Some Considerations in the Preparation of Radioiodoinsulin for Radioimmunoassay and Receptor Assay

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SUMMARY

^{125}I-insulins, prepared by iodination with chloramine T in marked excess or by stepwise, stoichiometric addition of the oxidizing agent, were compared with respect both to their molecular distribution of iodine and to their suitability for use in a cultured lymphocyte receptor assay. Iodination of insulin in aqueous solution results in the same distribution of iodine atoms, independent of experimental method and dependent only on the average iodine number. This distribution can be calculated on the basis of a Monte Carlo simulation. For insulin iodinated at an average of 0.8 I atoms per molecule, approximately 50 per cent of the radioactivity is in other than monoiodoinsulin. Purification methods that separate on the basis of charge, such as starch-gel electrophoresis, are then required, to obtain monoiodoinsulin. More highly iodinated insulins do bind to the lymphocyte receptor, although, as in radioimmunoassay, the overiodinated species are less satisfactory for use as tracers. The shelf life of iodinated insulin appears to be related better to the average iodine content than to any other factor, presumably because of decay catastrophe. There is no evidence to suggest that exposure to chloramine T in marked excess for a few seconds is deleterious to insulin. DIABETES *25*:260-67, April, 1976.

The current increase of interest in radioreceptor assay as a tool in polypeptide hormone research grows out of the potential of such assays to recognize biologically active molecules. The specific binding of labeled hormone to cell membrane or membrane preparations as observed in vitro has been suggested to parallel the initial step in the in-vivo action of polypeptide hormones. It is this assumption of the biologic significance of the in vitro binding that sets the radioreceptor assay apart from other competitive radioassays for polypeptide hormones and poses theoretical and practical considerations in the preparation of labeled hormones suitable for receptor assays. For example, stepwise, "stoichiometric" iodination procedures have been suggested for the production of monoiodinated, biologically active insulin presumed to be required in insulin receptor systems.[1-4]

In this report we compare labeled insulins prepared by iodination with chloramine T in marked excess or by stepwise, stoichiometric addition of the oxidizing agent with respect both to their suitability for use in a cultured lymphocyte receptor system and to the molecular distribution of their iodine content. Presented also is a theoretical approach to the determination of the distribution of iodine atoms following iodination of insulin in aqueous solution.

MATERIALS AND METHODS

Human insulin (Novo Laboratories) was iodinated with chloramine T employed as the oxidizing agent[5] by the method used routinely in our laboratory (standard iodination)[6,7] and by a method employing stepwise addition of chloramine T (stoichiometric iodination).[1] Both iodinations were performed at the same time using the same amount of human insulin (3 μg.) and the same lot of $^{125}I^-$(~1.1 millicuries ^{125}I,

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obtained from Union Carbide). If 100 per cent iodination were achieved, then the iodinated insulins would have contained an average of about one iodine atom per molecule. Both iodinations were carried out at room temperature, and the oxidizing (chloramine T, Eastman) and reducing (sodium metabisulfite, Fisher Scientific) agents were solubilized in 0.25 M phosphate buffer (pH 7.5). Iodinations were performed with six different lots of $^{125}I^-$, all from Union Carbide.

Standard iodination. The reagents were added in the order given. Following each addition, mixing was effected by gently bubbling air through the pipettes. To 20 μl. phosphate buffer there were added successively ^{125}I, insulin, chloramine T (52.5 μg.), Na metabisulfite (80 μg.), and human plasma (20 μl.). Before addition of plasma the volume of the reaction mixture was less than 60 μl. The total time from addition of the radioiodide until completion of the reaction and addition of the plasma was generally no more than 15 seconds. A minute portion of the iodination mixture was added to 50 μl. plasma and applied to paper for chromatoelectrophoresis to determine the percentage iodination,[8] and the remainder was purified on starch-gel electrophoresis.[6,7] Following electrophoresis at 100 volts for 14 hours, an autoradiograph of the gel was obtained. Three radioactive zones were cut out, frozen, thawed, and eluted with 0.02 M barbital buffer containing 1 per cent human serum albumin.

