5.5, and this solution was precipitated with 2 volumes of ethanol. This procedure was repeated three times. In order to eliminate traces of unincorporated radioactivity, which were present at this stage, the final solution was dialyzed for 8 hours against 0.01 M KI-0.01 M sodium thiosulfate and then dialyzed extensively against water.

The extent of iodination of the nucleotides composing sRNA was determined by digesting the labeled product with snake venom phosphodiesterase under the conditions employed by Nihei and Cantoni (10).2 The digests were chromatographed on strips of DEAE-formate-cellulose paper under the conditions described in the legend of Fig. 1. Three radioactive peaks were resolved. By reference to the chromatographic mobility of iodine-labeled nucleotides, we identified Peak I as 8-iodo-GMP, Peak II as 5-iodo-UMP, and Peak III as 5-iodo-CMP. The identity of these radioactive compounds was also established by converting them to nucleosides with the use of bacterial alkaline phosphatase. After dephosphorylation, radioactivity from Peak II was chromatographed on Whatman No. 3MM paper with the use of ethyl acetate saturated with 0.05 M potassium phosphate, pH 6, as the solvent. One radioactive peak was present having an RF value of 0.45, which is the same as that of 5-iodouridine in this solvent (13). Digests of Peaks I and III, when chromatographed on Whatman No. 3MM paper with the use of butanol-formic acid-water as the solvent, gave rise to single radioactive peaks having RF values of 0.18 and 0.15, respectively. These values should be compared with RF values of 0.2 and 0.15 observed with samples of 8-iodoguanosine and 5-iodocytidine which were prepared by iodinating the parent nucleosides directly.

A substantial quantity of iodinated sRNA was prepared and hydrolyzed in order to permit an identification of constituent iodonucleotides.3

1 The abbreviation used is: sRNA, soluble ribonucleic acid.
2 Enzymatic digestion was adopted to avoid the decomposition of iodinated bases that has been reported when iodinated nucleic acids are exposed either to alkali (11) or hot acid (12).
3 The agreement observed between the specific activities of these two iodonucleotides and the specific activity of the 125ICl employed in the iodination reaction proves that all of the radioactivity in the reagent was present either as 125ICl or as compounds capable of exchanging with this reagent. This might not have been the case if the 125ICl had contained substantial amounts of radioactivity either as iodate or bound to organic compounds. Iodate is frequently encountered in aqueous solutions of radioactive iodide (14).
be eluted by formic acid from Dowex 1-formate in a position following that of UMP. Approximately 0.14 μmoles of ribothymidine 5'-phosphate would have to be present to account for the excess ultraviolet absorbance measured. The identity and quantity of the nucleotides present were established. Peak a containedCMP, 3.1 μmoles; b, AMP, 2.11 μmoles; c, GMP, 2.5 μmoles; and d, UMP plus ϕ UMP, 1.23 μmoles. The properties of the fractions contained under Peaks A, B, and C, which were both ultraviolet-absorbing and radioactive, are described in Table I.

Radioactive components, in addition to the three iodinated nucleotides already resolved by DEAE-formate-paper chromatography, are apparent in Fig. 2. One peak of radioactivity was eluted between CMP and AMP, and this peak contained 3% of the radioactivity added to the column. It is of unknown origin. The radioactivity remaining on the column after extensive elution with 5 M formic acid could be eluted by 1 M perchloric acid. However, negligible ultraviolet absorbance was detected in these fractions. Upon treatment of these eluates with 0.001 M HIO₃, all of the radioactivity was extracted into CCl₄. It was concluded that this radioactivity (5% of the total that was added to the column) was due to iodide ions.

