A Study of some Factors that Influence the Iodination of Ox Insulin

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1. The influence of carrier iodide, iodine monochloride and pH on the labelling of ox insulin with ^{131}I by the iodine monochloride method have been studied. 2. The quantitative effect of the iodide in the radioactive iodine preparation was that predicted from a calculation of its specific activity. No other interfering factors were detected in the [^{131}I]iodide solutions used. 3. Increasing the molar ratio of iodine monochloride to insulin resulted in an increase followed progressively by a decrease in the proportion of ^{131}I bound, while the total iodine bound increased to an amount characteristic of pH and thereafter remained constant. 4. The influence of pH on the iodination of insulin with iodine monochloride was complex and the pH curve showed two maxima, at pH 2.8 and 6.4. At pH 2.8 it was not possible to exceed 8 atoms of iodine bound per molecule by increasing the molar ratio of iodine monochloride. Similarly, at pH 6.4 the substitution value of 11.5 atoms of iodine per molecule could not be exceeded. 5. Iodinated insulins containing an average of 1.96, 2.74, 6.0 and 7.0 atoms of iodine per molecule fully retained the ability to bind guinea-pig anti-(ox insulin) serum, and the ability to compete with unlabelled insulin for antibody sites only became significantly changed in the most highly substituted preparations and in the presence of large concentrations of unlabelled insulin. 6. The method for the iodination of insulin with 98% incorporation of ^{131}I by using chloramine-T is described. 7. ^{131}I -iodinated insulin prepared with graded quantities of chloramine-T in excess of that required for efficient labelling was less efficiently bound by guinea-pig anti-(ox insulin) serum than insulin labelled by the iodine monochloride method.

Although techniques that use proteins labelled with ^{131}I have been in existence for over 20 years (Fine & Seligman, 1944) and considerable progress has been made towards improving the efficiency of the labelling, small-scale protein iodinations are still subject to occasional and apparently unpredictable failure. The early methods, which used iodine in potassium iodide solution (Hughes & Straessle, 1950), iodide oxidized to a reactive state with nitrous acid (Pressman & Eisen, 1950) and iodine liberated from a mixture of iodide and iodate with acid (Francis, Mulligan & Wormall, 1951), have largely been superseded by methods that are more efficient with regard to ^{131}I or less harmful to the protein. The iodine monochloride method (McFarlane, 1958) has high theoretical efficiency and does not adversely affect the properties of the labelled protein. The method involving oxidation with chloramine-T (Bannerjee & Gibson, 1962; Greenwood, Hunter & Glover, 1963) is more efficient in the use of ^{131}I and can result in higher specific activities but in some cases results in

oxidative damage to the protein. Accordingly it was thought worth while to carry out a study of some of the factors influencing the small-scale iodination of proteins aiming to improve reliability of the process and to minimize chemical change in the product. The results presented here apply particularly to the iodination of insulin.

In recent years ^{131}I -iodinated insulin at high specific activity has been successfully prepared with iodine monochloride (Springell, 1961; Izzo, Bale, Izzo & Roncone, 1962) and by the chloramine-T method (Bannerjee & Gibson, 1962). Specific activities of the order of 1 c of ^{131}I /mg. of insulin have been obtained with chloramine-T although these preparations contained a high proportion of degraded insulin (which was attributed to radiation damage). Such preparations require further purification before use. With iodine monochloride, ^{131}I -iodinated insulin of specific activity up to 400 mc/mg. has been reported (Izzo *et al.* 1962) with little, if any, damage to the labelled protein. The maximum efficiency of labelling with ^{131}I reported

for the two methods is 90% with chloramine-T and 68% with iodine monochloride (Izzo, Bale, Izzo & Roncone, 1964a).

