Standardization of the Chloramine-T Method of Protein Iodination (34611)

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Many methods have been devised for labeling proteins with radioactive iodine. The iodine monochloride (ICl) method (1) and the chloramine-T method (2–4) have been most widely used. The iodine monochloride method becomes progressively less efficient as the amount of protein is reduced below 2–5 mg (5). High concentrations of chloramine-T may severely denature proteins (3), and low concentrations may either fail to label certain proteins (4) or may produce unpredictable efficiency of labeling. The following modifications of the chloramine-T method have permitted us to vary the amounts of protein and radioiodine over a wide range, to minimize the concentration of chloramine-T required, and to predict the degree of iodination within 5%. The method depends upon the basic observation that the rate at which iodine combines with protein is related to the "redox" potential, or emf, of the reaction mixture. The iodination reaction is run as a titration which uses the potential to measure the minimum amount of chloramine-T required for iodination.

Iodine-125 is usually supplied in aqueous solution as the salt $Na^+ {}^{125}I^-$. The primary reaction involved in labeling protein is the replacement of an H^+ ion on a tyrosine ring with the iodine of an H_2OI^+ ion (6). The H_2OI^+ ions are generated as follows:

$$2I^- \rightleftharpoons I_2 + 2e \qquad (1)$$

$$\text{emf} = E_{\text{ref}} + C \log \frac{I_2}{I^-} \qquad (2)$$

$$H_2O + I_2 \rightleftharpoons H_2OI^+ + I^- \quad K = 1.2 \times 10^{-11} \qquad (3)$$

The emf is the "redox" potential, E_{ref} and C, are constants within any given system, but may vary between systems (7). Equation (1) represents a "redox" half reaction which is governed by Eq. (2). Equation (2) states that the potential, emf, determines the ratio of I_2 to I^- (7). According to Eq. (3) this ratio determines the amount of H_2OI^+ ion generated (6). Consequently, the potential, emf, is a main factor in determining the amount of H_2OI^+ ion which is available for reaction with protein, and one might expect that the rate of reaction would depend upon potential. Our experiments have shown that the rate of iodination does depend upon potential, but the optimum potential is different for different proteins.

Materials and Procedures. The percentage of iodine which has combined with protein can be quickly estimated by a trichloracetic acid (TCA) precipitation test (8). This test is performed as follows: A small sample, about 0.01 ml of the iodinated protein solution is added to 1.0 ml of 0.01 *M* $Na_2S_2O_5$ solution. This reducing agent stops the iodination reaction and inhibits the absorption of unreacted iodine to protein. To this is added 0.5 ml of a 1/10 dilution of any normal serum as carrier and 0.5 ml of 30% TCA with thorough mixing. The precipitated protein is sedimented by centrifugation at 500*g* for 2 min and the supernatant fluid is decanted. The radioactivity in the supernatant fluid and the precipitate is counted to determine the percentage of iodine bound to protein. The precipitates can be washed with 1 ml of 6% TCA and recentrifuged to improve accuracy. However, we have found that the unwashed precipitate consistently contains about 5% of the non-protein-bound radioactivity. Consequently we ordinarily calculate

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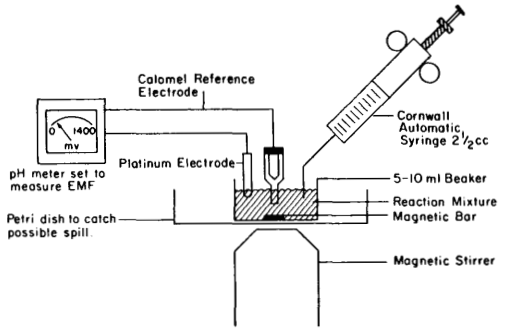

FIG. 1. Schematic diagram of the iodination apparatus used in our laboratory. All of the equipment, except the pH meter, is supported on a single ring stand.

the percentage of uptake as:

$$\% \text{ bound} = \frac{\text{cpm}_{(\text{precipitate})} - .05\ \text{cpm}_{(\text{supernatant})}}{\text{cpm}_{(\text{precipitate})} + \text{cpm}_{(\text{supernatant})}} \times 100$$

