High Specific Activity Iodination of \( \gamma \)-Globulin with Iodine-131 Monochloride

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SUMMARY

A technic, efficient in use, of I\( ^{131} \), has been developed for introducing I\( ^{131} \) into 4-mg. amounts of antibody or \( \gamma \)-globulin protein at a level of 10 mc. I\( ^{131} \) per mg. protein with a resulting iodine content of 2–3 atoms per protein molecule of 160,000 molecular weight. Oak Ridge I\( ^{131} \) was adjusted to pH 8 with borate buffer and the requisite amount of hydrochloric acid. To this solution was added Na\( _2 \)SO\( _3 \) to destroy H\( _2 \)O\( _2 \) produced by I\( ^{131} \) \( \beta \)-radiation. Excess sulfite was oxidized by aerating the solution in a boiling water bath. On the addition of iodine monochloride to the cooled solution, I\( ^{131} \)Cl (or HOI\( ^{131} \)) was produced by exchange, and following the rapid addition of this solution to protein, also in pH 8 buffer, coupling of approximately 60 per cent of the I\( ^{131} \) to protein occurred. When 10 mc. or less of Oak Ridge I\( ^{131} \) was used, the addition of Na\( _2 \)SO\( _3 \) and the aeration could be omitted, and a yield of about 60 per cent of I\( ^{131} \) attached to protein was still obtained.

To use antibodies with capacity to localize in malignant tumors as carriers of I\( ^{131} \) for radiation therapy, a method of introducing iodine with high specific radioactivity into the antibody is necessary. In particular, in a study of experimental radiation therapy of transplanted rat tumors (2), it is desirable to have 40-mc. antibody preparations containing 10 mc. of I\( ^{131} \) per mg. antibody protein. To avoid modification of the biological behavior of the antibody, evidence indicates that the number of iodine atoms attached to an antibody molecule of assumed 160,000 molecular weight should not much exceed two (5). Several factors, including the eventual cost of I\( ^{131} \) for use in human therapy, unite to make a high efficiency of I\( ^{131} \) incorporation desirable. McFarlane (7) has shown that the efficiency achieved with iodine monochloride as the iodinating agent is double that obtained with the more usually employed iodine in alkaline medium.

In the first instance the following reactions can be considered to occur:

\[
\text{ICl} + \text{NaOH} \rightarrow \text{HOI} + \text{NaCl} ;
\]

\[
\text{HOI} + \text{tyrosine residue} \rightarrow \text{iodinated tyrosine residue} + \text{H}_2\text{O} .
\]

On the other hand, in the reaction:

\[
\text{I}_2 + \text{NaOH} \rightarrow \text{HOI} + \text{NaI} ,
\]

50 per cent of the iodine is converted to iodide and thus becomes unavailable for reaction with protein.

Using McFarlane's procedure with tracer amounts of I\( ^{131} \) and 10 mg. of protein, we have been able to confirm his high degree of incorporation of I\( ^{131} \) into protein. With smaller amounts of protein the use of pH 8 borate buffer gave higher iodination efficiencies than those obtained with glycine buffers suggested by McFarlane.

Undiluted I\( ^{131} \) preparations as received by air shipment from the Oak Ridge National Laboratory contain free H\( _2 \)O\( _2 \) resulting from the action of \( \beta \)-radiation of I\( ^{131} \) on water containing dissolved oxygen. Sulfitie originally present is already oxidized to sulfate. This peroxide, unless removed, seriously impairs or completely inhibits iodination of protein by reducing ICl to iodide. The interfering action of peroxide can be eliminated by treating the Oak Ridge carrier-free I\( ^{131} \), after addition of borate buffer, with excess sulfite to reduce the peroxide and then oxidizing the excess by aerating.

