

## Criteria for Preparing, Evaluating, and Standardizing Iodinated Globulins for Radioimmunoassay Procedures

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Received for publication 19 August 1974

Procedures were examined for labeling immune globulins with radioactive iodine using chloramine-T as the oxidizing agent. The chloramine-T method was critically evaluated to establish the optimal conditions for preparing iodinated globulins with high specific radioactivities without impairing their immunospecificities for use in *in vitro* radioimmunoassays. The results showed that the use of 100  $\mu\text{g}$  of chloramine-T per ml, 500 to 1,000  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  per mg of protein, and a 10-min oxidation reaction time produced globulins of both high specific radioactivities and immunospecificities. Criteria were established for evaluating and determining optimal concentrations of iodine-labeled globulin for use in radioimmunoassays. The results of this investigation indicated that the amount of labeled indicator globulin used in radioimmunoassays should be based upon protein concentration rather than radioactivity.

Radioimmunoassay (RIA) methods are gaining acceptance as diagnostic tools for viral agents, and RIA will probably become the standard procedure for many serological determinations as definitive methods are established. Assays have been designed which are operationally simple yet provide sensitive, quantitative antibody determinations (8, 13; H. D. Hutchinson, D. W. Ziegler, G. A. Baer, P. A. Yager, J. S. Smith, and T. K. Lee, manuscript in preparation; D. W. Ziegler, H. D. Hutchinson, J. P. Koplan, and J. H. Nakano, submitted for publication).

The success of RIA methodology depends, however, upon the integrity of the immune globulin reagents. The radioactively labeled immune globulins must have high specific antibody activity and high specific radioactivity. After immune globulins of high antibody concentration are obtained, radioactive atoms must be introduced into the protein molecules without altering the immunological specificity.

Several methods of labeling protein with radioactive iodine have been described. Among these procedures are the electrolytic method of Rosa et al. (12), the microdiffusion method of Gruber and Wright (6), and the peroxidase methods of Thorell and Johansson (14) and David (3).

Because of their simplicity, chemical methods of converting anionic iodine to an oxidized state are used in many laboratories. Among the chemical methods are the iodine monochloride method described by McFarlane

(11) and the chloramine-T procedure described by Greenwood et al. (5). Both of these methods have gained considerable acceptance and are extensively used. The chloramine-T iodination procedure is easily controlled by precise termination of the oxidation period by addition of a reducing agent, sodium metabisulfite. This precludes the possibility of protein denaturation due to prolonged exposure of the protein to oxidizing conditions. Because of this distinct advantage of the chloramine-T method, it was adopted as the standard procedure in our laboratory.

The Greenwood chloramine-T oxidation procedure was designed for labeling microgram quantities of protein hormones with high specific radioactivities. McConahey and Dixon (10) later modified the procedure so that larger quantities of partially purified serum proteins, such as albumin and gamma globulin of various animal species, could be trace-iodinated with minimal denaturation for *in vivo* applications. The optimal conditions for iodinating these proteins may not be the same as those required for iodinating partially purified immune globulins for use in *in vitro* RIA procedures. Hence, in this study we determined the oxidation conditions and the quantity of radioactive iodine yielding high levels of iodination while maintaining satisfactory immunological specificity for use in *in vitro* serological determinations.

When radioactively labeled antiglobulin is used as an indicator in RIA, the sensitivity of the assay is greatly influenced by the quantity

of globulin added to the assay. Definitive information regarding the optimal quantity of iodine-labeled globulin for use in RIA is generally unavailable. To meet this need we investigated the effect of concentration of the labeled indicators on the RIA results. From this investigation, criteria for preparing, evaluating, and using RIA reagents are proposed.

#### MATERIALS AND METHODS

**Preparation of antiglobulins.** Antisera to human and rabbit globulins were prepared in guinea pigs. The guinea pigs were initially inoculated intradermally with 2 mg of sodium sulfate-precipitated human or rabbit globulin emulsified with Freund complete adjuvant. The animals received intraperitoneal booster inoculations of 2 mg of globulin every second week until high antiglobulin precipitin titers were obtained. The titers were tested with the Ouchterlony procedure of double diffusion in agar. Specificities of the globulins were tested by immunoelectrophoresis.

The globulin from the guinea pig antiglobulin serum was precipitated by the sodium sulfate method of Kekwick (9). After three sodium sulfate precipitations, the globulin was dissolved in borate saline buffer (0.15 M NaCl, 0.002 M  $H_2BO_3$ , pH 7.4) and dialyzed against 0.0175 M phosphate buffer, pH 7.4. Estimations of the protein concentrations of the partially purified globulins were made spectrophotometrically at 280 nm (extinction coefficient = 15.0).

