Some Quantitative Aspects of the Labelling of Proteins with $^{125}$I by the Iodine Monochloride Method

BY M. CESKA, A. V. SJÖDIN AND F. GROSSMÜLLER
Department of Biochemistry, Pharmacia AB, Uppsala, Sweden

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The labelling of proteins by the iodine monochloride method was studied by using a mathematical model. The equations used were primarily derived from the mass law equation of the isotopic exchange reaction between $[^{125}I]$iodide and iodine monochloride. For convenient application, all equations were programmed into a computing desk-top calculator. To support the validity of the theoretical model, a series of iodinations of insulin were performed under various labelling conditions. The results of these experiments compare well with the theoretically derived values. Deviations from the theoretical values occurring at molar ratios of $[^{125}I]$iodide to iodine monochloride $< 0.1$ and $> 4.0$ are explained and suggestions made about how to prevent them. The mathematical model was used to simulate the isotopic exchange, and the iodination reaction under various conditions, to study (a) the influence of the amount of $[^{125}I]$iodide on the amount of $[^{125}I]$iodine monochloride formed, (b) the influence of the specific radioactivity of $[^{125}I]$iodide on the amount of $[^{125}I]$iodine monochloride formed, and (c) the influence of the specific radioactivity of $[^{125}I]$iodide on the number of millicuries needed for labelling to a desired extent.

Techniques for the labelling of proteins with radioactive iodine have been known for over 25 years (Fine & Seligman, 1944). Since then considerable progress has been made towards improving the efficiency of labelling as well as minimizing damage to the protein during iodination. The iodine monochloride method (McFarlane, 1958) has high theoretical efficiency and does not adversely affect the properties of the labelled protein. Although this method was originally intended for efficient trace-labelling of proteins with radioactive iodine, it has been later extended and applied for high specific radioactivity labelling of proteins (Helmkamp, Goodland, Bale, Spar & Mutschler, 1960; Bale et al. 1962). A detailed investigation of this method of protein iodination has been reported by Helmkamp, Conteras & Bale (1967) and by Glover, Salter & Shepherd (1967).

Bale et al. (1962) made the first attempt to derive an expression for the labelling efficiency. They showed that the efficiency of iodination could be represented by $Y$ (wt. of iodine in iodine monochloride/[wt. of iodine as $I^- +$ wt. of iodine in iodine monochloride]) where $Y$ is the percentage of iodination by a given weight of iodine monochloride in the absence of iodide. The consequence of this is that adding an increasing amount of $[^{125}I]$iodide to an iodine monochloride iodination system results in a steadily decreasing percentage of $^{125}$I incorporation. These studies prompted us to investigate further the possibilities of quantitatively expressing the labelling of proteins by the iodine monochloride method. In our studies equations were derived which served as a mathematical model of the iodination reaction. By this means we were able to describe quantitatively the effects of certain factors that influence the iodination of proteins, making the course of the reaction more predictable and preventing failures of experiments. To support the validity of our model, a series of iodinations of insulin was performed under various labelling conditions and the results were compared with the theoretically derived data.

MATERIALS AND METHODS

Iodine monochloride reagent. An ICl solution was prepared by the method of Izzo, Bale, Izzo & Roncone (1964). Before use the ICl stock solution, which contained 2.54g of iodine/l, was diluted 79.6 times with 1 M NaCl-0.1 M HCl, in which ICl is known to be stable (Helmkamp et al. 1967).

Iodide. Potassium iodide was dissolved in 1 mM NaOH and diluted to the following concentrations (mM): 0.0251, 0.100, 0.251, 0.628, 1.26, 2.51, 5.02, 10.0, 25.1, 62.8 and 251.

Radioactive iodide. The $[^{125}I]$iodide (Union Carbide
Copenhagen, Denmark) was diluted 200 times in 1 mm-NaOH (1.2 μCi/μl), giving the trace iodide solution, which was used for the experiments.

**Insulin.** Pig insulin, ten times-recrystallized (Novo, Copenhagen, Denmark) was dissolved shortly before use in 5 mm-HCl to a concentration of 1 mg/ml.

**Outline of experiments.** To confirm experimentally the correctness of the theoretically derived equations, series of iodinations were performed under conditions where the concentrations of insulin and ICl were kept constant while the concentrations of added iodide, containing a trace amount of \(^{125}\text{I}\)iodide, were varied over a 10 000-fold range. The final molar ratios of iodide to ICl were: 0.01, 0.04, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 10, 25 and 100.