Stoichiometric iodination and purification of insulin was performed according to the general method of Gavin et al.[3] with minor modifications as recommended at a course in radioreceptor assays, NIH, November 27-29, 1973. To a glass test tube (13 × 100) the following reagents were added in the order given: 35 μl. of 0.25 M phosphate buffer, ~1.1 mCi. ^{125}I, 3 μg. insulin, and 10 μl. chloramine T (40 μg./ml.). After 15 seconds, less than 1 μl. of the reaction mixture was removed with the tip of a Pasteur pipette and added to a tube containing 1 ml. of phosphate buffer and 1 ml. of 10 per cent trichloracetic acid (TCA). The tube was centrifuged to determine the per cent of radioactivity that was TCA-precipitable. In our laboratory, generally about 40 per cent was TCA-precipitable at this stage of the procedure. Following further addition of 10 μl. chloramine T, about 60 per cent of the radioactivity was TCA-precipitable; another 5 μl. chloramine T was usually required in order to achieve 70 to 80 per cent TCA-precipitable radioactivity. Thus, the total amount of chloramine T was usually about 1 μg., or more than six times the molar concentration of insulin or ^{125}I. Following the final addition of chloramine T, 5 μl. of sodium metabisulfite (200 μg./ml.) was added, followed by 100 μl. of a 2.5 per cent solution of bovine serum albumin in 0.25 M phosphate buffer. The total time required for the iodination, including checking the precipitability with TCA, was 10 minutes. The percentage of iodination was also determined by paper chromatoelectrophoresis.[8]

Purification of the insulin labeled by the stepwise stoichiometric iodination is usually effected by adsorption to, and elution from, a column of Whatman cellulose powder.[9] The column is prepared of powdered cellulose packed in a Pasteur pipette to a height of about 4 cm. The iodination mixture is transferred to the column and forced into the body of the column with air pressure. The major fraction of the labeled insulin adsorbs to the cellulose and is washed free of unreacted iodide and damaged components with 7 to 8 ml. of 0.05 M barbital buffer. The labeled insulin is eluted with several successive 1.5-ml. portions of 12 per cent bovine serum albumin in the 0.25-M phosphate buffer. The labeled insulin in the second eluate is generally employed for receptor assays. The radioactivity in this eluate is generally 90 to 95 per cent precipitable by TCA and is adsorbed by 50 mg. talc from a 1.5-ml. volume to the extent of 90 to 95 per cent. For the purposes of the present study, a portion of this material was further fractionated on starch-gel electrophoresis as was a portion of the original stoichiometric iodination mixture.

Labeled insulins obtained from four of the six sets of iodinations were hydrolyzed into their component amino acids and the relative amounts of radioiodine contained in monoiodotyrosyl (MIT) and diiodotyrosyl (DIT) residues of the preparations were determined. This was accomplished by adding ~1 μCi. of each labeled insulin in 25 μl. of 0.02 M barbital buffer containing 0.25 per cent human serum albumin to 1 ml. of 0.25 M phosphate buffer containing 1 mg. of protease (Sigma). The reaction was allowed to proceed for 30 minutes at 37° C. Following the incubation, a portion (~50 μl.) of the reaction mixture was applied to the origin of a strip of Whatman no. 1 paper for descending chromatography using butanol-acetic acid-water (5:1:4) as solvent to effect separation of the labeled amino acids. The positions of authentic monoiodotyrosine and diiodotyrosine were determined by ninhydrin reaction, and their R_f values were used as standards. The chromatograms were scanned

for radioactivity with a strip scanner, and the relative areas under the peaks were determined as a measure of the relative MIT and DIT concentrations in each of the purified preparations.