Sulfate-precipitated guinea pig antirabbit or antihuman globulins were usually iodinated in 5.0-mg lots. If different amounts of protein were used, the volumes of the reactants were adjusted accordingly.

**Chloramine-T iodination.** Globulin was routinely iodinated by the McCahey and Dixon (10) modification of the Greenwood, Hunter, and Glover method (5) with chloramine-T as the oxidizing agent. Chloramine-T and sodium metabisulfite solutions were prepared in 0.05 M phosphate buffer, pH 7.5, at a concentration of 500  $\mu\text{g}/\text{ml}$ . Fresh solutions were prepared on the day of each iodination.

Samples of 5.0 mg of antiglobulin were diluted to 4.0 ml with 0.05 M phosphate buffer. The dilution and iodination were performed in a 12-ml Wheaton vial fitted with a rubber-sleeve stopper and containing a small magnetic stirring bar. The diluted protein sample was chilled in an ice bath on a magnetic stirrer before the iodination reaction was started, and it was maintained in the ice bath during the reaction. All of the reagents were injected through the sleeve stopper with syringes.

Carrier-free  $Na^{125}I$  (New England Nuclear) was added at a concentration of 1,000  $\mu\text{Ci}/\text{mg}$  of protein with a Hamilton syringe. Immediately after the addition of  $Na^{125}I$ , 1.0 ml of chloramine-T (500  $\mu\text{g}/\text{ml}$ ) was injected slowly (about 30 s) into the reaction mixture. The mixture was stirred vigorously for 10 min. Finally, 1.0 ml of sodium metabisulfite (500  $\mu\text{g}/\text{ml}$ ) was added, and the reaction mixture was stirred for 1 min.

The iodinated protein was then transferred to dialysis tubing (8 mm diameter) and dialyzed against 0.1 M phosphate buffer (pH 7.0) to remove the unreacted  $Na^{125}I$ . This procedure was adopted as the standard method after variations of the conditions were investigated to ascertain optimal conditions. To determine the amount of protein-bound  $^{125}I$ , we added a portion of iodinated protein to 0.2 ml (approximately 2 mg) of carrier protein and precipitated it with 0.2 ml of 10% trichloroacetic acid. The precipitate was then washed with three 1.0-ml portions of 10% trichloroacetic acid. The radioactivity in the precipitate, the supernatant, and the three rinses was measured. If 90% or more of the radioactivity was present in the precipitate (protein-bound), the protein was considered satisfactory for use in the RIA procedure.

**RIA procedure.** The RIA procedures used in this study have been described by Hutchinson and Ziegler (8), Hutchinson et al. (manuscript in preparation), and Ziegler et al. (submitted for publication). Either *Escherichia coli*, vaccinia virus, or rabies virus was used as the primary antigen in the reaction and was prepared as described by these authors. The RIA procedures were performed either on cover slips (8) or in microtiter wells (Hutchinson et al., manuscript in preparation; D. W. Ziegler, H. D. Hutchinson, J. P. Koplán, and J. H. Nakano, submitted for publication). In each case the RIA was an indirect test in which specific rabbit or human antiserum was allowed to react with the antigen which was affixed to the cover slips or microtiter wells.

After the primary antigen-antibody reaction, a dilution of guinea pig antirabbit or antihuman  $^{125}I$ -labeled globulin was allowed to react with the primary antigen-antibody complex. The guinea pig antiglobulin used was specific for the antiserum species reacting in the primary immunological reaction. After the second reaction, the excess antiglobulin was removed, and the cover slips or microtiter plates were washed with phosphate-buffered saline (pH 7.2), dehydrated with 95% ethanol, and air dried. The samples were then transferred to counting vials, and the incorporated radioactivity was measured in an autogamma scintillation spectrometer. The results of antiserum titrations by the RIA were recorded as the counts per minute per cover slip or counts per minute per microtiter well (counts min per sample).

#### RESULTS AND DISCUSSION

RIA methodology depends upon the availability of indicator globulin of both high specific radioactivity and high immunospecificity. Therefore, it was desirable to evaluate available methods for iodination. Several methods have been reported but were not evaluated in this investigation because they required unique equipment and materials.

In this study the optimal conditions for iodination of protein with the McCahey and Dixon (10) modification of the chloramine-T procedure were critically evaluated. The choices

of the optimal concentrations of the reagents and conditions for the reactions were based on two considerations: the amount of iodine introduced into the protein and the *in vitro* immunospecificity. Factors which may alter the efficiency of iodination of the protein or its immunospecificity include: (i) the chloramine-T concentrations, (ii) the  $\text{Na}^{125}\text{I}$  concentration, and (iii) the oxidation reaction time. Each of these factors was considered in this investigation.