**Procedure for labelling.** To a mixture of 5 μl of iodide and 5 μl of \(^{125}\text{I}\)iodide (6 μCi), 50 μl of diluted ICl solution (containing 1.59 μg of iodine) was added. After equilibration for 1 min, 50 μl of the iodide-ICl mixture was injected quickly into 200 μl of 0.4 M-borate-carbonate buffer, pH 9.1, containing 0.02% NaNO₃ and 30 μl of insulin (30 μg), followed immediately by rapid mixing. After 2 min, 20 μl of 0.1 M-sodium thiosulphate, 50 μl of 2% (w/v) KI and 200 μl of 5% (w/v) human serum albumin in 50 mm-borate-carbonate buffer, pH 9.1, were added. All solutions were kept cold and the iodination was performed at 0°C.

**Separation and radioactivity measurements.** Samples (5 or 10 μl) of the final iodination mixtures were applied to a 2.5 cm from the bottom of filter paper strips (14 cm × 0.6 cm). These were placed in test tubes (11 cm × 1.6 cm) which contained 1 ml of 0.5 M-trichloroacetic acid. Whereas labelled insulin and carrier protein remained at the point of application, unchanged iodide moved up with the solvent front. After complete separation (5 min) the paper strips were cut into 14 pieces of 1 cm length and the radioactivity was counted with a γ-counter. Duplicates were run on all samples.

**Derivation of equations**

The isotopic exchange reaction between radioactive iodide and ICl can be presented as follows:

\[ ^{127}\text{ICl} + ^{125}\text{I}^- \leftrightarrow ^{125}\text{ICl} + ^{127}\text{I}^- \]

By applying the mass law equation to the isotopic exchange reaction eqn. (1) is derived:

\[ \frac{[^{125}\text{ICl}]}{[^{127}\text{ICl}]} \cdot \frac{[^{127}\text{I}^-]}{[^{125}\text{I}^-]} = k \]  

(1)

For all practical purposes the equilibrium constant may be assumed to be 1.00. Regarding the molar content of a certain volume, eqn. (1) can be re-written to give eqn. (2):

\[ \frac{n_{^{125}\text{ICl}, \text{eq}}}{n_{^{125}\text{ICl}}} = \frac{n_{125}}{n_{127} + n_{125} + n_{125}} \]  

(2)

where \( n \) is the number of mol of the different species before equilibrium. (The index 'eq.' indicates the number of mol of the reactants at equilibrium.) The molar ratio between radioactive ICl formed at equilibrium to the amount of non-radioactive ICl originally present is equal to the molar ratio of added radioactive iodide to the total amount of all species containing iodine.

The terms in eqn. (2) are expressed in a convenient form to be applied to protein iodination.

\[ ^{125}\text{ICl}, \text{eq} = \frac{m_{125}}{M_{^{125}\text{ICl}}} \cdot n_{125} \]  

(3)

\( m_{125} \) = weight of substance to be labelled; \( M_{125} \) = mol. wt. of that substance; \( n_{125} \) = number of atoms of \(^{125}\text{I}\) to be incorporated per protein molecule; \( \eta_{125} \) = iodination efficiency (%).

\[ ^{127}\text{ICl} \]

**Outline of experiments.** To confirm experimentally the correctness of the theoretically derived equations, series of iodinations were performed under conditions where the concentrations of insulin and ICl were kept constant while the concentrations of added iodide, containing a trace amount of \(^{125}\text{I}\)iodide, were varied over a 10 000-fold range. The final molar ratios of iodide to ICl were: 0.01, 0.04, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 10, 25 and 100.

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(1)

For all practical purposes the equilibrium constant may be assumed to be 1.00. Regarding the molar content of a certain volume, eqn. (1) can be re-written to give eqn. (2):

\[ \frac{n_{^{125}\text{ICl}, \text{eq}}}{n_{^{125}\text{ICl}}} = \frac{n_{125}}{n_{127} + n_{125} + n_{125}} \]  

(2)

where \( n \) is the number of mol of the different species before equilibrium. (The index 'eq.' indicates the number of mol of the reactants at equilibrium.) The molar ratio between radioactive ICl formed at equilibrium to the amount of non-radioactive ICl originally present is equal to the molar ratio of added radioactive iodide to the total amount of all species containing iodine.