Investigations on both of these methods are reported here. Of the two the iodine monochloride method, although giving the product that has suffered less chemical change, is more sensitive to variations in reaction conditions and hence has been studied in greater detail. Particularly the influence of pH of the reaction mixture, carrier iodide and interfering factors in the ^{131}I iodide solution have been studied. The influence of the number of iodine atoms bound per molecule of insulin on its immunological properties has been determined. The quantitative relationship between chloramine-T and the binding of ^{131}I by insulin has been examined and the conditions necessary to achieve a binding efficiency of 98% have been determined. The immunological properties of the chloramine-T iodinated insulin have been compared with those of insulin iodinated with iodine monochloride.

MATERIALS AND METHODS

Ox insulin. Specially purified ox insulin with a biological activity of 24.3 units/mg. was obtained as a gift from the Wellcome Research Laboratories, Beckenham, Kent.

^{131}I iodine. ^{131}I iodine without added reducing agent (The Radiochemical Centre, Amersham, Bucks., Catalogue no. IBS 3) was prepared by neutron bombardment of purified natural tellurium dioxide and recovered from the target by distillation into 0.02N-NaOH soln.

Iodine monochloride. Iodine monochloride in glycine buffer, pH 1.35, containing 97.5 μg . of iodine/ml., when diluted for the iodination, was prepared as described by Hales & Randle (1963). In experiments to determine the influence of pH on the iodination, the final dilution of iodine monochloride was made in buffer of the appropriate pH immediately before mixing with insulin.

Buffers for iodine monochloride iodinations. Borate buffer, pH 9.5, was prepared by adjusting 0.025M-borax to the required pH with 0.1N-NaOH. Phosphate buffers, pH 6.4 and 7.7, were prepared by adjusting 0.1M- KH_2PO_4 with 0.1N-NaOH, acetate buffers, pH 3.8 and 4.9, by adjusting 0.1N-sodium acetate with 0.1N-acetic acid, glycine buffer, pH 2.8, by adjusting 0.1M-glycine with 0.1N-HCl, and chloride, pH 1.4 and 1.7, by addition of 0.1N-HCl to 0.1M-KCl. Glycine buffer, pH 1.35, contained 0.06M-glycine, 0.1N-HCl and 0.24M-NaCl. The pH values were determined by means of an Ingold combined glass and reference electrode (type 401-NS; W. G. Pye and Co. Ltd., Cambridge).

Buffer for chloramine-T iodinations. Barbitone-acetate-HCl buffer, pH 8.6 (Michaelis, 1931), ionic strength 0.1, was used.

Phosphate buffer. The phosphate buffer, pH 7.4, for dilution of insulin and use in immunological studies contained 0.1% bovine serum albumin (Difco Laboratories, Detroit 1, Mich., U.S.A.) and 0.6mm-thiomersal (sodium ethylmercurithiosalicylate). A concentrated ($\times 10$) stock buffer was prepared by dissolving anhydrous KH_2PO_4 (108.88g.) in water, adding 640ml. of N-NaOH and thiomersal (4.856g.) and making up to 2l. This stock buffer

keeps for at least 2 months at 2–4°. A working strength buffer was prepared by diluting the stock buffer 1 in 10 and including 3.4ml. of 30% (w/v) bovine serum albumin in each litre of diluted buffer. The final pH should be 7.4.

Catalase. For the removal of hydrogen peroxide, 0.5 μg . of catalase (L. Light and Co. Ltd., Colnbrook, Bucks.) in 100 μl . of 0.05M-phosphate buffer, pH 6.8, with an activity of 1.75 Keil units was added per 100 μl . of ^{131}I iodide soln. and maintained at 25° for 10 min. Before use in the iodination, 200 μl . of glycine buffer, pH 1.35, was added.

Salts. All salts were of analytical reagent grade.

