$[^{125}I]$Diiodoinsulins

Binding Affinities, Biologic Potencies, and Properties of their Decay Products

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SUMMARY

Insulin was iodinated with 0.3–0.4 mol ^{125}I/mol insulin using the lactoperoxidase method. About one-third of the radioactivity incorporated into insulin was in diiodoinsulins and about 40% of these molecules contained diiodotyrosine in residue 14 of the A chain. Most of the remaining molecules contained one A14-monoiodotyrosine and one monoiodotyrosine in either position A19, B16, or B26. The binding affinity and biologic potency of this heterogeneous diiodoinsulin preparation was not significantly different from that of A14-$[^{125}I]$monoiodoinsulin in rat adipocytes, whereas it was slightly reduced in hepatocytes and IM-9 lymphocytes. From the iodine distribution and previous data on the binding affinity of each of the four monoiodoinsulin isomers it was calculated that A14-diiodotyrosine-insulin possesses full binding affinity and biologic potency in adipocytes.

Diiodoinsulins isolated from another iodoinsulin preparation (iodate method) contained 58% A19-diiodotyrosine-insulin, and most remaining molecules contained one A19-monoiodotyrosine. The binding affinity of this mixed diiodoinsulin preparation was approximately one-fourth of that of A14-monoiodoinsulin in adipocytes, IM-9 lymphocytes, and hepatocytes. It was calculated that A19-diiodotyrosine-insulin is nearly devoid of binding affinity.

The diiodoinsulins (lactoperoxidase method) decayed to iodide (probably from diiodotyrosine-insulin) or to polymers with little specific but a markedly increased nonspecific binding. In addition, the polymers had a marked tendency to adsorb to cellulose acetate filters.

Conclusions: 1. The binding affinities of diiodoinsulins range from very low values to values at least as high as that of insulin depending on the positions of the iodine moieties. 2. The relative binding affinities vary among tissues. 3. Polymeric decay products give high nonspecific binding. **DIABETES 31:634–640, July 1982.**

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Frequently, $[^{125}I]$iodoinsulins are used as tracers for insulin. Monoiodoinsulins of quite high specific activity[1] or practically "carrier-free"[2–4] have been prepared. However, most investigators have used heterogeneous ^{125}I-insulin preparations iodinated with essentially carrier-free ^{125}I (2.1 Ci/μmol) to a specific activity of 0.9–1.2 Ci/μmol, that is to a degree of iodination of 0.4–0.6 (for examples see refs. 5–10).

These preparations contain appreciable amounts of diiodoinsulins whose biologic properties are unknown. In addition, the diiodoinsulins will decay to monoiodinated products of hitherto unknown nature. The purpose of the present study is to determine the binding affinities and biologic potencies of the diiodoinsulins and to examine the properties of their decay products.

MATERIAL AND METHODS

Chemicals. Highly purified porcine insulin was prepared as described previously.[11] ^{125}I (IMS 30) with a specific activity (as indicated by the manufacturer) of 2.0–2.1 Ci/μmol and [U-^{14}C]glucose (250 Ci/mol) were obtained from The Radiochemical Centre, Amersham. Hydrogen peroxide (30%, Perhydrol) was from Merck. Lactoperoxidase (L 2005), α-chymotrypsin (C-7762), trypsin (T8253), and bovine serum albumin (fraction V) were from Sigma. Pronase (53702) was from Calbiochem. Collagenase (type 1) was from Worthington. Cellulose acetate filters (type EA, 1.0 μm) were from Millipore; cellulose (CF-11) and filter paper wicks (No. 3MM-2835) were from Whatman.