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the solution in a boiling water bath. On the addition of ICl, the formation of I\(^{131}\)Cl (or HOI\(^{131}\)) occurs by a rapid exchange reaction, and immediate mixing with the buffered protein solution results in I\(^{131}\) introduction into the protein. After iodination at high I\(^{131}\) levels, some measure, such as dilution with protective inert protein, is essential to prevent protein damage from self-irradiation. Unbound I\(^{131}\) is removed by an ion-exchange resin. With this method I\(^{131}\) can be coupled to \(\gamma\)-globulin or antibody protein at a level of 10 mc. of I\(^{131}\) per mg. of protein with an efficiency of approximately 60 per cent.

MATERIALS AND METHODS

Iodide-131 solutions.—Samples of carrier-free I\(^{131}\) from Oak Ridge contained about 80 mc. at time of use, usually 2–3 days after shipment. Activity at time of shipment ranged from 20 to 50 mc/ml. The pH varied from 8 to 10. The chemical form was given as Na\(_2\)SO\(_3\) solution, since in the final purification step at Oak Ridge National Laboratory radioactive iodine was distilled into 6 per cent H\(_2\)SO\(_4\) and the acid was then neutralized by NaHCO\(_3\). However, tests (3) showed the absence of sulfite but the presence of H\(_2\)O\(_2\) and traces of nitrile. The iodide content (radioactive plus nonradioactive) of the Oak Ridge preparations was not determined. The efficiency achieved in introducing I\(^{131}\) into 4 mc. of protein suggests that the amount in an 80-mc. sample is usually small compared with the 12.7 \(\mu\)g. of iodine in the four equivalents of ICl used in our iodinations.

Stock iodine monochloride solutions.—A convenient stock solution was 0.02 \(\mu\)m in ICl, 2.0 \(\mu\)m in NaCl, 0.09 \(\mu\)m in KCl, and 1.0 \(\mu\)m in HCl. It can be prepared by the reaction:

\[
2 \text{KI} + \text{KIO}_3 + 6 \text{HCl} \rightarrow 3 \text{ICl} + 3 \text{KCl} + 3 \text{H}_2\text{O}.
\]

To a solution of 0.5550 gm. of KI (0.00394 mole), 0.3567 gm. KIO\(_3\) (0.00107 mole), and 29.23 gm. of NaCl were added 21 ml. of concentrated HCl (sp. gr., 1.18) and the necessary water to make the volume 250 ml. Since the weight of KI is slightly in excess of that stoichiometrically required to react with the KIO\(_3\) and an additional trace of iodide is commonly present in the NaCl, the ICl solution contained a small amount of free iodine. This was removed by repeatedly shaking the solution with CCl\(_4\) until the latter remained colorless. About five extractions with 10-ml. portions of CCl\(_4\) have been found necessary. After careful separation of the CCl\(_4\) used in this final extraction, a current of air, saturated with water vapor, was passed through the ICl solution to volatilize suspended and dissolved CCl\(_4\). The free iodine could also be removed by aerating the solution with moist air for several hours. The exact molarity of the solution was determined by adding an excess of KI to an aliquot and titrating the liberated iodine with a standardized thiosulfate solution. When prepared as described, the solution had a molarity within 1 per cent of that calculated on the basis of the KIO\(_3\) used. Such a stock solution showed no significant change in molarity after 6 months.

For iodinations of 4 mc. of protein a 0.0005 \(\mu\)m ICl solution was commonly used. This was prepared prior to use by dilution of a portion of the stock solution with 2 \(\mu\)m NaCl and was then immersed in ice water until needed. Four mc. of protein of assumed 100,000 molecular weight corresponds to \(2.5 \times 10^{-8}\) moles, and accordingly 0.2 ml. of the above diluted ICl solution provided four molar equivalents of iodine.

Borate buffers.—Borate buffer of pH 8, designated 1X, was prepared by adjusting a distilled water solution of 0.16 \(\mu\)m NaCl and 0.20 \(\mu\)m H\(_2\)BO\(_3\) to pH 8 by the addition of 1.6 \(\mu\)m NaOH to a final concentration of approximately 0.04 \(\mu\)m NaOH \(8\). Borate buffer with the same constituents in twice these concentrations has a pH of 7.65 and is termed 2X borate buffer.