**Optimal concentration of chloramine-T.** Although chloramine-T is a mild oxidizing agent, it may alter the protein and result in a diminution of the immunospecificity of the globulin (7). In these trials conditions were explored which would consistently produce  $^{125}\text{I}$ -labeled indicator globulin of high specific radioactivity and freedom from denaturation. The optimal concentration of chloramine-T for iodination of immunoglobulin was estimated by iodinating six separate 2-mg portions of a single lot of guinea pig antirabbit globulin and using variable amounts of chloramine-T (0.5 to 250  $\mu\text{g}/\text{ml}$ ). The volume of the reaction mixture was 2.0 ml in each trial. For each trial reaction, carrier-free  $\text{Na}^{125}\text{I}$  was added at a concentration of 100  $\mu\text{Ci}/\text{mg}$  of protein. Each reaction was terminated by adding a reducing agent, sodium metabisulfite, in the amount equal to the amount (weight/weight) of chloramine-T. Inasmuch as chloramine-T has a higher molecular weight than sodium metabisulfite (281.7 and 190.1, respectively), an excess of the reducing agent was assured when equal concentrations of the two reactants were used.

The results (Table 1) show that the efficiency of iodination increased from 0.19 to 20.7%, an increase of approximately 100-fold, as the concentration of chloramine-T was increased from 0.5 to 250  $\mu\text{g}/\text{ml}$ . The maximal efficiency of iodination was only 20.7% with the highest concentration of chloramine-T; this suggests that even greater concentrations of chloramine-T could increase the efficiency of the reaction.

However, in addition to the efficiency of iodination, the immunological integrity of the iodinated protein must be considered. Therefore, the effect of chloramine-T on the immunospecificity of the labeled antiglobulin was tested by RIA with each of the six antiglobulin samples (Table 1) which were iodinated with different concentrations of chloramine-T. Each iodinated antiglobulin was tested by RIA with *E. coli* as the antigen and a 1:10 dilution of specific *E. coli* antiserum. In the controls, phosphate-buffered saline diluent was substi-

tuted for the antiserum in the primary reaction.

The immunospecificity was revealed by comparing the  $^{125}\text{I}$ -labeled antiglobulin adsorbed by the specific reaction with that adsorbed by the control reaction (Fig. 1). The specific and control reactions adsorbed about the same amounts of radioactivity when the antiglobulins

TABLE 1. Results of iodinating protein with varying concentrations of chloramine-T

| Chloramine-T concn ( $\mu\text{g}/\text{ml}$ ) <sup>a</sup> | Protein-bound $^{125}\text{I}$ <sup>b</sup> (counts/min per mg of protein) | Efficiency of iodination (%) <sup>c</sup> |
|---|--|---|
| 0.5   | $2.10 \times 10^5$   | 0.19                                      |
| 2.5   | $1.69 \times 10^6$   | 1.50                                      |
| 10.0  | $7.62 \times 10^6$   | 6.73                                      |
| 40.0  | $1.46 \times 10^7$   | 13.0                                      |
| 100   | $1.78 \times 10^7$   | 15.8                                      |
| 250   | $2.34 \times 10^7$   | 20.7                                      |

<sup>a</sup>The concentration represents that which was present in the reaction mixture during the oxidation reaction. The volume and protein concentrations were constant in each reaction.

<sup>b</sup>Over 90% of the  $^{125}\text{I}$  was protein bound as determined by precipitation with 10% trichloroacetic acid.

<sup>c</sup>The efficiency of iodination was calculated as the ratio of the amount (counts/min) of radioactivity which was protein bound versus the amount (counts/min) of radioactivity which was added to each sample.  $\text{Na}^{125}\text{I}$  was added to each sample to obtain a concentration of 100  $\mu\text{Ci}/\text{mg}$  of protein. After adjusting for the counting efficiency of the scintillation spectrometer,  $\text{Na}^{125}\text{I} = 1.12 \times 10^8$  counts/min per mg of protein.