All labeled insulin preparations were tested for suitability as a tracer in the cultured human leukemic lymphoblast (NIH line IM 9) system as described by Gavin et al.[3] Cells were grown in Eagle's minimal essential medium (GIBCO) fortified with 10 per cent fetal calf serum, 2 per cent l-glutamine, and 0.2 per cent penicillin-streptomycin, and were fed 48 hours prior to use. For studies of the binding of the iodoinsulins to the receptor, approximately 10^7 cultured human leukemic lymphoblasts were incubated with the labeled insulin either in the presence or in the absence of unlabeled insulin. Following one hour of incubation at 15° C., bound and free ^{125}I-insulins were separated by centrifugation. Maximum displaceable binding (MDB) and nonspecific binding were determined. Nonspecific binding is defined as the fraction of the labeled hormone bound in the presence of a large excess (2 μg./ml.) of unlabeled insulin. MDB, i.e., that specifically bound, is calculated as the difference between the total bound and that nonspecifically bound.

The relative MIT and DIT contents of the total labeled insulin bound to the receptor as well as that nonspecifically bound and that dissociable from the receptor were determined by proteolytic digestion of the appropriate cellular precipitates or supernates and chromatographic analysis of the solubilized amino acids.

The calculation of the theoretical distribution of iodine atoms in the labeled insulin preparations was made on the basis of a Monte Carlo simulation. This analysis was based on the assumptions that at an average of one iodine atom per molecule or less, there is exclusive iodination of the two A-chain tyrosyl residues, that there is equal probability of iodinating each, and that the presence of one iodine atom on a tyrosyl residue is nondirecting—that is, it does not affect the probability of a second iodine atom's attaching to the same residue. These assumptions are consistent with the experimental observations of Massaglia et al.[10] on the electrolytic iodination of insulin in aqueous solution. The calculation was made initially by considering that there were 250 molecules each containing four sites of equal probability for iodine incorporation. The percentages of molecules with zero, one, two, three, or four iodine atoms were then calculated on the assumption that a total of 50, 100, 150, 200, 250 iodine atoms were distributed among the 1,000 sites completely on the basis of a random process. To smooth out statistical uncertainties the calculation was repeated for 25,000 molecules and the same relative number of iodine atoms. The calculated distribution frequencies were in agreement to better than 0.1 per cent.

RESULTS

Paper chromatoelectrophoretic analysis of the products of one set of iodinations is shown in figure 1. There was no significant difference between the extent of iodination of the standard (figure 1, top left) and stoichiometric iodinations (figure 1, top right). Unreacted iodine generally averaged about 20 per cent. The final specific activity of each preparation averaged about 300 microcuries per microgram, corresponding to an average of about 0.8 iodine atoms per molecule of insulin.

Autoradiographs of the starch gel following electrophoresis of the labeled preparations showed three distinct prealbumin peaks. The most cathodal is designated zone 1 and the most anodal as zone 3 (figure 1, bottom). A similar pattern was observed on the autoradiograph when the cellulose column eluate of the stoichiometric iodination was repurified on starch gel. The distribution of radioactivity among the zones was independent of prior purification on the cellulose column and did not differ significantly for the two methods of iodination. Zone 1 and zone 2 each contained approximately 45 per cent of the radioactivity and zone 3 contained virtually all of the remaining 10 per cent.

Scans of paper radiochromatograms of the products following enzymatic proteolysis of the cellulose-column eluate and the starch-gel eluates of the iodina-

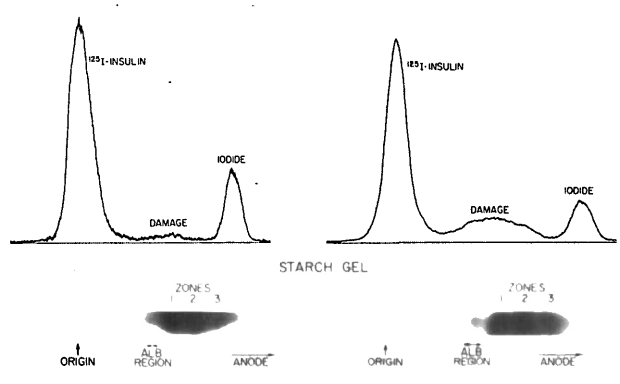

FIG. 1. Scans of paper chromatoelectrophoresis strips (top) and autoradiographs of starch-gel electrophoresis (bottom) of standard (left) and stoichiometric (right) iodination mixtures.