Preparation of diiodoinsulins. Insulin was iodinated to 0.3–0.4 mol I/mol insulin using lactoperoxidase. In brief: 10 μl (1.0 mCi) $^{125}I^-$ in NaOH (as supplied by the manufacturer) was transferred to a 10-×-7-mm round-bottom polystyrene tube followed by the addition of 10 μl 0.4M phosphate buffer

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(pH 7.4), 10 μl insulin (1.0 mg/ml) in 0.04N HCl, 5 μl 3 × 10^{-4}M H_2O_2 and 5 μl phosphate buffer with lactoperoxidase 0.2 mg/ml. The solution was stirred for 5 min at 22°C, and 40 μl 40% sucrose was added before the mixture was applied to a polyacrylamide gel. Incorporation of ^{125}I into protein was above 90% as measured by precipitation in 10% trichloroacetic acid. ^{125}I-iodoinsulin, prepared by the iodate method as described previously,[12] was obtained from Novo, Bagsvaerd. It contained about 0.1 mol iodine/mol insulin. Iodination (lactoperoxidase method, 0.15 mol I/mol insulin) in the presence of 6M urea was carried out as described in detail elsewhere.[13]

Fractionation of the heterogeneous iodination mixtures was performed using polyacrylamide gel electrophoresis (18-cm rods, pH 9.15), as described.[4,11]

Determination of the iodine distribution in the four tyrosine groups. The [^{125}I]diiodoinsulins were subjected to oxidative sulfitolysis[14] in the presence of nonradioactive carrier iodoinsulin (heterogeneous preparation, 1 mol I/mol insulin) in analogy with the method described for [^{125}I]monoiodoinsulin.[4] Chymotryptic cleavage of the eluted S-sulfo A chain[15] was carried out as previously described.[4] The eluted S-sulfo B chain was cleaved with trypsin[15] as follows: The lyophilized B chain was dissolved in 1.0 ml of 0.02M phosphate buffer and the pH was readjusted to 8.4. Thirty micrograms of trypsin in 30 μl phosphate buffer was added and the reaction carried out at 5°C for 60 min. The reaction was stopped by adding 40 μl acetic acid to 200 μl of the hydrolytic mixture.

The cleavage peptides A_{1-14} and A_{15-21} were separated using paper electrophoresis in 30% acetic acid[16] or using gel filtration on Sephadex G-25 F in 1M acetic acid.[4,16] The cleavage peptides B_{1-22} and B_{23-29} were separated using paper electrophoresis in 0.2M phosphate-8M urea, pH 6.5.[15] The paper strips were dried with cold air and cut into 5-mm shreds for counting of radioactivity. The bands were identified by staining with Pauly's reagent.

Determination of the iodine as monoiodotyrosine or diiodotyrosine. Diiodoinsulins or cleavage peptides were digested to amino acids using incubation for 24 h with pronase (0.05 mg/ml) in 0.3M Tris buffer with 1 mM calcium acetate (pH 8.0) containing 2.2 mM carrier monoiodotyrosine and diiodotyrosine. Labeled monoiodotyrosine and diiodotyrosine were identified by adsorption chromatography using Sephadex G-25, fine, with 0.5M acetic acid, 0.1% albumin as the eluent.

Characterization of the decay products. Wick chromatography of diiodoinsulin was carried out as described by Ørskov,[17] and precipitation with an excess of insulin antibodies in 82% ethanol (final concentration) was performed as described by Heding.[18] Precipitation of protein was carried out using 13% trichloroacetic acid (final concentration) in the presence of carrier albumin (1 mg/ml, final). $^{125}I^-$ in the trichloroacetic acid supernatant was identified by oxidation to iodine followed by extraction in chloroform. In brief, 4 mg of carrier KI and 100 μl 30% H_2O_2 was added to 2 ml supernatant followed by the addition of 4 ml chloroform. After shaking for 5 min and centrifugation, aliquots of the chloroform phase (containing the iodine) and the water phase (containing iodotyrosine) were assayed for radioactivity.