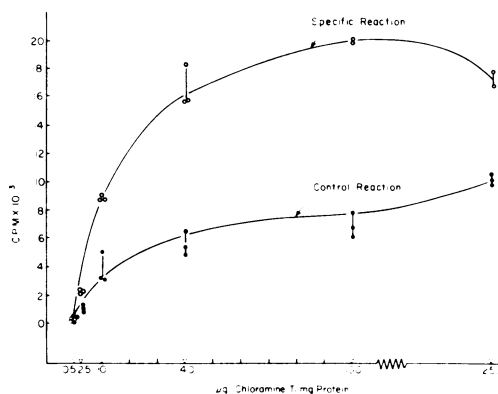


FIG. 1. Estimation of the optimal chloramine-T concentration. Guinea pig antirabbit globulin was iodinated with the same amount of  $\text{Na}^{125}\text{I}$  (100  $\mu\text{Ci}/\text{mg}$ ) but with different concentrations of chloramine-T. Each lot of  $^{125}\text{I}$ -labeled indicator globulin was reacted with an *E. coli* antigen-antibody immune complex. Symbols: ●, control reaction (diluent); ○, specific antigen reaction. CPM, counts/min.

iodinated with 0.5 and 2.5  $\mu\text{g}$  of chloramine-T per ml were used as indicators. When antiglobulins iodinated with 10, 40, and 100  $\mu\text{g}$  of chloramine-T per ml were used, the iodine bound in both the specific and control reactions increased as the concentration of chloramine-T was increased. However, the specific reactions adsorbed proportionately more of the  $^{125}\text{I}$ -labeled antiglobulin than the controls at these concentrations of chloramine-T. Antiglobulin iodinated with 250  $\mu\text{g}$  of chloramine-T per ml exhibited a decreased specific adsorption of  $^{125}\text{I}$ -labeled antiglobulin and an increased adsorption in the control reaction. Thus, the results suggest that immunoglobulins iodinated with 250  $\mu\text{g}$  of chloramine-T per ml are partially denatured, with a concomitant loss of immunospecificity.

Despite the higher efficiency of labeling at this chloramine-T concentration, it was necessary to sacrifice iodination efficiency so that the immunological specificity of the protein could be retained. Therefore, chloramine-T was used at a concentration of 100  $\mu\text{g}/\text{ml}$  of protein in the standard procedure.

**Optimal concentration of  $\text{Na}^{125}\text{I}$ .** In a similar trial we examined the relative effects of varying concentrations of  $\text{Na}^{125}\text{I}$  on the iodination of globulins. The optimal concentration of  $\text{Na}^{125}\text{I}$  was estimated by varying the concentration of  $\text{Na}^{125}\text{I}$  from 25 to 500  $\mu\text{Ci}/\text{mg}$  of protein in the iodination reaction. The antiglobulin (2 mg), chloramine-T (100  $\mu\text{g}/\text{ml}$ ), and sodium metabisulfite (100  $\mu\text{g}/\text{ml}$ ) concentrations were constant in each iodination reaction with the different  $\text{Na}^{125}\text{I}$  concentrations.

The results show that as  $\text{Na}^{125}\text{I}$  was increased from 25 to 500  $\mu\text{Ci}$ , the specific activity of the iodinated protein increased about 20-fold (Table 2). Likewise, the efficiency of iodination increased from 20 to 31% as the concentration was increased from 25 to 50  $\mu\text{Ci}/\text{mg}$  of protein. Although 50, 100, and 250  $\mu\text{Ci}$  showed about the same efficiencies of iodination, the efficiency declined about 10% with 500  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  per mg of protein. These results suggest that in terms of maximal iodination efficiency 50 to 250  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  per mg of protein represent the near optimal concentration of iodine. In this experiment we observed that when  $\text{Na}^{125}\text{I}$  concentrations were varied (Table 2) the efficiencies of iodination were higher than the efficiencies attained when chloramine-T concentrations were varied (Table 1). This apparent discrepancy may be caused by the use of different lots of  $\text{Na}^{125}\text{I}$ . Anomalous iodination reactions have been attributed to unknown contam-

TABLE 2. Results of iodinating protein with varying concentrations of  $\text{Na}^{125}\text{I}$

| $\text{Na}^{125}\text{I}$ concn<br>( $\mu\text{Ci}/\text{mg}$ ) | Protein-bound $^{125}\text{I}$ <sup>a</sup><br>( $\mu\text{Ci}/\text{mg}$ ) | Efficiency of<br>iodination (%) <sup>b</sup> |
|---|---|--|
| 25  | 5.05  | 20.1   |
| 50  | 15.7  | 31.2   |
| 100   | 32.0  | 32.0   |
| 250   | 69.2  | 27.5   |
| 500   | 100.0   | 20.0   |

<sup>a</sup> Over 90% of the  $^{125}\text{I}$  was protein bound as determined by precipitation with 10% trichloroacetic acid.