tion set depicted in figure 1 are shown in figure 2. For both iodinations, zone 1 was free of DIT and the

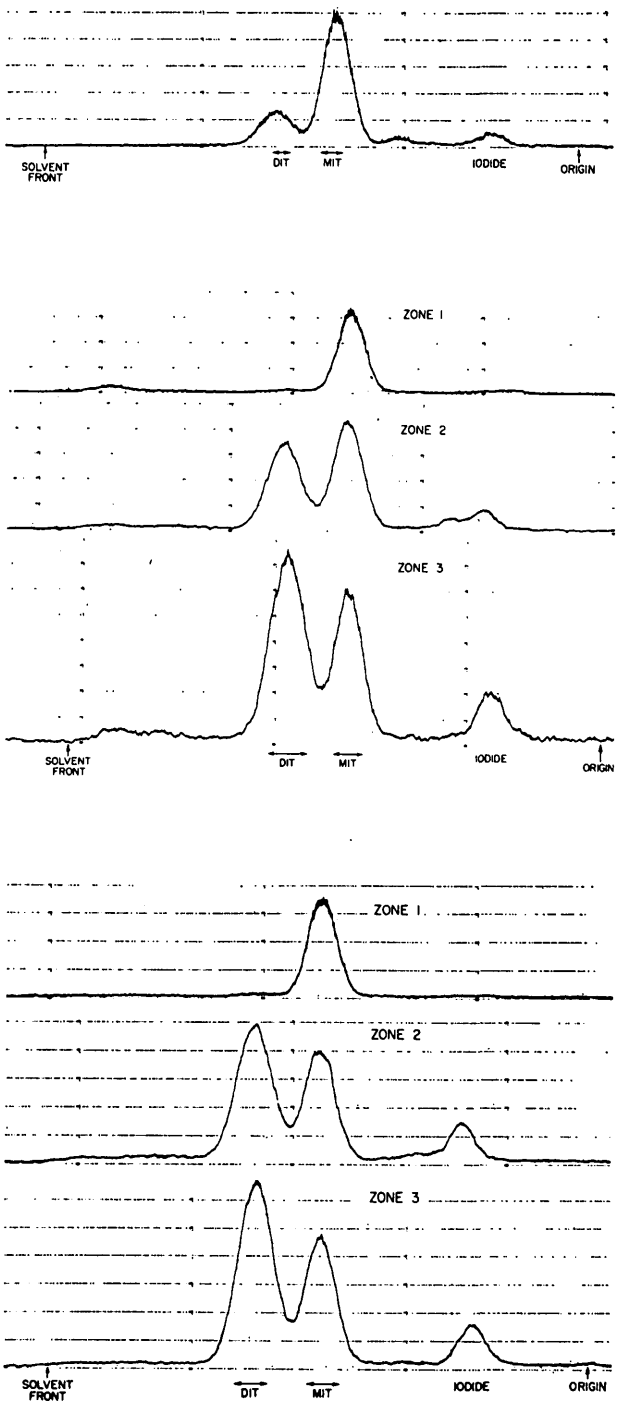

FIG. 2. Scans of descending chromatography strips of protease-hydrolyzed eluates of ^{125}I-insulin from cellulose column purification of stoichiometric iodination (top), from zones 1, 2, and 3 of starch gel electrophoresis purification of stoichiometric iodination (middle), and of standard iodination (bottom).

TABLE 1

Distribution of radioactivity between MIT and DIT in hydrolyzed eluates, and binding characteristics of purified radioiodoinsulins

Iodination	Radioactivity in DIT	MDB*	Nonspecific binding
Standard			
Zone 1	0%	38%	10%
Zone 2	58	24	10
Zone 3	65	15	10
Stoichiometric			
Zone 1	0	28	11
Zone 2	50	20	12
Zone 3	60	14	10
Cellulose eluate	19	24	11

*Maximum Displaceable Binding (MDB)

fraction of the total radioactivity in DIT increased progressively in zones 2 and 3 (table 1). On the basis of the fraction of radioactivity in each zone and the distribution of radioactivity between MIT and DIT in each zone, the calculated mean percentage MIT in the cellulose-column eluate should have been 71 per cent, in reasonably good agreement with the observed value of 81 per cent.