To insure homogeneity of the labeled protein preparation, it is usually desirable to use an average of one or two iodine atoms per molecule of protein (6). If this were all radioactive iodine, the specific activity could be much higher than necessary. Consequently, we usually label protein with a mixture of radioactive and nonradioactive iodine. The following constants facilitate calculation of the amounts of $^{125}I^-$, KI, and protein required to give any desired specific activity and degree of substitution (9): Carrier-free $^{125}I^-$ has 2.58×10^{14} iodine atoms per millicurie. KI has 3.66×10^{18} molecules per milligram. Protein has 6.03×10^{20}/mol wt molecules per milligram. Thus flagellin (mol wt 30,000) has 2.01×10^{16} molecules per milligram. In a typical experiment, we might iodinate 2 mg of flagellin (4.02×10^{16} molecules) with 10 mCi $^{125}I^-$ (2.58×10^{15} atoms) and 0.02 mg KI as carrier (7.32×10^{16} molecules). If 80% of the iodine combined with the protein, the product would have a specific activity of 4 mCi/mg and an average substitution rate of 1.5 atoms of iodine per molecule of flagellin.

The protein, KI, and $^{125}I^-$solutions are made up in 0.15 *M* phosphate-buffered saline, pH 7.5, (PBS). These reagents in a total volume of 1–5 ml are mixed at room temperature in a small (5–10-ml) beaker with a magnetic stirring rod (Fig. 1). The pH is adjusted to 7.5 with 0.1 *N* NaOH using indicator paper sensitive in the pH 6–8 range. Next, calomel and platinum electrodes are immersed in the beaker and are connected to a pH meter set to record potential (EMF). We use a Beckman Model G pH meter. A "Cornwall" automatic syringe (2.5-cc) (Becton, Dickenson and Co., Rutherford, N.J.) is filled with chloramine-T (Eastman Organic Chemicals, Rochester, N.Y.) 2 mg/ml in distilled water. The iodination reaction is started by adding the chloramine-T slowly to the reaction mixture by twisting the volume adjustment on the Cornwall syringe. The rise in potential is monitored on the pH meter.

Since chloramine-T dissociates slowly (3), the potential will continue to rise for several minutes after the addition of chloramine-T. During this time, small samples of the reaction mixture, 0.01 ml or less, are removed at 1- 2-min intervals for TCA precipitation tests to determine the percentage of the idoine bound to protein. These values are then plotted against the potential to show the iodination characteristics of the protein. Representative curves of this type are shown in Fig. 2. It can be seen that, at a given potential, the rate at which iodination occurs is different for different proteins, and the amount of chloramine-T required to reach a given potential may vary substantially between iodinations of a single protein; but the potential at which any given protein undergoes iodination appears to be constant. The lowest potential at which a protein undergoes iodination at an acceptable rate is the optimum potential for the iodination of that protein. Once the optimum potential for a given system has been determined from the curve of percent uptake vs. potential, chloramine-T need be added only in quantity sufficient to produce that potential. This is the minimum amount which will cause acceptable iodination of the protein. The optimum potentials for iodination of several different proteins are recorded in Table I. The actual values may vary with different meters and electrodes, but

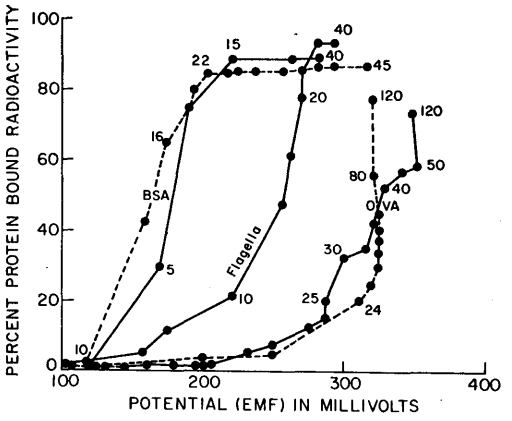

FIG. 2. The iodination characteristics of three proteins, BSA, *Salmonella* flagella, and OVA. In each case, 1 mg of protein was labeled with 50 μCi $^{125}I^-$ and 0.20 mg KI in a volume of 3 ml. The time in minutes after the start of iodination is shown beside some of the points. The experiments represented by solid lines used the following amounts of chloramine-T: BSA, 50 μg, flagella, 500 μg, OVA, 20 μg. In the experiments shown by the dashed lines 200 μg of $Na_2S_2O_5$ was added to the reaction mixtures, and 700 μg and 750 μg of chloramine-T were required for iodination of BSA and OVA, respectively.

the relative positions should remain unchanged.