<sup>b</sup> The efficiency of iodination was calculated as the ratio of the amount ( $\mu\text{Ci}/\text{mg}$ ) of radioactivity which was protein bound versus the amount ( $\mu\text{Ci}/\text{mg}$ ) of radioactivity which was added to each sample.

inants in different lots of  $\text{Na}^{125}\text{I}$  when lactoperoxidase is used for iodination of protein (4). It is conceivable that chloramine-T iodinations might be similarly affected.

To further assess the optimal concentration of  $\text{Na}^{125}\text{I}$  for labeling protein we evaluated the specificity of  $^{125}\text{I}$ -labeled indicator globulin by measuring the competition between radioactively labeled and unlabeled antiglobulin for the specific antibody bound in the primary reaction. In these studies the primary reaction consisted of a 1:10 dilution of antigen-specific antiserum and a diluent control which was adsorbed to the antigen. The RIA procedure was the same as the routine RIA method, except that unlabeled indicator globulin in varying concentrations was combined with the  $^{125}\text{I}$ -labeled indicator globulin. The  $^{125}\text{I}$ -labeled indicator globulin was maintained at a constant concentration (0.02 mg/0.1 ml), whereas the concentration of unlabeled globulin was varied (0.01 mg/0.1 ml to 0.167 mg/0.1 ml). The results of the competitive titrations were recorded graphically by a semilogarithmic plot. The radioactivity (counts/min per sample) was plotted on the ordinate, and the logarithm of the concentration of the unlabeled globulin (milligrams per sample) was plotted on the abscissa.

The adsorption of  $^{125}\text{I}$ -labeled indicator globulin was inhibited by the simultaneous addition of unlabeled antiglobulin. With each separate lot of antiglobulin labeled with either 100, 250, or 500  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  per mg of protein, the quantity of adsorbed  $^{125}\text{I}$ -labeled antiglobulin decreased as the amount of unlabeled antiglobulin was increased (Fig. 2). Although the controls are not plotted in Fig. 2, the minimal adsorption of the  $^{125}\text{I}$ -labeled indicator globulin by the specific reaction was always three- to fourfold greater than the diluent control reaction. Moreover, none of the controls showed

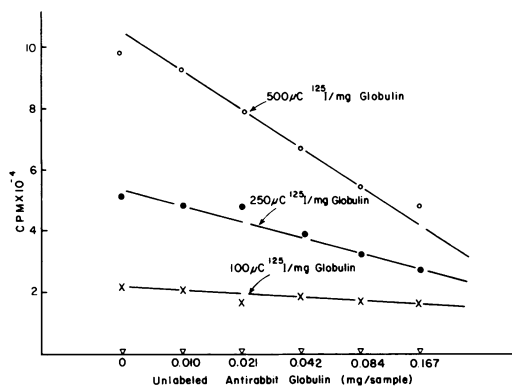


FIG. 2. Reactivity of  $^{125}\text{I}$ -labeled guinea pig anti-rabbit globulin labeled with different concentrations of  $\text{Na}^{125}\text{I}$ . Antirabbit globulin labeled with different concentrations of  $^{125}\text{I}$  and unlabeled antirabbit globulin were allowed to competitively react with immune complexes of *E. coli* and its specific antiserum. A constant amount of  $^{125}\text{I}$ -labeled indicator globulin (0.02 mg/reaction) and varied amounts of unlabeled antirabbit globulin (amount indicated on the abscissa) were added to the immune complexes on cover slips. Symbols:  $\times$ , 100  $\mu\text{Ci}$  of  $^{125}\text{I}$ /mg of globulin;  $\bullet$ , 250  $\mu\text{Ci}$  of  $^{125}\text{I}$ /mg of globulin;  $\circ$ , 500  $\mu\text{Ci}$  of  $^{125}\text{I}$ /mg of globulin.

marked inhibition when the  $^{125}\text{I}$ -labeled indicator globulin was combined with increased concentrations of unlabeled globulin.

The inhibition of the specific reaction was linear over the entire range (16-fold) tested for each of the three  $^{125}\text{I}$ -labeled antiglobulin preparations. However, the competition between labeled and unlabeled globulin appeared to be related to the amount of iodine introduced into the globulin molecule. This is indicated by the changes in the slopes of the curves in Fig. 2. The globulin preparation labeled with 500  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  per mg of protein had a negative inhibition slope of  $1.62 \times 10^4$ , whereas those labeled with 250 and 100  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  per mg of protein had negative slopes of  $8.23 \times 10^3$  and  $1.10 \times 10^3$ , respectively. The smaller negative slope with decreasing specific activities is directly related to a decrease in the sensitivity of the globulin preparation. As the specific activity of the iodinated preparation decreases, fewer molecules of protein are labeled; hence, there are fewer labeled molecules to compete with the unlabeled globulin for the antibody-binding sites. The linear regression lines suggest that the antibody specificity was not altered. Therefore, greater quantities of  $\text{Na}^{125}\text{I}$  could be added to the protein without impairing its immunological integrity.