Proteins for iodination.—The preparation of purified antibody solutions for iodination is described elsewhere (2). They were dialyzed against pH 8 borate buffer and iodinated at concentrations of approximately 1 mc. protein/ml. Solutions of normal rabbit \(\gamma\)-globulin or antibody protein at a level of 10 mc. were prepared prior to use by dilution of a portion of the stock solution with 2 \(\mu\)m NaCl and was then immersed in ice water until needed. Four mc. of protein of assumed 100,000 molecular weight corresponds to \(2.5 \times 10^{-8}\) moles, and accordingly 0.2 ml. of the above diluted ICl solution provided four molar equivalents of iodine.

Ion exchange columns.—Dowex 1-X4 resin (50–100 mesh) was allowed to stand in contact with 1 \(\mu\)m HCl for several hours, washed successively with 20 per cent NaCl solution and 0.85 per cent NaCl solution until the filtrate was neutral, and then stored under the latter. It was found that a column of 2 cc. of resin in a 2-ml. expendable glass or plastic syringe containing a small plug of glass wool removed all iodide ion from the iodinated protein solution and that no significant amount of iodide ion was eluted on subsequent rinsing of the column with 1.5 ml. of normal rabbit serum or 0.5 ml. of normal rabbit serum followed by 1 ml. of saline. Despite this rinse, however, 4–8 per cent of the iodinated protein was still retained by the resin.

Procedure for high I\(^{131}\) level iodination.—Oak
Ridge I\textsuperscript{131} solutions vary in volume, pH, and amount of dissolved solids. It seems, therefore, preferable to forego describing individual iodinations carried out by the method which we at present regard to be most satisfactory at a high level of activity (80 mc.), and to give instead the essential features of this procedure in a form sufficiently general to allow for such variations. The I\textsuperscript{131} solution was transferred to a 2 X 15-cm. pyrex test tube. If the volume was less than 2 ml., at least 2 ml. of 2X borate buffer was added. For volumes of 2-4 ml. an equal volume of buffer was used. If the pH was greater than 8 by indicator paper, it was adjusted to this value by the addition dropwise of 1 N HCl. To this solution was then added 0.3 ml. of a freshly made 0.05 M sodium sulfite solution to destroy H\textsubscript{2}O\textsubscript{2} that was present. After 5 minutes the mixture was placed in a boiling water bath covering most of the test tube and aerated with moist air (two to three bubbles per second emerging from a drawn out glass tube with an approximately 0.08-cm. opening) for 15 minutes to oxidize excess sulfite, while at the same time destroying any newly formed H\textsubscript{2}O\textsubscript{2}. If at the end of 10 minutes of aeration the volume of the solution was less than 3 ml., 2X borate buffer was added to bring the volume to about 4 ml. At the end of 15 minutes of aeration the solution was immersed for 2 minutes in ice and water, and during this time the pH was readjusted, if required, to approximately 8 by addition of HCl. The calculated amount of cold ICl solution was then introduced, rapidly mixed, and the total solution immediately jetted into the protein solution to be iodinated by means of the apparatus shown in Chart 1. After a 5-minute interval 1 ml. of protective protein solution was added—usually, in our experiments, normal rabbit serum or a 6.25 per cent solution of human albumin. The solution was then aspirated into a pipette by means of a connected screw-type Lucite syringe of 20-ml. capacity and passed through the ion exchange column at about 1-1.25 ml/min followed by 1.5 ml. of protective protein rinse. (The wait of 5 minutes between iodination and the addition of the protective protein was doubtlessly much longer than necessary, since it was demonstrated at the tracer level that iodination is virtually complete within seconds after the iodinating solution has been jetted into the protein.)