Methods of iodination

Iodine monochloride method. The method of iodination was based on a modification of McFarlane's iodine monochloride method (Springell, 1961) with further modifications. The ^{131}I iodine (50 μl ., 10–20 mc) was dispensed into a Pyrex glass tube, 6mm. $\times$ 50mm., contained within a 10ml. glass vial, the tube being firmly embedded in modelling clay. To this solution was added 20 μl . of the diluted iodine monochloride (containing 1.95 μg . of iodine) and 50 μg . of crystalline ox insulin in 10 μl . of glycine buffer, pH 1.35, immediately followed by rapid mixing with a Vortex mixer [Vortex Junior Mixer, Scientific Industries International Inc. (U.K.) Ltd., London, N.W. 4]. After 5 min. 20 μl . of 0.1N- $\text{Na}_2\text{S}_2\text{O}_3$ was added, Vortex mixing was repeated and was followed by the addition of 0.5ml. of 2% (w/v) NaI and 0.5ml. of the 0.04M-phosphate buffer, pH 7.4. The contents of the tube were mixed and transferred to a 10ml. vial containing 0.5ml. of the same phosphate buffer. In tracer-level experiments to determine the influence of pH and the weight of iodine monochloride used, it was necessary to use larger tubes (10mm. $\times$ 50mm.) and the buffer and diluted ICl (combined volume 480 μl .) were dispensed into the tube before the addition of diluted ^{131}I iodide. In these cases also, 0.2M-phosphate buffer, pH 7.4, was required for the transfer to maintain the final pH at the desired value.

Chloramine-T method. The method of iodination of insulin with chloramine-T was based on experience gained in the iodination of other proteins during the course of several years. To 50 μl . of ^{131}I iodide solution (10–20 mc), contained in a reaction tube of the type described above, were added 20 μl . of KI soln. (6.9%, w/v), 70 μl . of barbitone-acetate-HCl buffer, pH 8.6, ionic strength 0.1, and 10 μl . of 0.104% chloramine-T in the same buffer. After Vortex mixing, 50 μg . of crystalline ox insulin in 10 μl . of buffer, pH 8.6, was added, Vortex mixing was repeated and was followed by the addition of 10 μl . of 0.03N- $\text{Na}_2\text{S}_2\text{O}_3$. After further mixing, 5% (w/v) bovine serum albumin in 0.04M-phosphate buffer, pH 7.4, was added to reduce radiation damage. This method consistently gave better than 95% binding of the total ^{131}I . ^{131}I -iodinated insulin preparations were freed from ^{131}I iodide by gel filtration on a 2.8cm. $\times$ 18.0cm. column of Sephadex G-25 (bead form, coarse grade, Pharmacia, Great Britain Ltd., London, W. 13) with 0.04M-phosphate buffer, pH 7.4 (composition as above), as the solvent and a flow rate of 3.5 ml./min.

Electrophoresis. Reaction mixtures and ^{131}I -iodinated insulin solutions were diluted to approx. 100 μC /ml. with the 0.04M-phosphate buffer and spots (10 μl .) were applied close to the cathode and subjected to electrophoresis on horizontal strips (5cm. $\times$ 36cm.) of Whatman no. 1 paper, at

12v/cm. for 1hr., in barbitone buffer, pH8.6 (Flynn & de Mayo, 1951). Samples of labelled insulin were also mixed with carrier crystalline insulin (25mg./ml.) and subjected to electrophoresis in the same way. In the absence of carrier insulin all of the undamaged labelled insulin became bound to the paper and remained at the origin, whereas in its presence it travelled as a single peak coincident with the carrier, which was located by staining with naphthalene black or light green. Precautions were taken to avoid loss of ^{131}I -iodinated insulin due to absorption on glassware by pretreatment of sampling tubes with phosphate buffer containing bovine serum albumin (0.1%).

For the measurement of radioactivity the strips were dried at 100° for 20 min., then passed through an automatic radioactivity scanning recorder with an end-window Geiger tube, relating radioactivity to distance along the strip, the area of the peaks being proportional to the ^{131}I content of the components. From the total area of the peaks, measured by planimetry, it was possible to determine the proportion of ^{131}I bound to insulin.