Incubation of cells and calculation of the relative binding affinities. Preparation and incubation of adipocytes, hepatocytes, and cultured human lymphocytes of the IM-9 line followed previously published methods.[4] When necessary, details are given in the legends to figures and tables. Preliminary experiments showed no marked difference between the rate of association or dissociation of A14 monoiodoinsulin and diiodoinsulins prepared by the lactoperoxidase method. Also, there was no difference in the percentage of the bound ligand that was degraded. It was therefore considered legitimate to calculate relative affinities using a fixed incubation time of 45 min at 37°C. The average binding affinity of a diiodoinsulin preparation as a percentage of that of A14-monoidoinsulin was calculated as [(B/F)diiodoinsulin × 100]/[(B/F)monoiodoinsulin], where B/F is the concentration of specifically bound analogue divided by the concentration of free analogue. This ratio of specific tracer binding is practically identical with the ratio of affinities because the ^{125}I-labeled tracers were used at concentrations (5–15 pM) very much lower than the average K_d (about 2 nM) for binding in either of the three cell types.[3,13] In each experiment the molar concentration of diiodoinsulin was the same as that of monoiodoinsulin, i.e., the concentration of radioactivity was twice as high. The nonspecific binding averaged 2% of the total binding of tracer A14-monoiodoinsulin in adipocytes, 10% in hepatocytes, and 10% in IM-9 lymphocytes. The coefficient of variation on binding of tracer was about 4% in adipocytes and about 10% in hepatocytes and lymphocytes.

The biologic potency, relative to that of A14-monoiodoinsulin, was determined from the enhancement of conversion of glucose to lipids in rat adipocytes as described previously.[4]

The results are based on two diiodoinsulin preparations using the lactoperoxidase method (radioactivity in diiodoinsulins, 31% and 34%), one preparation using the iodate method (radioactivity in diiodoinsulins, 8%), and one preparation using lactoperoxidase in the presence of urea (radioactivity in diiodoinsulins, 9%). In each case the receptor binding (adipocytes) of the A14-[^{125}I]-monoiodoinsulin from the iodination carried out for the preparation of diiodoinsulins was compared with that of A14-monoiodoinsulin prepared as described previously,[4] and they were not distinguishable.

Calculations of decay of total ^{125}I-activity, decay of molecules containing two ^{125}I-atoms, and rate of formation of decay products containing one ^{125}I-atom. The decay of ^{125}I-activity follows the equation:

$$y_t = y_0 \cdot e^{-kt} \quad (1)$$

where y_t is the amount of radioactivity at time t, y_0 the amount at time zero, and k the decay constant. This is 0.0115 · day^{-1} for ^{125}I (half-life 60.0 days). Molecules containing one ^{125}I atom (monoiodoinsulin or monoiodotyrosine) will decay according to Eq.(1).

The ^{125}I-activity in molecules containing two ^{125}I-atoms (y^*) decreases following the equation:

$$y^*_t = y^*_0 \cdot e^{-2kt}, \text{ where } y^*_0 = y_0 \quad (2)$$

Note that the rate constant for disappearance of a particular species of molecules with two ^{125}I-atoms is 0.023 · day^{-1}. The equation applies to diiodoinsulin and diiodotyrosine under the assumption that the isotopic abundance is 100%.

The decrease in molecules with two ^{125}I atoms in fraction

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of the total radioactivity follows the equation:

$$\frac{y_t^*}{y_t} = \frac{y_0 \cdot e^{-2kt}}{y_0 \cdot e^{-kt}} = e^{-kt} \quad (3)$$

The amount of ^{125}I-monoiodo decay products at time t (P_t) is the difference between the amount of radioactivity (y_t) and the amount of molecules containing two ^{125}I atoms at that time (y_t^*). Hence:

$$P_t = y_0 \cdot e^{-kt} - y_0 \cdot e^{-2kt} = y_0 \cdot e^{-kt}(1 - e^{-kt}) \quad (4)$$