**Procedure for I\textsuperscript{131} iodinations at levels below 10 mc.**—In labeling 4-mg. amounts of protein with I\textsuperscript{131} activities up to 10 mc., it was found that without the addition of sulfite and subsequent aeration very satisfactory incorporation of fresh Oak Ridge I\textsuperscript{131} into the protein could be obtained. The pH of 1-4-ml portions of I\textsuperscript{131} solution was adjusted to approximately 8 by the addition of 1 ml. of 2X borate buffer and, if necessary, the requisite number of drops of 1 N HCl. The calculated amount of 0.0005 M ICl was then added, and the same procedure followed as outlined for high level iodination.

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**Chart 1.**—Apparatus for jetting iodination mixture into protein solution. A and C are 2 X 15-cm. Pyrex test tubes. B is a 1-mm. i.d. capillary tube. Tube D (6 mm.) is connected through a closed stopcock to a filtering flask attached to an aspirator. To iodinate, the capillary tube B was inserted into the I\textsuperscript{131} solution in tube A, and the solution was jetted into the protein solution in tube C by opening the stopcock in the suction line.

In our tracer level studies Oak Ridge I\textsuperscript{131} solutions were diluted in 0.05 N NaOH to 0.1-0.2 \( \mu \)c/ml. Dilutions made with distilled water alone tended to lose activity, presumably by oxidation and volatilization of I\textsuperscript{131} or by its adsorption on glass. For iodinations at this level by the McFarlane procedure, the required glycine-NaOH buffer solutions were prepared by diluting 8 parts by volume of a solution 1 M in glycine and 0.25 M in NaCl with 2 parts of 1 N NaOH (pH \( \sim 9.2 \)) and 9 parts of the glycine-NaCl solution with 1 part of 1 N NaOH (pH \( \sim 8.8 \)).
Scopoletin-peroxidase test for hydrogen peroxide.—The capacity of H₂O₂ in the presence of peroxidase to oxidize scopoletin in aqueous solution at pH 4.5 has been made the basis of a very sensitive method for the detection and quantitative determination of H₂O₂ (1). In the test, as used by us, a drop of the solution under investigation was added to a mixture of 0.02 μg. of scopoletin in 0.2 ml. of water and 0.2 ml. of 1 mg. per cent horseradish peroxidase in equal volumes of 0.1 M HAc and 0.1 M NaAc. After 1–2 minutes the mixture was made alkaline by the addition of 2 ml. of 2X borate buffer and then compared under ultraviolet illumination with a blank. Under these conditions it was found possible to identify readily, by the absence of fluorescence, the amount of H₂O₂ in a drop of a solution of 1 part of 30 per cent H₂O₂ to 2,500,000 parts of water (i.e., 0.005–0.01 μg. at a dilution of 1:8,000,000).

TABLE 1
Per cent of activity recovered in γ-globulin on iodination at tracer level (McFarlane’s procedure)

<table>
<thead>
<tr>
<th>γ-globulin (mg.)</th>
<th>Molar ratio of ICl to protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION
Iodinations at levels below 10 mc.—In Table 1 are listed results obtained in iodinations according to McFarlane’s procedure with tracer quantities of I²¹³ and varying amounts of iodine monochloride and ɣ-globulin. The calculated volume of ICl was added to a mixture of 1 ml. of tracer solution of I²¹³ in 1 ml. of 9:1 glycine-NaOH buffer. The mixture was immediately transferred to and mixed with 1 ml. of 8:2 glycine-NaOH buffer containing 3–10 mg. ɣ-globulin. In these experiments I²¹³ not bound to protein was removed by dialysis.