Immunological properties of iodinated insulin. The proportion of ^{131}I -iodinated ox insulin which was sufficiently similar to unlabelled ox insulin to bind to anti-(ox insulin) serum was determined by method C of Hales & Randle (1963). With the same method, various treatments could be assessed by comparing the competition of ^{131}I -iodinated insulin with unlabelled insulin for antibody sites. Anti-(ox insulin) serum raised in guinea pigs (batch D/6, Wellcome Research Laboratories) was diluted serially in the range 1:8000 to 1:128000 with the 0.04M-phosphate buffer, pH7.4, to which had been added (1:5000) normal guinea-pig serum (Wellcome Research Laboratories). The diluted anti-(ox insulin) serum (50 μl . of each dilution) was Vortex-mixed with 50 μl . of antibodies to whole guinea-pig serum raised in rabbits (Wellcome Research Laboratories). The concentration of the precipitating antiserum, which produced maximum binding with anti-(ox insulin) serum (approx. 1:12 to 1:24), was previously determined. After incubation at $2-4^\circ$ for 24hr., 100 μl . of the 0.04M-phosphate buffer, pH7.4, containing 0.9% NaCl, or 100 μl . of unlabelled insulin in this buffer, was added with Vortex mixing. After further incubation at $2-4^\circ$ for 6hr., 100 μl . of ^{131}I -iodinated insulin (diluted to 2.5 μg ./ml. with 0.04M-phosphate buffer, pH7.4) was added, again with Vortex mixing, and incubation was continued for a further 18hr. at $2-4^\circ$. The antibody precipitates containing bound labelled insulin were filtered as described by Hales & Randle (1963) and counted with a Nuclear-Chicago model 725 liquid- β -scintillation counter. Total radioactivity was determined by measuring 100 μl . of ^{131}I -iodinated insulin in the scintillant. This assay was carried out in duplicate at each dilution of anti-(ox insulin) serum.

RESULTS

Influence of components of ^{131}I iodide on iodine monochloride iodinations. In the iodination of proteins by use of iodine monochloride the weight of iodine that can be incorporated is determined by the amount of iodine chloride available (in excess of that which is used up in reaction with peroxides, adventitious material, non-inert surfaces etc.). Increasing the weight of iodide that is present will

not increase the amount of iodine that becomes protein-bound and the percentage binding will in fact fall. This has been pointed out by Bale *et al.* (1962), who suggested that the efficiency of iodination could be represented by the following expression:

$$\frac{\text{Wt. of I in ICl}}{\text{Wt. of I as I}^- + \text{wt. of I in ICl}} \times Y$$

where Y is the percentage iodination by that weight of iodine monochloride in the absence of iodide.

The consequence of this is that adding an increasing amount of ^{131}I iodide to an iodine chloride iodination system results in a steadily decreasing percentage incorporation and an optimum amount of ^{131}I iodide for the maximum radiochemical yield and hence maximum specific activity. This is illustrated by the results of the series of iodinations shown in Fig. 1. The effect in this case was produced by the iodide in the ^{131}I -iodide solution used. This preparation was obtained by neutron irradiation of natural tellurium dioxide, which contains ^{126}Te (18.7%), ^{128}Te (31.8%) and ^{130}Te (34.5%), which undergo nuclear reactions to form ^{127}Te , ^{129}Te and ^{131}Te , which decay rapidly to ^{127}I (stable), ^{129}I (half-life 1.6×10^7 years) and ^{131}I (half-life 8.04 days) respectively. The relative proportion of these isotopes of iodine in the ^{131}I preparation depend upon the irradiation time, neutron-capture cross-section, neutron flux and storage time and for any given set of conditions the isotopic abundance of each nuclide can be calculated.

Fig. 1. Influence of the amount of ^{131}I on the yield of ^{131}I -iodinated insulin.