RESULTS

Fractionation of diiodoinsulins. The distribution of radioactivity in the polyacrylamide gel after electrophoresis of a heterogeneous iodination mixture (lactoperoxidase method, 0.30 mol I/mol insulin) is shown in Figure 1, top panel. The slices corresponding to the hatched bars (26% of the radioactivity) contained diiodoinsulins. Control experiments (not shown) demonstrated that reelectrophoresis of A19-monoiodoinsulin (R_f 0.75–0.76) or A14-monoiodoinsulin (R_f 0.78–0.79) gave no radioactivity in gel slices with R_f values higher than 0.81. This is in agreement with previous results (Figure 2, panel 4, ref. 4), and it is concluded, therefore, that the radioactivity shown in the hatched bars represents essentially only diiodoinsulins. In addition, the slice corresponding to R_f about 0.80–0.81 contained mainly diiodoinsulins. Reelectrophoresis of material corresponding to the hatched bars (diiodoinsulins) showed 20% radioactivity in the R_f 0.80–0.81 fraction (Figure 1, lower panel), whereas reelectrophoresis of A14-monoiodoinsulin showed only 2% of the radioactivity in this fraction, in broad agreement with previous results.[4] The radioactivity in diiodoinsulins was therefore estimated as 31%. This is considered to be a minimum estimate since the content of diiodoinsulins in slices with R_f values of less than 0.80 (cf. Figure 1, lower panel) was neglected. In addition, the radioactivity in slices with R_f values higher than 0.86 was neglected. This may in part represent triiodoinsulins. Finally, it should be noted that diiodoinsulins are well separated from unlabeled insulin, which has an R_f value of 0.73.[3,4] The diiodoinsulins were eluted from the slices corresponding to the hatched bars. The further studies were carried out on two diiodoinsulin preparations from two iodinations using the lactoperoxidase method (that shown in Figure 1 and one from an iodination with about 0.4 mol I/mol insulin), one preparation using the iodate method, and one using lactoperoxidase in the presence of 6M urea.

Distribution and properties of diiodoinsulin isomers at the time of preparation. The binding affinity and biologic activity of the diiodoinsulin preparations were assessed shortly after their preparation (days 2–3). On the other hand, the procedures involved to identify the location of the mono and diiodotyrosine residues required time. In order to calculate back to the distribution at the time of preparation it was necessary to know whether [^{125}I]diiodotyrosine decayed to [^{125}I]monoiodotyrosine or to other radioactive products. This was evaluated by digestion of diiodoinsulin with pronase followed by adsorption chromatography on Sephadex at two different dates. The distribution of ^{125}I-activity in diiodoinsulins treated with pronase 15 days after its preparation using the lactoperoxidase method is shown in Figure 2, top panel.

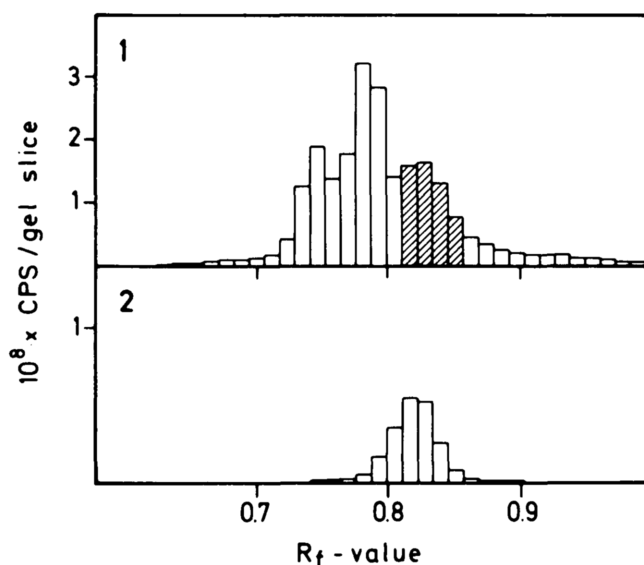

FIGURE 1. Top panel: The distribution of radioactivity in polyacrylamide gel slices after iodination (0.30 mol I/mol insulin) using lactoperoxidase. The position of each fraction was calculated relative to the tracking dye (bromphenol blue, R_f = 1.00). The hatched bars represent the diiodoinsulins. Bottom panel: Reelectrophoresis of diiodoinsulins. For further explanation, see text.