The quality of the various labeled insulin preparations prepared at the same time was evaluated for usefulness in the receptor assay (table 1). Nonspecific binding to IM-9 human leukemic lymphoblasts generally was between 10 and 12 per cent per 10^7 cells, independently[1] of the method of preparation. However, it seemed to depend on the lot of radioiodide employed. The iodination products associated with an occasional lot of radioiodide (vide infra) seemed to manifest a much lower nonspecific binding than the others. Specific binding (MDB) was greatest for radioiodoinsulin from zone 1 and diminished progressively for radioiodoinsulins from the more anodal zones. In figure 3 are shown linear and semilogarithmic curves of competitive inhibition for several of these preparations obtained from a single set of iodinations. In the study shown, zone 1 of the standard iodination contained the most suitable preparation for the receptor assay—that is, it showed the highest MDB. The MDB (24 per cent) of the cellulose-purified preparation behaved as would be expected from the weighted mean of the starch-gel-purified material from the same iodination.

To determine whether radioiodoinsulin that contains DIT, and is therefore certainly not monoiodoinsulin, can bind specifically to the receptor, the labeled

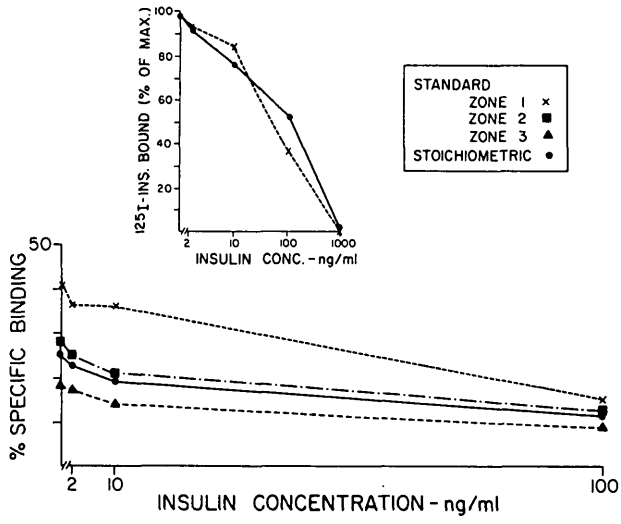

FIG. 3. Standard curves of competitive inhibition of ^{125}I-insulin prepared by standard or by stoichiometric iodination: (bottom) plot of the per cent specific binding for various labeled insulins as a function of unlabeled-insulin concentration, (top) plot of the same data for two of the labeled preparations in terms of the maximal specific binding and as a function of the log of the unlabeled insulin concentration.

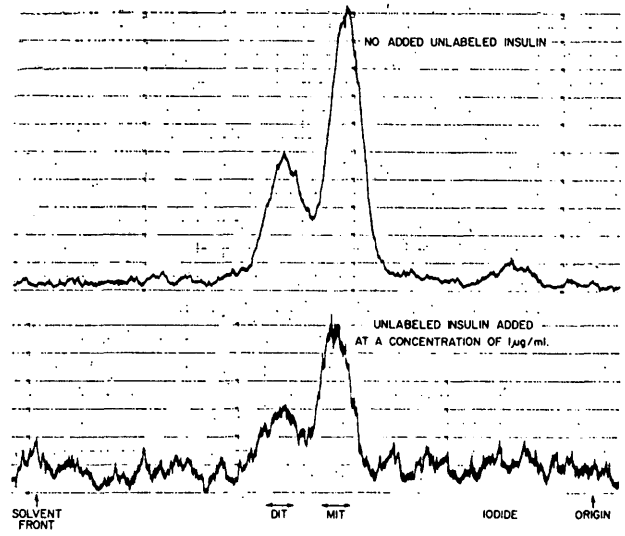

FIG. 4. Scans of descending chromatography strips of protease-hydrolyzed insulin from standard iodination zone 2, which was bound to cells in the absence of unlabeled insulin (top) and in the presence of unlabeled insulin (bottom).