The effect which iodide has upon the efficiency of protein iodination offers a way of checking the calculated specific activity of ^{131}I preparations. To do this two series of iodinations were carried out. The first employed each time a constant amount (1.0mc) of ^{131}I iodide, and an increasing weight of carrier iodide, which caused the expected fall in efficiency of iodination. The second series of iodinations employed an increasing amount of ^{131}I iodide preparation to which no carrier was added in any iodination. The weight of iodine (^{127}I , ^{129}I and ^{131}I) in each of these samples was calculated from the irradiation and decay data, no allowance being made for successive neutron capture. The fall in efficiency of iodination in this series was then correlated with the calculated weight of iodide present and this curve was compared with the one in the first series where the weight of iodide is known rather than calculated. As can be seen from the results shown in Fig. 2 the two curves are almost superimposable. The preparation of ^{131}I iodide used represented the most aged material normally despatched from The Radiochemical Centre (i.e. 1 day after distillation from the target, which had been removed from the reactor 11 days previously) and the specific activity used in the calculations was 9.09mc/ μg ., representing approx. 7.2% isotopic abundance of ^{131}I . Previous examination of the effect of the removal of peroxide by the method recommended by Bale *et al.* (1962) had resulted in no significant improvement in iodination efficiency and no steps were taken in this work to remove this substance or any other complicating factor. The ^{131}I solution in fact behaved identically with a solution of non-radioactive sodium iodide of the appropriate concentrations as calculated from the physical data.

Although the effect of the presence of varying amounts of iodide has qualitatively the effect described by Bale *et al.* (1962) for iodination of insulin and by Greene, Stang & Hillman (1963) for the iodination of γ -globulin, there are important quantitative differences. Bale's data, when plotted as percentage incorporation against $\text{ICl}/(\text{I} + \text{ICl})$, showed a straight line passing through the origin with a maximum incorporation of approx. 65%. The data presented here (Fig. 3) gave a sigmoid curve with a maximum at 78%. Other quantitative differences are presented in the following section.

Influence of pH and the weight of iodine monochloride on the efficiency of iodination of insulin. The effect of varying the weight of iodine monochloride used in the iodination of 50 μg . of insulin has been studied over a wide range of pH values. These iodinations were carried out with a tracer (0.3mc) level of ^{131}I to minimize the effect of any contribution

Fig. 2. Comparison of the effect of carrier iodide and ^{131}I iodide (9.1mc/ μg . of iodine) on the incorporation of ^{131}I into insulin.

Fig. 3. Influence of iodide on the incorporation of ^{131}I into iodinated insulin.

to the results by components of the ^{131}I iodide. As has been said in the previous section, increasing the weight of iodine monochloride present caused an increase in the proportion of ^{131}I which became bound to protein up to a certain point, beyond which a decrease occurred. The maximum percentage incorporation that could be obtained (and hence the maximum specific activity) was very dependent on the pH at which the iodination took place. A series of curves showing the efficiency of iodination as a function of the weight of iodine monochloride at different pH values is shown in Fig. 4. A corollary of this observation is that when the results were plotted as atoms of iodine incorporated into each molecule of insulin it was found (Fig. 5) that for each pH value there was a characteristic substitution level which could not be exceeded even by large excesses of iodine monochloride. At pH values where iodination is inefficient, e.g. pH 4.9, the iodine monochloride levels employed did not reach this maximum level.

The influence of pH on the proportion of ^{131}I bound when 50 $\mu\text{g.}$ of insulin was iodinated with varying weights of iodine monochloride is shown in Fig. 6. Two definite optimum pH values were

Fig. 4. Influence of the amount of iodine monochloride on the proportion of ^{131}I bound to insulin at various pH values: A ($\circ$), pH 1.40; B (Δ), pH 1.71; C ($\square$), pH 2.86; D ($\bullet$), pH 3.79; E ($\blacktriangle$), pH 4.92; F ($\blacksquare$), pH 6.43; G ($\circ-\circ$), pH 7.73; H ($\bullet-\bullet$), pH 9.48.

observed for each weight of iodine monochloride used, one in the acid region around pH 2.8, the other between pH 6.5 and 7.0. The iodine substitution levels corresponding to these results are shown in Fig. 7. At pH 2.8 the maximum degree of substitu-