In subsequent iodination trials with higher

concentrations of  $\text{Na}^{125}\text{I}$  (250 to 1,000  $\mu\text{Ci}$ /mg of protein), the mean efficiencies of iodination have remained about the same. However, the variation in the iodination efficiencies among the individual trials with higher  $\text{Na}^{125}\text{I}$  concentrations was greater than the variation among individual iodination trials with lower concentrations of  $\text{Na}^{125}\text{I}$ . Nevertheless, when antiglobulins iodinated with higher concentrations of  $\text{Na}^{125}\text{I}$  (750 or 1,000  $\mu\text{Ci}$ /mg of protein) were tested in competition RIA procedures, it was shown that the immunological integrity of the labeled protein was maintained.

High specific radioactivities are required because the labeled antiglobulin must be diluted to obtain low nonspecific adsorption and high specific adsorption in RIA methods. Subsequent results will show (Fig. 4) that either excess or insufficient concentrations of  $^{125}\text{I}$ -labeled antiglobulin markedly depressed the sensitivity of the RIA. Because high specific radioactivities are required for the RIA procedure, we adopted 500 to 1,000  $\mu\text{Ci}$ /mg as the standard  $\text{Na}^{125}\text{I}$  concentration.

**Effect of varying the oxidation reaction time on the efficiency of iodination.** Another factor considered in the investigation of the iodination of proteins was the oxidation reaction time. A single sample of protein was iodinated with chloramine-T as the oxidizing agent. After 2, 5, 10, 20, and 30 min, samples of the protein-iodine reaction mixture were transferred to vials containing sodium metabisulfite. The samples were dialyzed, and trichloroacetic acid precipitations were used to determine the protein-bound iodine of each.

Samples removed at 2, 5, 10, 20, and 30 min after the start of the oxidation reaction contained 81, 84, 97, 92, and 95  $\mu\text{Ci}$  of  $^{125}\text{I}$  per mg of protein, respectively. Because reaction times beyond 10 min produced no additional increases in the quantity of  $^{125}\text{I}$ -labeled protein, this reaction time was used routinely.

The immunological integrity of these radioiodinated proteins was assessed in two RIA experiments. First, the specificity was tested by competitively inhibiting the  $^{125}\text{I}$ -labeled globulin with unlabeled globulin as described above (see Fig. 2). Pronounced differences were not exhibited by the five labeled globulins in the competition assay. Next, each of the five  $^{125}\text{I}$ -labeled globulins were used in RIA serum titrations. A human antiserum of known anti-vaccinia potency was titrated with each of the  $^{125}\text{I}$ -labeled globulin preparations. The range of titers obtained with five preparations was 80 to 102, with an arithmetic mean titer of 100. The

titers observed for the five samples did not exhibit any apparent pattern as a function of iodination reaction time. This suggests the globulin was not altered by increases in the iodination reaction time.

**Criteria for determining the optimal concentration of  $^{125}\text{I}$ -labeled globulin for use in RIA.** After the optimal conditions for iodination of globulin protein had been investigated, it appeared advisable to study and subsequently standardize the procedures used for determining the optimal concentration of  $^{125}\text{I}$ -labeled antiglobulin which should be used in indirect RIA. Investigators have shown that dilution of the radioactively labeled globulin reduced the nonspecific adherence and yielded increased titers (13). Some laboratories have reported that the amount of radioactively labeled globulin they added to their RIA procedures was based on the amount of radioactivity in the labeled indicator (1, 2). However, the basis for the addition was not established.

To develop a criterion for determining the optimal concentration of an  $^{125}\text{I}$ -labeled indicator globulin for use in RIA, we prepared three separate lots of  $^{125}\text{I}$ -labeled guinea pig antihuman globulin for trial reactions with antigen-human antibody complexes. Two of the lots (A and B) were prepared from the same globulin preparation, but with different shipments of  $\text{Na}^{125}\text{I}$ . The third lot (C) was prepared from a different antihuman globulin preparation. The antihuman titers as determined by immunodiffusion were the same for each of the purified unlabeled globulin preparations. After the proteins were iodinated, lots A and B were evaluated in reactions with vaccinia-human antibody complexes. Lot C was evaluated in a similar reaction, but with rabies-human antibody complexes.