insulin from zone 2 of a standard iodination was incubated with IM-9 cells with and without excess unlabeled insulin. The cells were precipitated by centrifugation and subjected to proteolysis, and the hydrolysates were chromatographed to determine the DIT/MIT ratio in the radioactivity that was nonspecifically bound (figure 4, bottom) and that which represented the sum of nonspecific and specific binding (figure 4, top). The pattern shows no increase in the fraction of DIT in the nonspecifically bound radioinsulin (figure 4, bottom), as would have been the case if the insulin-containing DIT were unable to bind specifically to the receptor sites.

In a subsequent standard iodination with another lot of ^{125}I, radioiodoinsulin from zone 2 showed 20 per cent specific binding and only 1 per cent nonspecific binding per 10^7 cells. In view of the high quality of this preparation the question of specific binding of DIT-insulin was re-examined. Shown in figure 5 are the proteolytic hydrolysates of the ^{125}I-insulin from zone 2 (figure 5, top), of the labeled insulin that did not bind to the receptor (figure 5, second from top), of that which remained bound to receptor following addition of excess unlabeled insulin (figure 5, third from top) or of that which was dissociated from the receptor by the excess insulin (figure 5, bottom). Each of the strips was scanned at least three times and the relative areas under the MIT and DIT peaks were determined planimetrically. There were no significant differences in MIT/DIT ratios in these hydrolysates.

Earlier studies had demonstrated that on starch-gel electrophoresis there was an increase in the fraction of radioactivity in the more anodal components as the mean iodination number for insulin (average iodine

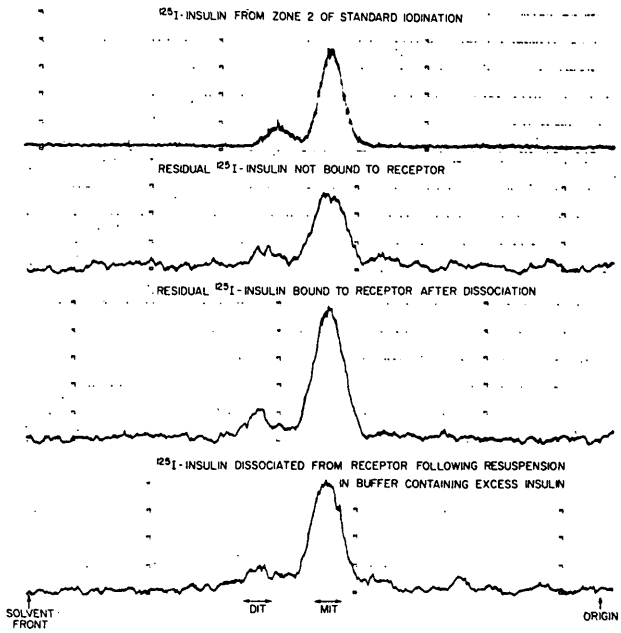

FIG. 5. Scans of descending chromatography strips of protease-hydrolyzed ^{125}I-insulin. The experimental conditions are shown on the scans.

atoms per molecule) increased.[11] In fact the distribution of radioactivity in the various zones as a function of average iodine number observed in the earlier study[11] and in the current study are in excellent agreement. However the hypothesis that zones 1, 2, 3, and 4 contained mono-, di-, tri-, and tetraiodoinsulins respectively had not been unequivocally tested. The Monte Carlo simulation predicts that at an average of 0.8 I atoms per molecule about 50 per cent of the radioactivity should be incorporated in monoiodoinsulin, 38 per cent in diiodoinsulin, and about 10 per cent in triiodoinsulin (figure 6). This predicted distribution is in good agreement with the fraction of radioactivity actually found in zones 1, 2, and 3, respectively (vide supra). Furthermore, were zone 1 to contain exclusively monoiodoinsulin, it should contain only MIT-insulin, and this was in fact observed (figure 2). If zone 2 were to contain only diiodoinsulin, on a theoretical basis one would expect the fraction of radioactivity in MIT and DIT to be equal, and this agrees well with the observed fraction of radioactivity in DIT in zone 2 (table 1). If zone 3 were to contain exclusively triiodoinsulin, the radioactivity in DIT should be twice that in MIT, i.e., approximately 60 per cent observed. Thus, the predictions from the Monte Carlo simulation and the hypothesis that the starch-gel zones contain insulins increasing in iodine content stepwise anodally are in good agreement with the experimentally observed amounts of radioactivity in the successive anodal zones and the relative distribution of radioactivity in monoiodotyrosyl and diiodotyrosyl residues.