Iodination of DNA—A maximum extent of iodination of DNA was obtained by denaturing the native polymer prior to formation of the hexadeethyltrimethylammonium salt. Denaturation was effected by heating a solution containing 30 μg of DNA per ml in 0.015 M NaCl and 0.0015 M trisodium citrate to 100° for 10 min and then cooling the solution rapidly in an ice bath. The volume of the solution was concentrated under reduced pressure with a rotary evaporator until a concentration of approximately 300 μg per ml was obtained. The hexadeethyltrimethylammonium salt was then prepared and dissolved in dimethylformamide to a concentration of 4 μmoles of nucleotides per ml. This solution was combined with 0.05 volume of 0.15 M ¹²⁵ICl in CCl₄, allowed to react for 18 hours, and then combined with 0.25 volume of 3 M NaCl containing 0.06 M sodium thiosulfate. The precipitate of DNA was dissolved in 0.015 M NaCl containing 0.0015 M trisodium citrate and dialyzed extensively against this same buffer.

Iododeoxyribonucleotides were isolated from this preparation by digesting 2 μmoles of DNA nucleotides, dissolved in 0.1 ml of 0.1 M Tris (pH 8.5) and 0.01 M magnesium acetate, with 5 μg of DNase for 15 min at 37°. After addition of 2 μmoles of CaCl₂ and 10 μg of snake venom phosphodiesterase, the incubation was continued at 37° for 24 hours. The digest was applied to DEAE-formate-cellulose paper and developed with formic acid under the conditions described in the legend of Fig. 1. Two major radioactive regions, having values of 0.27 and 0.83, contained 97% of the radioactivity present in the digest. The remaining radioactivity was found between the origin and Rₚ 0.1. Since the preparations of 8-iodo-dGMP and 5-iodo-dCMP synthesized directly from the corresponding nucleotides had Rₚ values of 0.26 and 0.84, respectively, it was concluded that these two iodinated nucleotides had also been formed in DNA upon exposure to ICl. The amounts of radioactivity migrating in the position of

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**TABLE I**

Properties of radioactive fractions isolated from ¹²⁵I-labeled sRNA

<table>
<thead>
<tr>
<th>Peak designation</th>
<th>Nucleotide presumed present</th>
<th>Spectral properties</th>
<th>Quantity of nucleotide recovered&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proportion of parent nucleotide iodinated&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specific radioactivity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed&lt;sub&gt;λmax&lt;/sub&gt;</td>
<td>Repeated&lt;sub&gt;λmax&lt;/sub&gt;</td>
<td>μ mole</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>5-Iodo-CMP</td>
<td>308</td>
<td>307</td>
<td>0.29</td>
<td>8.5</td>
</tr>
<tr>
<td>B</td>
<td>5-Iodo-UMP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>283</td>
<td>280</td>
<td>0.60</td>
<td>30.2</td>
</tr>
<tr>
<td>C</td>
<td>8-Iodo-GMP</td>
<td>200</td>
<td>200</td>
<td>0.24</td>
<td>8.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculations are based on extinction coefficients previously reported (1, 5, 11).

<sup>b</sup> Amounts of each of the four major nucleotides recovered from the column are reported in legend to Fig. 2.

<sup>c</sup> Value quoted is that reported for 5-iodo-CMP (1).

<sup>d</sup> Reasons are cited in “Results” for regarding this fraction as containing substantial amounts of ribothymidine 5'-monophosphate in addition to 5-iodo-UMP.

<sup>e</sup> Value quoted is that reported for 5-iodo-UMP (II).

<sup>f</sup> Value quoted is that reported for 8-iodoguanosine (5).
8-iodo-dGMP and 5-iodo-dCMP represent 1.7 and 14%, respectively, of the parent nucleotides originally present in DNA. These two radioactive regions were further identified by eluting and digesting them with alkaline phosphatase. Radioactivity from these digests, chromatographed on Whatman No. 3MM paper with the butanol-formic acid-water solvent, was found to have the same mobility as standards of iododeoxyguanosine and iododeoxyctydine which had been synthesized directly from the corresponding nucleosides.