The optimal concentrations of each of the three lots of  $^{125}\text{I}$ -labeled globulin were determined by reacting serial dilutions of lots A, B, and C with antigen-human antibody complexes. As controls, diluent was substituted for the human antiserum in the primary reaction for each dilution of  $^{125}\text{I}$ -labeled antihuman globulin. After appropriate dilutions each lot of  $^{125}\text{I}$ -labeled antiglobulin were reacted, the radioactivity adsorbed by both the specific reactions and the control reactions was measured. A ratio of the specific and the control radioactivity (counts/min) observed at each dilution was calculated and evaluated by two procedures.

First, the ratios were evaluated on the basis of the radioactivity of  $^{125}\text{I}$ -labeled globulin (counts/min) added to each sample. The calcu-

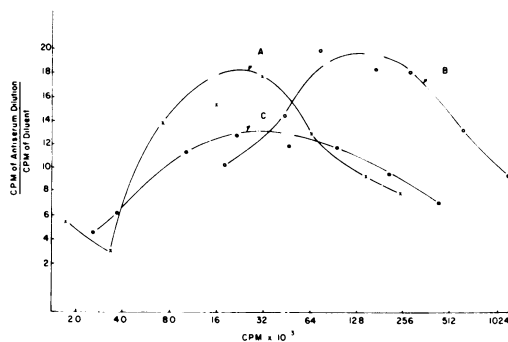


FIG. 3. Radioactivity as a criterion for selecting optimal amounts of  $^{125}\text{I}$ -labeled indicator globulin for use in RIA. The optimal amounts for each of three lots of  $^{125}\text{I}$ -labeled guinea pig antihuman globulin were the concentrations which produced the highest specific (counts/min) to nonspecific (counts/min) ratios of radioactivity adsorption with radioactivity (counts/min) used as a basis for comparison. A constant amount of each immune complex or control reaction was reacted with the amounts (counts/min) of  $^{125}\text{I}$ -labeled indicator globulin indicated on the abscissa. Symbols: (A)  $\times$ , vaccinia-human antivaccinia reaction measured by using  $^{125}\text{I}$ -labeled antihuman globulin with a specific activity of  $70 \mu\text{Ci}$  of  $^{125}\text{I}$  per mg of globulin; (B)  $\circ$ , vaccinia-human antivaccinia reaction measured by using  $^{125}\text{I}$ -labeled antihuman globulin with a specific activity of  $280 \mu\text{Ci}$  of  $^{125}\text{I}$  per mg of globulin; (C)  $\bullet$ , rabies-human antirabies reaction measured by using  $^{125}\text{I}$ -labeled antihuman globulin with a specific activity of  $85 \mu\text{Ci}$  of  $^{125}\text{I}$  per mg of globulin.

lated ratios were plotted on the ordinate, and the added radioactivity was plotted on the abscissa (Fig. 3). The amount of radioactivity yielding the maximal ratio was different for each lot of  $^{125}\text{I}$ -labeled indicator globulin. Lot B had the highest specific radioactivity ( $280 \mu\text{Ci}/\text{mg}$ ), yet it required a larger amount of radioactivity to attain the maximal ratios. Lots A and C had lower specific activities ( $A = 70 \mu\text{Ci}/\text{mg}$ ,  $C = 85 \mu\text{Ci}/\text{mg}$ ), and they required approximately equal but smaller amounts of radioactivity to attain maximal ratios.

Next, the same data were examined by plotting the ratios on the ordinate and the protein concentration of the  $^{125}\text{I}$ -labeled antiglobulin on the abscissa (Fig. 4). These plots show that the maximal ratios for each lot of  $^{125}\text{I}$ -labeled antiglobulin were attained at approximately the same protein concentration.

It is important to note that the optimal concentration of the indicator globulin in the reaction with the primary antigen-antibody complex is independent of the antigen involved in the complex. This was shown by the fact that