No significant differences were noted in shelf lives of iodoinsulins eluted from comparable zones of the starch gel irrespective of whether or not excess chloramine T was employed in the iodination procedure. Iodoinsulins from zone 1 were satisfactory for up to four weeks without repurification. In agreement with our earlier studies,[12] radioiodoinsulins containing two or more radioactive atoms undergo spontaneous damage due to decay catastrophe, and therefore the labeled insulin from the more anodal zones manifest marked damage within several weeks.

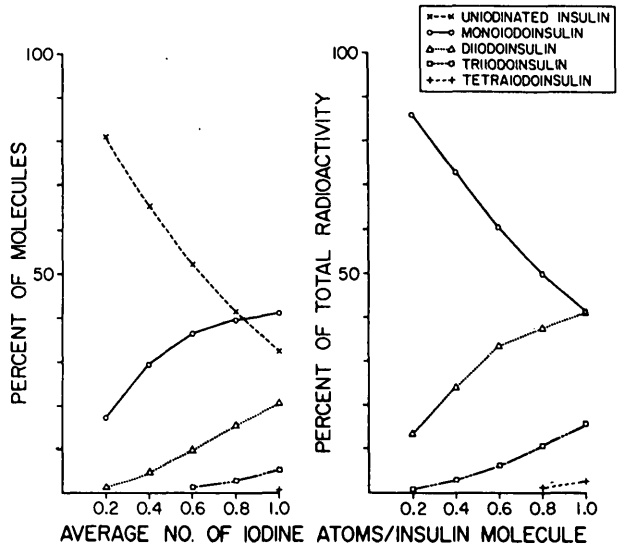

FIG. 6. Theoretical distribution of radioactivity calculated from Monte Carlo simulation based on assumption of exclusive A-chain iodination and random distribution of ^{125}I among the four reactive sites on the two A-chain tyrosines: (left) per cent of insulin molecules with zero to four iodine atoms as a function of the average number of I atoms/molecule; (right) per cent of the total radioactivity in each of the iodinated species as a function of the average number of I atoms/molecule.

DISCUSSION

Numerous papers have suggested that the advantage of radioreceptor assay over radioimmunoassay is that the potency of a hormone to compete for binding to its cellular receptor gives an estimate of its biologic potency (see ref. 1 for review). However, several papers by Cuatrecasas and associates[13,14] suggest some reconsideration as to whether all tissue-receptor assays measure substances according to their biologic potency. These investigators appear to have demonstrated that the binding of ^{3}H-norepinephrine to intact cells and microsomes can be inhibited equally as well by biologically inactive as by biologically active isomers.[13] They have also demonstrated[14] that the interactions of ^{125}I-insulin with nontissue materials such as talc and silica exhibit binding characteristics that share, at least superficially, such criteria as saturability, specificity, high affinity, and reversibility commonly attributed to "specific" hormone-receptor interactions.

In the present study we have no independent evidence concerning the biologic activity of the iodinated insulins used in our study of ^{125}I-insulin-receptor interactions. However, we have demonstrated that iodoinsulin containing even diiodotyrosyl residues can be specifically bound to lymphocyte receptor sites and therefore that there is no absolute requirement for the use of monoiodoinsulin in lymphocyte receptor assays. Nonetheless, more highly iodinated insulin preparations are less satisfactory when used as a tracer for binding in the receptor system. This is in keeping with their behavior in radioimmunoassay procedures

where lesser immunoreactivity and greater susceptibility to damage has been characteristic of overiodinated preparations.[6,11,12,14]