A higher incorporation of I²¹³ in protein was found to occur when iodinations were carried out in a similar manner but with both protein and I²¹³ in pH 8 borate buffer. In a long series of such iodinations with 4-mg. quantities of ɣ-globulin, 4 equivalents of ICl, and tracer amounts of I²¹³, with removal of unbound I²¹³ by ion exchange, 64–74 (mean, 68) per cent of the activity initially used was recovered coupled to protein in the effluent from the ion exchange column. The efficiency in similar iodinations with quantities of I²¹³ in the 1–10-mc. range was about 60 per cent. As in these iodinations, so in all others to be reported 4-mg. quantities of protein and 4 molar equivalents of ICl were routinely used.

It might be emphasized that in the iodination procedure there is some sacrifice of quantitative technic to convenience and rapidity, and that the iodination values obtained do not represent the percentage of the iodine which can be incorporated into protein by use of ICl in competition with other oxidation reactions which ɣ-globulin can undergo. There was a loss of several per cent of I²¹³ in the jetting operation. Some of the iodinated protein was retained by the resin and a small amount by the pipette used in the transfer of the solution. In addition to manipulative losses of activity, there were those which resulted from reactions of I²¹³ in which the protein was not involved. Whereas the interchange of I²¹³ with the iodine of the ICl (or the HOI formed on its hydrolysis) was evidently very rapid, it was not established that an equilibrium condition was attained prior to the introduction of the iodinating mixture into the protein solution. To the extent to which HOI²¹³ disproportionated into iodide and iodate, it was lost for iodination. It was found that an interval of a minute or more between the addition of ICl to the I²¹³ solution and the jetting of the iodinating mixture into the protein could substantially decrease the iodination efficiency. The presence of traces of iodide and other substances which reduce “positive” iodine diminished the amount of HOI available for iodination. Since these sources of loss probably accounted for much of the activity not present in the protein in the effluent from the resin column, it would appear that the amount of I²¹³ lost through any oxidation of the protein which might occur concurrently with iodination was relatively small. For this reason and because of the possibility of damaging antibody, preoxidation of the protein by iodine at pH 4.5, as suggested by McFarlane, was not extensively investigated.

Hydrogen peroxide interference in iodination.—With amounts of I²¹³ substantially larger than 10 mc. the percentage incorporated in protein fell to low values. This was initially attributed to the sulfite assumed to be present in the undiluted Oak Ridge solutions. However, spot tests never indicated the presence of sulfite ion. On the other hand, H₂O₂ (formed by β-radiation from the I²¹³ in the solution) was identified by the scopoletin-peroxidase test. In basic medium the peroxide would oxidize sulfite while its reducing action on HOI would decrease the iodination yield.
The formation of $\text{H}_2\text{O}_2$ by radiation was confirmed by subjecting a tracer solution of $\text{I}^{131}$ at pH 7–8 to 100,000 rad with 250 kv x-rays. The test for $\text{H}_2\text{O}_2$ was negative before radiation, positive after radiation. When the radiated solution was used in protein labeling, percentage of incorporation of $\text{I}^{131}$ was negligible. A crude quantitative test made 8 days after shipment of a 2.4-ml lot of $\text{I}^{131}$ from Oak Ridge with an initial activity of 37.2 mc/ml showed a total $\text{H}_2\text{O}_2$ content in the range of 1 to $2 \times 10^{-4}$ millimoles. Results of a study of the effect of traces of $\text{H}_2\text{O}_2$ of this order of magnitude on iodinations at tracer levels of $\text{I}^{131}$ are shown in Table 2.

**Elimination of hydrogen peroxide.**—Finely divided platinum, prepared by reducing Adam’s hydrogenation catalyst with hydrogen, will rapidly and completely decompose $\text{H}_2\text{O}_2$. However, it will also absorb practically all the carrier-free $\text{I}^{131}$. This sometimes took as long as 30 minutes. Since there was evidence that the complete oxidation of sulfite to sulfate is more readily accomplished by heating and aeration than is the elimination of hydrogen peroxide by this treatment, it appeared to be more practical to reduce all hydrogen peroxide present by the addition of a large excess of sulfite and then to oxidize the excess by aerating the hot solution. The iodination procedure was therefore modified as described in the section "Materials and Methods." Three iodinations of 4 mg. of protein ($\gamma$-globulin or purified antibody) were carried out by this procedure, in each instance with approximately 80 mc. Oak Ridge $\text{I}^{131}$ Iodinations of 59, 61, and 60 per cent were achieved.