**Iodination of Synthetic Polynucleotides**—After exposure of solutions of the hexadecyltrimethylammonium salt of polyuridylic acid in N-ethylacetamide to ICl for 2 hours, an average incorporation of 0.4 atom of iodine per nucleotide residue was observed. This product was digested to nucleotides with pancreatic ribonuclease and treated further with alkaline phosphatase. Paper chromatography of the digest revealed the presence of only two ultraviolet-absorbing regions; one had the spectrum of uridine, and the other, that of 5-iodouridine. Treatment of solutions of the hexadecyltrimethylammonium salt of polycytidylic acid in N-ethylacetamide with $^{125}$IICl resulted in the incorporation of a maximum of 0.01 atom of iodine per nucleotide residue. When a phosphodiesterase digest of this product was submitted to chromatography on DEAE-formate-cellulose paper, a single radioactive region was detected at a $R_f$ of 0.82, the position of 5-iodo-CMP.

**DISCUSSION**

The treatment of nucleic acids with ICl results in the introduction of iodine into uracil, guanine, and cytosine residues. Direct proof that a substitution reaction had occurred, as would be expected under the anhydrous conditions employed, was obtained in the case of iodinated sRNA by examining the properties of the nucleotides separated by ion exchange chromatography from enzymatic hydrolysates. In this analysis, which succeeded in identifying 97% of the radioactivity and 95% of the ultraviolet-absorbing materials added to the column, the bulk of the radioactivity was present in three peaks. The ultraviolet absorbance spectra of these peaks corresponded to those of 5-iodo-CMP, 5-iodo-UMP, and 8-iodo-GMP. The formation of the first two derivatives is in accord with the results for similar substituted products of Lipkin et al. (5) in their experiments with cytidine and uridine.

Our claim that iodine-substituted nucleotides are also formed when DNA, polyuridylic acid, and polycytidylic acid are allowed to react with ICl is based upon the chromatographic mobilities of radioactive nucleotides and nucleosides isolated from the labeled polynucleotides after enzymatic digestion.

It is of interest to compare the extent of reaction of the different bases in sRNA with Br$_2$ and with ICl. Weil has shown that Br$_2$ reacts mainly with guanine and to a lesser extent with uracil and cytosine residues (16). The present results show uracil to be iodinated to an extent 4 to 6 times higher than that of guanine and cytosine. Neither in our experiments nor in those of Weil (16) was it possible to obtain complete halogenation of the susceptible bases. Secondary structure of the polynucleotide in the organic solvent may play some role in this limited reaction as is illustrated by our finding that DNA which is not denatured prior to the formation of the hexadecyltrimethylammonium salt incorporates only 23% of the radioactivity observed to attach to denatured DNA. Substantial secondary structure has, in fact, been demonstrated in native DNA hexadecyltrimethylammonium salts dissolved in organic solvents (17).

Iodination in organic solvents with ICl provides a means of introducing high levels of radioactivity into RNA and DNA after they are isolated. This technique would find greatest application in experiments involving higher organisms of large size or low metabolic activity which might preclude the use of radioactive precursors for labeling nucleic acids biosynthetically. Such labeled polynucleotides could be used in techniques which employ renaturation (18, 19) to establish the extent to which nucleic acids have regions that are complementary to each other. Iodination may also be used to test the biological role in polynucleotides of those bases which react with ICl. An example of such an investigation is provided by the interesting observations of Weil and Ebel (9) on the extent of polynucleotide-stimulated transfer of amino acids into protein from sRNA which had been treated with Br$_2$ in organic solvents. In the case of DNA, iodination provides the sole means of halogenating cytosine and guanine. Studies of the properties of DNA containing 5-iodouracil (20) suggest that DNA treated with ICl may become sensitized to ultraviolet and ionizing radiation. However, before such experiments can be performed, partial degradation of DNA, which was observed under the conditions of this study, must be prevented. To this end, milder reaction conditions are currently being explored.

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**REFERENCES**

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