Fig. 5. Influence of the amount of iodine monochloride on the degree of substitution of insulin with iodine at various pH values: A ($\circ$), pH 1.4; B (Δ), pH 1.71; C ($\square$), pH 2.86; D ($\bullet$), pH 3.71; E ($\blacktriangle$), pH 4.92; F ($\blacksquare$), pH 6.43; G ($\circ-\circ$), pH 7.74; H ($\bullet-\bullet$), pH 9.48. Values were also obtained with 1.46 $\mu\text{g.}$ and 2.92 $\mu\text{g.}$ of iodine (as ICl) but these have been omitted for clarity.

Fig. 6. Influence of pH on the proportion of ^{131}I bound to insulin with various amounts of iodine monochloride (expressed in terms of iodine): $\circ$, 1.46 $\mu\text{g.}$; Δ , 2.92 $\mu\text{g.}$; $\square$, 5.85 $\mu\text{g.}$; $\bullet$, 11.7 $\mu\text{g.}$; $\blacktriangle$, 23.4 $\mu\text{g.}$; $\blacksquare$, 46.8 $\mu\text{g.}$

tion, an average of 8 atoms/molecule, was reached with 11.7 $\mu\text{g.}$ of iodine monochloride and could not be exceeded even with a fourfold increase in the weight of iodine monochloride used. At pH 6.5, the substitution level tended to a maximum at an average of 11.5 atoms of iodine/molecule of insulin. Even when very large excesses of iodine monochloride were used (49, 98 and 195 $\mu\text{g.}$ of iodine), 11.5 atoms/molecule of insulin was not exceeded at pH 6.43.

Immunological properties of ^{131}I -iodinated insulin. The binding of equal weights of iodinated insulins of comparable specific activity (about 100 mc/mg.), containing 1.96, 2.74, 6.0 and 7.0 atoms of iodine/molecule, by antibody to insulin by using Hales & Randle method C is shown in Fig. 8. The curves for the different iodinated insulin preparations were not significantly different and showed that a maximum 88–95% was bound to anti-(ox insulin) serum under optimum conditions. Competition between three of these preparations and unlabelled insulin for antibody sites is shown in Fig. 9. This shows the results of using these samples of iodinated insulin to prepare standard curves for immunoassay with pure insulin solutions. An inverse relationship between substitution level and ability to compete for binding sites was demon-

strated, but the magnitude of this effect became serious only in the presence of high concentrations of unlabelled insulin.

Efficient ^{131}I -labelling of insulin with chloramine-T. Chloramine-T is a reactive agent that potentially can chemically modify proteins and it is clearly desirable that it should not be used in unnecessary excess. Our experience with other proteins has shown a stoichiometry between the weight of chloramine-T used and the percentage incorporation of iodine in the protein under fixed conditions.

Accordingly, the efficiency of the iodination of insulin as a function of the weight of chloramine-T used was studied. Fig. 10 shows the relationship between the proportion of the ^{131}I bound to 50 $\mu\text{g.}$ of insulin at pH 8.6 and the weight of chloramine-T. In each case 10 mc of [^{131}I]iodide and 1.7 $\mu\text{g.}$ of total

Fig. 7. Influence of pH on the degree of substitution of insulin with iodine with various amounts of iodine monochloride (expressed in terms of iodine): $\circ$, 1.46 $\mu\text{g.}$; Δ , 2.92 $\mu\text{g.}$; $\square$, 5.85 $\mu\text{g.}$; $\bullet$, 11.7 $\mu\text{g.}$; $\blacktriangle$, 23.4 $\mu\text{g.}$; $\blacksquare$, 46.8 $\mu\text{g.}$

Fig. 8. Influence of the degree of iodination of ox insulin on its binding with anti-(ox insulin) serum. $\bullet$, 2.0 atoms of I/molecule of insulin; $\circ$, 2.7 atoms of I/molecule of insulin; Δ , 6.0 atoms of I/molecule of insulin; $\square$, 7.0 atoms of I/molecule of insulin.