**Interfering substances in the protein solution.**—Low iodination efficiencies were obtained in several instances in which the antibody solution was concentrated by a stream of air directed against the solution in dialysis tubing (cellulose casing, Visking Company, Chicago, Illinois.) A significant reduction of iodination values was observed likewise when $\gamma$-globulin solutions were dialyzed only a short time. Investigation revealed that the dialysis tubing released a material which reduced ICl. This material is removable by dialysis. It was our experience that 5–10 ml. of protein solution should be dialyzed against 2 liters of buffer at 0°C for a minimum of 4 hours to eliminate this reducing agent.

| TABLE 2 |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **IODINATIONS IN BORATE BUFFER AT $\text{I}^{131}$ TRACER LEVEL IN THE PRESENCE OF HYDROGEN PEROXIDE** |
| Amount of protein: 4 mg.; equivalents of ICl: 4 |

<table>
<thead>
<tr>
<th>Millimoles of $\text{H}_2\text{O}_2$ added to tracer solution $\times 10^{-4}$</th>
<th>2.25</th>
<th>1.12</th>
<th>0.56</th>
<th>0.28</th>
<th>0.14</th>
<th>0.007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent iodination:</td>
<td>0.4</td>
<td>1</td>
<td>16</td>
<td>44</td>
<td>56</td>
<td>92</td>
</tr>
</tbody>
</table>

**Protection of protein against damage by self-irradiation.**—At a level of 10 mc/ml the self-irradiation from the $\beta$-rays of $\text{I}^{131}$ in the solution is about 4000 rad/hour. To investigate the effect of ionizing radiation on antibody protein, antibody to rat fibrin labeled with $\text{I}^{131}$ was radiated with Co$^{60} \gamma$-radiation or lightly filtered 250 kv x-radiation. In dilute solutions (less than 0.1 mg protein/ml) 10,000 rad Co$^{60} \gamma$-radiation reduced the uptake of labeled antibody by rat fibrin about 17 per cent and 40,000 rad about 47 per cent. Dosage of 200,000 rad Co$^{60} \gamma$-rays or 100,000 rad x-ray reduced the specific uptake virtually to zero. At this latter dosage the presence of normal rabbit serum protein at a level of 8 mg/ml of radiated solution reduced the radiation effect to approximately that produced by 10,000 rad.

Since the incorporation of $\text{I}^{131}$ into protein at pH 8 occurs very rapidly, it was possible to minimize self-irradiation damage by adding to the protein solution, immediately after iodination, substances which protect against such damage; thereupon inorganic iodide could be quickly removed by ion exchange resin. The rapidity of protein iodination at pH 8 was demonstrated by experiments at the tracer level of activity. The presence of sulfite in manifold the molar concen-
tation of the protein completely inhibited iodination. On the other hand, if the sulfite was added to the protein solution 10 seconds after the iodinating mixture, the iodination efficiency was not affected.

Application to large animal and human cancer therapy.—If I\textsuperscript{131} attached to antibody or other proteins is to be used in experimental cancer therapy in large animals or in radiation therapy of human cancer, it will probably turn out that there will be needed protein preparations with substantially higher specific radioactivities and total amounts of several hundred mc. of incorporated I\textsuperscript{131}. The methods and results reported here indicate that this can be achieved and that at these high radioactivity levels radiation damage to the antibody can be kept negligible. For such iodinations I\textsuperscript{131} solutions much lower in inorganic salts than the present Oak Ridge preparations will probably be desirable. Such solutions can doubtless be produced by distillation or other suitable technic.

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