At the end of the reaction period, the potential is lowered to the starting level by the dropwise addition of sodium metabisulfite (5 mg/ml). In our experience, *Salmonella* flagella are ordinarily labeled with about 85% of the iodine in 20 min at a potential of 225–250 mV. The reaction proceeds faster at a higher potential. We have consistently observed this pattern of reaction even though the quantity of flagella has been varied from 10 μg to 10 mg. After the iodination reaction has been terminated, the unbound iodine can be removed by any one of several methods (5). We dialize our preparations for 24–48 hr against several changes of saline.

Discussion. The method of protein iodination described in this paper has greatly increased our ability to trace antigens and antibodies. It is more complex than most other methods, but it has the advantages of consistancy and reliability. It depends upon the observations that the rate at which iodine combines with protein is dependent on the EMF or "redox" potential of the reaction mixture and that different proteins require different potentials for the same rate of reaction. Once the behavior of a protein has been established, the rate of iodination reaction can be controlled using potential to determine the amount of chloramine-T needed. If the reaction is run slowly, a TCA precipitation test can be run during the course of the iodination to be sure that it is going as planned. Alternatively, if one desires to label a protein rapidly or does not have immediate access to a gamma counter, the reaction may be stopped at the predicted time by the dropwise addition of enough sodium metabisulfite to reduce the potential to the starting levels. If the labeling then proves to be insufficient, the reaction can be continued by raising the potential again with chloramine-T.

The routine use of this method in our laboratory has virutally eliminated iodination failures. There are, however, some pitfalls which must be avoided. Some materials, notably polyacrilamide gel and formalin, inhibit the iodination reaction. Consequently, the probability of labeling an unfamiliar material can be increased substantially by dializing it against PBS before iodination. If the inhibi-

TABLE I. Optimum Potentials for Iodination.

Protein	Optimum potentials for iodination[a] (mV)	Time at optimum potential to reach 70–90% uptake (min)
Bovine serum albumin	150–200	5–10
Bovine gamma globulin	150–200	5–10
Rabbit gamma globulin	150–200	5–10
Myoglobin	150–200	15–20
S. thyphi flagella	200–250	15–25
Epstein-Barr virus particles	250–300	15–20
Horse ferritin	250–300	30–40
Ovalbumin	300–350	60–90
Proteus mirabilis flagella	300–400	40–60

[a] These potentials were derived from curves such as those in Fig. 2. They represent the lowest range of potential values in which protein iodinates fairly rapidly. If the potential is raised above these levels, the reaction will proceed faster.

tor can not be eliminated from the system, the method may be modified slightly to improve the probability of labeling. Polyacrilamide probably interferes with iodination by reacting with chloramine-T. If polyacrilamide or similar-reacting material is present, the potential rises and falls within a few seconds after addition of chloramine-T. In this situation, we have not achieved uniformly good results, but the chances of success seem to be improved by delaying the addition of iodine until enough chloramine-T has been added to neutralize the inhibitor and produce a slowly rising or sustained elevated potential.

Finally, the damage done to a protein during iodination must be assessed relative to the needs of each situation. Low concentrations of chloramine-T have been found to produce results comparable with other methods (4, 10, 11). The present modifications of the method do not affect the amount of damage done to a protein, but they do allow one to more precisely define and reproduce the conditions which produce acceptable results in a given system.

Summary. A method is described for the controlled iodination of proteins. The potential, EMF, of the reaction mixture is monitored and used to determine the minimum amount of chloramine-T required for acceptable iodination. The method has been applied to the iodination of bovine serum albumin, ovalbumin, *Salmonella* flagella, and other proteins.

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