Fig. 9. Influence of the iodine substitution level of iodinated insulin on competition with unlabelled insulin for antibody sites. O, 2.7 atoms of I/molecule of insulin; Δ , 6.0 atoms of I/molecule of insulin; $\square$, 7.0 atoms of I/molecule of insulin.

Fig. 10. Relationship between the weight of chloramine-T and the binding of ¹³¹I by insulin.

iodide was used. Maximum binding (98%) was reached with 10.4 μg. of chloramine-T under these conditions. Amounts in excess of this value cannot result in a more efficient labelling but could result in a more highly damaged preparation. This is studied in the following section.

Fig. 11. Influence of chloramine-T (O, 10.4 μg.; Δ , 20.8 μg.; $\square$, 41.6 μg.; $\bullet$, 83.2 μg.) on the binding of iodinated insulin with anti-(ox insulin) serum. $\blacktriangle$, Insulin iodinated with iodine monochloride.

Immunological properties of insulin iodinated by the chloramine-T method. Insulin was iodinated by using the minimum amount of chloramine-T necessary to achieve quantitative incorporation of iodine (10.4 μg. of chloramine-T/50 μg. of insulin) and also with graded excesses of chloramine-T. All preparations were carried out under otherwise similar conditions, achieving the uniform specific activity of 200 mc/mg. and substitution levels of 1.7 atoms/molecule of insulin. All these preparations of iodinated insulin and a batch prepared by the iodine monochloride method at similar specific activity and substitution levels were compared immunologically.

The results shown in Fig. 11 demonstrate clearly that, whereas 96% of labelled insulin prepared by the iodine monochloride method was bound to anti-(ox insulin) serum (which is in agreement with the results obtained with higher substitution levels), only 66–70% of that labelled under the best condition with chloramine-T was bound. Increasing the ratio of chloramine-T to insulin caused a further slight reduction in binding.

DISCUSSION

The results show that the pH at which insulin is iodinated has an important effect on the efficiency of binding of ^{131}I and on the degree of substitution. The observed results show that the maximum number of iodine atoms that can be bound to insulin is determined by the pH, since for each pH value tested a maximum substitution level was reached, which could not be exceeded by increasing the ratio of iodine monochloride to insulin. The pH curve was complex and showed two optimum values for the incorporation of ^{131}I into insulin, at pH 2.8 and 6.4, the minimum value being approx. pH 5. At pH 2.8 not more than 8 atoms of iodine/molecule of insulin could be bound, whereas at pH 6.4 it was possible to achieve, but not exceed, 11.5 atoms of iodine/molecule, assuming a molecular weight of 6000 for insulin. If it is accepted that each tyrosyl residue has only two sites that will react with iodine (Hughes & Straessle, 1950; Springell, 1961), the maximum number of iodine atoms bound to tyrosyl residues in each molecule of insulin would be 8. It seems reasonable therefore to suggest that at pH 6.4 other residues, most probably histidyl, can be substituted. The maximum number of 11.5 iodine atoms/molecule observed at pH 6.4 is consistent with disubstitution of both histidyl residues in addition to all four tyrosyl residues. In this respect these results are in agreement with the evidence obtained with serum albumin that a proportion of the tyrosyl residues reacted concomitantly with histidyl (Hughes & Straessle, 1950). Similarly, the maximum of 8 atoms of iodine/molecule at pH 2.8 is consistent with disubstitution of all four tyrosyl residues.