The terms in eqn. (2) are expressed in a convenient form to be applied to protein iodination.

\[ ^{125}\text{ICl}, \text{eq} = \frac{m_{125}}{M_{^{125}\text{ICl}}} \cdot n_{125} \]  

(3)

\( m_{125} \) = weight of substance to be labelled; \( M_{125} \) = mol. wt. of that substance; \( n_{125} \) = number of atoms of \(^{125}\text{I}\) to be incorporated per protein molecule; \( \eta_{125} \) = iodination efficiency (%).

\[ ^{125}\text{ICl} \]
Placing \( N \) (as calculated from equation I) into equation (II), one can study what percentage of the total ICl would be radioactive under the conditions given in equation (I).

*Simulation of the iodination reaction by a computing calculator.* To facilitate the computation of the various terms, equations (I) and (II) were programmed into a Hewlett-Packard computing desk top calculator, model 9100A. This program can be used directly to simulate the iodination of proteins under various conditions.

**RESULTS**

To confirm the validity of the theoretical model a series of iodinations with insulin was planned at various molar ratios of \([^{125}\text{I}]\)iodide to iodine monochloride. However, for convenience, all iodinations were actually performed with \([^{127}\text{I}]\)iodide to which a trace amount of \([^{125}\text{I}]\)iodide was added. Since radioactive and non-radioactive iodide behave chemically identically the results of the experiments were therefore interpreted as if only \([^{125}\text{I}]\)iodide had been used.

The experimental data were plotted as % of radioactivity (added as \([^{125}\text{I}]\)iodide together with \([^{127}\text{I}]\)iodide) that was bound to insulin versus the molar ratios of iodide to iodine monochloride (these are shown as the points in Fig. 1). The curve in Fig. 1 represents the calculated values corrected for an iodination efficiency of 95.2%. This iodination efficiency was computed from the mean of six iodinations (molar ratios of iodide to iodine monochloride 0.1–4.0). In this range the iodination efficiencies were almost constant whereas above and below these ratios deviations occurred from the theoretical curve. The iodination efficiencies were calculated by dividing the experimentally obtained data (% of added radioactivity bound to protein) by those theoretical values computed for a 100% labelling efficiency.

Deviations from the theoretical curve occurring at molar ratios very much above 4.0 are very likely caused by non-specific adsorption of iodide to protein (these deviations cannot be seen clearly on the plot in Fig. 1). However, they are more of theoretical than of practical importance owing to the large waste of radioactive iodide under these conditions. Deviations from the theoretical curve occurring at molar ratios below 0.1 are of more practical importance, since optimum iodination conditions are very often studied by so called ‘trace’-labelling. In such experiments only a trace amount of \([^{125}\text{I}]\)iodide is added to an iodine monochloride solution, so that \([^{125}\text{I}]\)iodide is practically completely incorporated into the iodine monochloride. When adding such a mixture to a buffered protein solution hypiodous acid is formed from hydrolysis of iodine monochloride. The relatively high pH of the iodination mixture (pH \(\geq 8\)) promotes a fast disproportionation of hypiodous acid into iodine and IO\(^{-}\) (Helmkamp et al. 1967). As a result, a certain percentage of hypiodous acid will not be available for protein iodination. To explain the lower labelling efficiencies at lower molar ratios the reaction between iodide and iodine monochloride in sodium chloroide–hydrochloric acid solutions was studied. The results indicated that at molar ratios of iodide to iodine monochloride \(<1.0\), mainly iodine is formed, whereas at molar ratios of iodide to iodine monochloride \(>1.0\) mainly I\(^{-}\) is formed. In other words, when increasing the molar ratio of iodide to iodine monochloride (e.g. starting from 0.01) formation of iodine is increased at the same rate as the amount of iodine monochloride is decreased. Therefore less hypiodous acid is formed in the iodination mixture from the remaining iodine monochloride and thus less hypiodous acid is lost for iodination by disproportionation. In addition an increasing amount of iodine, being one of the products of hypiodous acid disproportionation, represses the latter reaction.