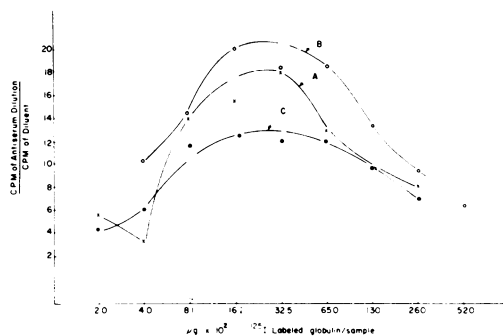


FIG. 4. Protein concentration as a criterion for selecting optimal amounts of <sup>125</sup>I-labeled indicator globulin for use in RIA. The same data (Fig. 3) were evaluated for each of three lots of <sup>125</sup>I-labeled guinea pig antihuman globulin with protein concentration (µg) used as a basis for comparison. A constant amount of each immune complex or control reaction was reacted with the amounts (µg) of <sup>125</sup>I-labeled indicator globulin indicated on the abscissa. The specific activities of the <sup>125</sup>I-labeled indicator globulins were: (A) 70 µCi of <sup>125</sup>I per mg of globulin; (B) 280 µCi of <sup>125</sup>I per mg of globulin; and (C) 85 µCi of <sup>125</sup>I per mg of globulin. Symbols: (A) ×, vaccinia-human antivaccinia reaction; (B) O, vaccinia-human antivaccinia reaction; and (C) ●, rabies-human antirabies reaction.

lots A and B were reacted with complexes of vaccinia and its specific antibody, whereas lot C was reacted with complexes of rabies antigen and its antibody.

The results of these studies indicate that the quantity of <sup>125</sup>I-labeled indicator globulin which should be used in RIA is very important. Either insufficient or excess amounts of <sup>125</sup>I-labeled antigen will impair the sensitivity of the test. Thus, an arbitrary choice of an amount equivalent to a specific radioactivity content may cause failure of the RIA. The optimal amount, based upon radioactivity as in Fig. 3, could be determined; however, each lot of iodinated antigen would have to be evaluated. Furthermore, periodic adjustments for decay of the radioactivity would be necessary.

An alternate and better procedure would be to determine the optimal amount of <sup>125</sup>I-labeled antigen on the basis of its protein content (Fig. 4). By this procedure an initial determination of the optimal protein concentration of an iodinated antigen lot will be valid for subsequent iodinations of the same antigen.

**Relationship of the concentration of <sup>125</sup>I-labeled indicator globulin to RIA titers.** After the importance of adding <sup>125</sup>I-labeled indicator globulin in optimal amounts was observed, the

effect of using less than or greater than optimal amounts in an actual RIA procedure was considered. The antivaccinia titer of a human serum was determined by using six different concentrations of <sup>125</sup>I-labeled guinea pig antihuman globulin as indicator. After the primary antigen-antibody reaction, the <sup>125</sup>I-labeled indicator globulin was added to the complexes in twofold increments, from 0.16 to 5.2 µg/sample. Thus, an antiserum titration curve for each concentration of <sup>125</sup>I-labeled indicator globulin was established. The results obtained with each concentration of <sup>125</sup>I-labeled indicator globulin were evaluated by plotting the logarithm of the radioactivity (counts/min) observed for each antiserum dilution on the ordinate versus the logarithm of the antiserum dilution factor on the abscissa. From these antiserum titration curves the slope and titer were calculated at each <sup>125</sup>I-labeled indicator globulin concentration (Table 3).

The results showed that with decreasing concentrations of the radioactively labeled protein the slope of the antiserum titration curves increased. Concomitant with the increased slopes of the titration curves, the radioactivity adsorbed by the diluent control (nonspecific adsorption) decreased. The increased slopes and decreased nonspecific adsorption resulted in increased antiserum titers. In fact, at the three highest <sup>125</sup>I-labeled indicator globulin concentrations (5.2, 2.6, and 1.3 µg/sample) the nonspecific adsorption was so great that erroneous negative or nonsense titers were obtained. As the slope of the titration curves increased from 1.04 to 2.76, the apparent titers increased from <10 to 70 (Table 3). Thus, to accurately

TABLE 3. Relationship of <sup>125</sup>I-labeled indicator globulin concentration to titers and slopes of antiserum dilution curves

| Concn of <sup>125</sup> I-labeled indicator globulin <sup>a</sup> | Slope <sup>b</sup> | Titer <sup>c</sup> |
|---|--------------------|--------------------|
| 5.2   | 1.04               | <10                |
| 2.6   | 1.56               | <10                |
| 1.3   | 1.49               | <10                |
| 0.65  | 2.06               | 38                 |
| 0.32  | 2.44               | 48                 |
| 0.16  | 2.76               | 70                 |

<sup>a</sup> Quantity (µg of <sup>125</sup>I-labeled indicator globulin/sample) added to each primary antigen-antibody complex and to each diluent control.

<sup>b</sup> The method of least squares was used to determine the slope of each titration curve.

<sup>c</sup> The titer is the antiserum dilution factor which adsorbed twice the radioactivity (counts/min) adsorbed by the control.

titrate sera with RIA methods, it is necessary to select an optimal concentration of the  $^{125}\text{I}$ -labeled antiglobulin as well as to use iodinated globulins of high specific activity and immunological integrity.

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