Increasing the number of atoms up to 7 did not significantly alter the behaviour of insulin iodinated with iodine monochloride at pH 2.8 in the immunological tests applied and such preparations gave satisfactory assays of standard insulin solutions when used in method C of Hales & Randle (1963). Similar results have been reported for insulin iodinated by iodine monochloride at pH 8 (Izzo, Roncone, Izzo & Bale, 1964b). These results for iodine monochloride iodinations are quantitatively different from those reported by Ooms & Arquilla (1966), who found distinct changes in immunological behaviour of insulin, which had been iodinated electrolytically in this range of substitution level. These findings can be compared with the observation that serum albumin can be iodinated to a higher substitution level by the electrolytic method (Bianchi *et al.* 1966) before metabolic changes are detectable than with the iodine monochloride method (Freeman, 1959). It is a matter for speculation whether this difference arises because insulin is more easily damaged by electrolytic iodination than by

iodine mono-chloride, where the reverse is true for albumin, or whether immunological integrity is more easily impaired by electrolytic iodination than by iodine chloride iodination and the reverse of this is true when metabolic quality is the criterion.

It has been shown (Bale *et al.* 1962) that the percentage incorporation of ^{131}I into γ -globulin during iodine monochloride iodinations at pH 8 can be predicted by the expression:

$$\frac{\text{Wt. of I in ICl}}{\text{Wt. of I as I}^- + \text{wt. of I in ICl}} \times Y$$

where Y is the percentage found in the absence of any carrier iodide. Under these conditions Y is approximately 65%, although there seems no fundamental justification for this value. Indeed, ideally, where none of the iodine monochloride is used up in extraneous reactions and all the iodine in this reagent is in the chemical form of iodine monochloride, one would expect Y to be 100%. Changing the pH of the iodination clearly introduces complexities as this change alone can alter the percentage binding in tracer iodinations from > 5% to 79% by using a constant amount of iodine monochloride and iodine. Further, at pH 2.86 three levels of iodine monochloride employed resulted in percentage incorporations that exceeded the implied maximum of 65%, which Bale *et al.* (1962) find characteristic of iodinations in borate buffer. The expression put forward by these authors explains the effects of carrier iodide in the system and is useful in predicting this quantitatively under their conditions. However, it is clear that this holds true only when the only parameters which are changing are the weight of iodine and iodine chloride present in the system. Changing other factors (among which pH and the nature of the protein are important) introduces new variables. The effect of microgram amounts of iodide on the efficiency of iodine monochloride iodinations has been used to assess the specific activity of the [^{131}I]iodide preparations used. This was very close to the calculated values and no allowance for the presence of peroxide or other interfering substances needed to be made.

Excess of chloramine-T has been shown to affect the immunological properties of insulin iodinated by use of this reagent. This is acceptable when highest attainable specific activities are demanded since methods exist for separating the insulin from that which has been chemically changed. Further, the criterion for suitability of an iodinated hormone in immunoassay is that it competes for antibody sites in an identical manner with the standard hormone and with the endogenous hormone being assayed respectively. This criterion is more likely to be reliably achieved if chemical damage is minimized. It has been demonstrated that insulin iodinated

with iodine monochloride has suffered less change (as judged by its immunological properties) than a similar preparation obtained by chloramine-T iodination. The advantage of using chloramine-T in iodinations is, of course, the higher specific activity that can be obtained by this method. These specific activities are, however, achieved at a cost of damage to the iodinated insulin. Since the specific activity obtainable by use of iodine monochloride (450mc/mg.) is more than sufficient for many purposes (especially when relatively large samples can be counted in the immunoassay, e.g. by the double antibody technique) freedom from chemical damage makes this an attractive method of preparing iodinated hormones.

In iodinations with iodine monochloride, the use of a fixed weight of this reagent automatically maintains the chemical substitution level at a known value which is independent of the contribution of iodide carrier in the isotope preparation or from any other source. This is not so in chloramine-T iodinations unless the appropriate weight of the latter is carefully determined. With iodinated insulin for use in immunoassay it has been shown that even full iodination produces only marginal changes. Iodinated proteins for other purposes (and even different iodinated hormones for use in immunoassay) differ in this respect and when the degree of iodination needs to be carefully controlled the iodine monochloride method has clear advantages.

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