**Influence of the amount of \([^{125}\text{I}]\)iodide on the amount of \([^{125}\text{I}]\)iodide monochloride formed.** At the point of equal molar ratios of \([^{125}\text{I}]\)iodide to iodine monochloride, 50% of the added radioactive iodide (with a specific radioactivity of 17.5 mCi/\(\mu\)g) is incorporated into iodine monochloride (see Fig. 2). In most iodination experiments one tries to achieve this molar ratio since at this point one also reaches the point of optimum utilization of the radioisotope with regard to the incorporation of \([^{125}\text{I}]\)iodide into iodine monochloride (and thus into protein) as compared with the amount of \([^{125}\text{I}]\)iodide that cannot react with protein. However, one may achieve any other degree of formation of \([^{125}\text{I}]\)iodide monochloride that is greater than 50% by addition of

![Fig. 1. The portion of the total added \([^{125}\text{I}]\) that is bound to insulin (% as a function of the molar ratio of \([^{125}\text{I}]\) to ICl. The points represent the experimental data, the curve the theoretical values corrected for an iodination efficiency of 95.2% (see the text).](image-url)
more $^{125}$I iodide, but at the cost of an increasing fraction of added radioactive iodide that cannot be utilized for labelling.

On the other hand, it can be seen that decreasing the molar ratio of $^{125}$I to iodine monochloride results in an increasing fraction of the total added radioactive iodide being incorporated into iodine monochloride. However, in view of the better utilization of the radioactive iodide one should be aware of the fact that in this case less $^{125}$I iodine monochloride will be formed; i.e. the specific radioactivity of the iodinated protein will be correspondingly lower. In the case where the molar ratios of $^{125}$I iodide to iodine monochloride are 0.01 or less one may neglect the amount of free $^{125}$I iodide ions and assume for all practical purposes that essentially all the added $^{125}$I iodide will be incorporated into iodine monochloride. Under these conditions the reaction between iodine monochloride and protein alone can be studied, the influence of any added $^{125}$I iodide being neglected.

**Influence of the specific radioactivity of $^{125}$I iodide on the amount of $^{125}$I iodide monochloride formed.** When using radioactive iodide of lower specific activities than 17.5 mCi/µg, $^{125}$I iodine monochloride will be formed in lower amount (see Fig. 2). This effect is especially pronounced at molar ratios of $^{125}$I iodide to iodine monochloride $>$ 1.0. In fact, each specific radioactivity reaches a 'plateau' where additional radioactive iodide of that specific radioactivity does not significantly increase the formation of $^{125}$I iodine monochloride. On the other hand, when working at molar ratios of $^{125}$I iodide to iodine monochloride $<$ 0.1 (approaching 'trace' conditions) the specific radioactivity of the radioactive iodide does not have any practical implications.

**DISCUSSION**

The iodination of proteins by the iodine monochloride method can be divided into two major steps: (a) the reaction of $^{127}$I iodine monochloride with $^{125}$I iodide to form $^{125}$I iodine monochloride (isotopic exchange reaction); (b) the reaction of $^{125}$I- and $^{127}$I- iodine monochloride with the protein (iodination reaction).
Since only the iodine monochloride is acting as the protein-iodinating agent, it is apparent that the degree of iodination with $^{125}$I is dependent on the position of the equilibrium of the isotopic exchange reaction. If this cannot be controlled, then the actual degree of incorporation of $^{125}$I into protein cannot be controlled either. The isotopic exchange reaction as well as of the chemical reaction between $[^{125}\text{I}]$iodide and iodine monochloride in an acidic sodium chloride solution is far more complex than is indicated in eqn. (1). However, it proved to be sufficient to describe the isotopic exchange reaction and thus the iodination of proteins in a quantitative way. The derived equations proved to be very useful not only in studying the various factors that influence the iodination of proteins, but they also can be used as a practical tool and guide for the design of labelling experiments. In addition these equations assist in the preparation of labelled proteins containing the desired ratios of $^{125}$I to $^{127}$I atoms throughout a wide range. From the results it can also be seen that when studying the optimum conditions of protein iodinations at a 'trace'-level of $[^{125}\text{I}]$iodide (i.e. at a molar ratio of $[^{125}\text{I}]$iodide to iodine monochloride less than 0.1) lower labelling efficiencies are obtained than otherwise would be expected. As a conclusion, protein iodinations performed at a 'trace'-level of $[^{125}\text{I}]$iodide should be carried out with $[^{127}\text{I}]$iodide containing a trace amount of $[^{125}\text{I}]$iodide. The ratios of $[^{127}\text{I}]$iodide to iodine monochloride should be close to the expected molar ratios of $[^{125}\text{I}]$iodide to iodine monochloride that are to be used when 'hot'-labelling under otherwise identical conditions.

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REFERENCES