It has been suggested that when the iodination of insulin takes place under conditions in which ^{125}I, chloramine T, and insulin are reacted in approximately equal molar ratios, the introduction of more than one atom of ^{125}I per molecule of insulin is minimized.[3] The experimental observations reported here demonstrate that the distribution of radioactivity among the iodoinsulins is independent of whether the chloramine T is added in excess or whether the iodination proceeds stepwise with limiting amounts of chloramine T and is dependent only on the insulin:iodine ratio achieved. Furthermore, our observed distribution is virtually the same as that obtained by Massaglia et al.,[10] who employed an electrolytic method, and by de Zoeten and deBruin,[16] who used KIO_3 as the oxidizing agent. It appears, therefore, that, in aqueous solution, iodination up to an average of one iodine atom per molecule insulin proceeds on a random basis, irrespective of the iodination method employed, and that a statistical method, the Monte Carlo method, can be used to predict the distribution of iodine atoms incorporated in the iodoinsulin. At an average of 0.2 iodine atoms per molecule just over 85 per cent of the radioactivity is in monoiodoinsulin, at 0.6 iodine atoms per molecule only 60 per cent of the radioactivity is in monoiodoinsulin, and at an average of one iodine atom per molecule the radioactivity is distributed about equally between mono- and diiodoinsulin, with almost 20 per cent of the radioactivity in more highly iodinated species. To assure that following the iodination procedure, more than 90 per cent of the radioactivity is incorporated in monoiodoinsulin requires iodination at about an average of 0.1 iodine atom per molecule insulin or less. To obtain high-specific-activity monoiodoinsulin, therefore, requires separation of this form from uniodinated insulin and/or the more highly iodinated species using methods such as gradient chromatography on DEAE-cellulose[2] or starch-gel electrophoresis. The question might be asked whether high-specific-activity preparations are in fact required for most receptor assays. In some insulin assays depending on the inhibition of binding to lymphocytes[3] or fat cell or liver membranes,[2,4] concentrations of insulin at least as large as 250 pg./ml., and generally 1 ng./ml. or more, are the minimal detectable in contrast to the radioimmunoassay system, where sensitivities of 5 to 10 pg./ml. are routine and as little as 1 pg./ml. is achievable in some laboratories.[7] Since in assay systems there is no necessity for the concentration of the labeled substance to be much smaller than the minimally detectable hormone concentration, for many receptor assays that have been described relatively low-specific-activity preparations will suffice.

Prolonged exposure to high concentrations of chloramine T has been shown to result in damage to peptide hormones.[15] For this reason we routinely employ a reaction time of only a few seconds. The stoichiometric iodination method[1,3] does have the advantage of exposing the hormone to be iodinated to a lower concentration of chloramine T. However, since the ^{125}I preparations used in the procedure frequently contain an unknown and variable amount of reducing substances, the required chloramine T is empirically determined by a stepwise procedure that often requires as long as 10 minutes or more. This may be disadvantageous because of radiation exposure and potential adsorption to glassware of basic peptide hormones during the prolonged time in an alkaline buffer without protein protection. For the preparation of labeled insulin suitable for the lymphocyte radioassay system, either chloramine T method appears to suffice; the shelf life of the preparation is better related to the average iodine content than to any other factor. However the determination of which method or combination of methods is optimal may require individual evaluation for other peptide hormones.

In receptor assays, as in all competitive radioassays, there is no requirement for identity of labeled and unlabeled hormone. It is simply required that labeled hormone bind to the receptor and that the binding be inhibited by unlabeled hormone. However, the quality and characteristics of the labeled preparation are often important in determining the sensitivity and reproducibility of the assay. From the studies presented here it would appear that insulins of higher iodine number are less suitable for receptor assays, as they have earlier been shown to be less suitable for radioimmunoassays.[6,7,11] Since the sites on the hormone molecule for binding to the receptor or to the antibody probably differ and may well reside in other than the region of the tyrosyl residues, a possible interpretation is simply that overiodination results in changes in the general configuration of the insulin molecule.

ACKNOWLEDGMENT

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