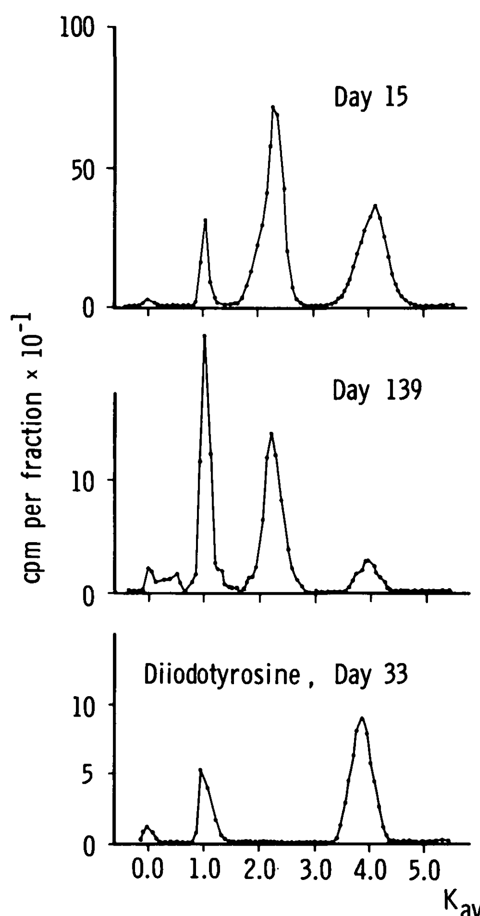

FIGURE 2. Distribution of diiodotyrosine, monoiodotyrosine, and iodide in heterogeneous diiodoinsulins prepared using the lactoperoxidase method. The diiodoinsulin was treated with pronase and the digest chromatographed on a Sephadex G-25, fine, column (0.9 × 60 cm) at 4°C using 0.5M acetic acid, 0.1% albumin as the eluent. Top panel: 15 days after preparation of diiodoinsulin. Middle panel: 139 days. Bottom panel: Diiodotyrosine recovered from an experiment as that shown in the top panel was rechromatographed 33 days later.

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TABLE 1
Distribution of mono- and diiodotyrosine in diiodoinsulins prepared using the lactoperoxidase method

	Position	A14	A19	B16	B26
Distribution (percentage of total radioactivity)	Monoiodotyrosine	30.0 (34.6)	12.0 (13.8)	8.2 (9.4)	4.4 (5.0)
	Diiodotyrosine	43.5 (35.5)	1.7 (1.4)	0.3 (0.2)	0.1 (0.1)

Distribution was measured in the fragments of A and B chain at day 30, and the results are shown in parentheses. The distribution of [^{125}I]monoiodotyrosine and [^{125}I]diiodotyrosine was calculated back to time zero using Eqs. (1) and (2), respectively (see METHODS). The preparation is the same as that used in the experiment of Figure 1.

Three main peaks were obtained: iodide (K_{av} = 1.0, 9% of the total radioactivity, monoiodotyrosine K_{av} = 2.3, 51%, and diiodotyrosine K_{av} = 4.0, 39%), in addition to a small peak in the high-molecular-weight fraction (1%). The middle panel shows the distribution at day 139. It appears that monoiodotyrosine constitutes nearly the same fraction (48%) of the total radioactivity as at day 15. This suggests that [^{125}I]diiodotyrosine does not decay to [^{125}I]monoiodotyrosine. Diiodotyrosine now constituted about 10% of the radioactivity and iodide 34%. The radioactivity of the high-molecular-weight fraction had increased to 8%. The actual decrease in diiodotyrosine agrees well with the theoretical value for 139 days of 39% × 0.20 = 7.8%; cf. Eq. (3). The sum of the percentage of diiodotyrosine plus the increment in the percentage of iodide (10 + 25) agrees reasonably well with the 39% in diiodotyrosine at day 15. The data suggest that the radioactive product formed when the [^{125}I]diiodotyrosine residue in the diiodoinsulin molecule decays is largely [^{125}I]$^-$. This conclusion is further supported by the observation (Figure 2, panel 3) that [^{125}I]diiodotyrosine decays almost exclusively to iodide, whereas no [^{125}I]monoiodotyrosine is formed. Also, it was found in other experiments (not shown) that [^{125}I]monoiodotyrosine alone (K_{av} 1.9–2.4, Figure 2, panel 1) did not cause the formation of other radioactive compounds as judged by rechromatography on Sephadex G-25 after storage for 50 days. Having done this analysis it appears that the iodide peak at day 15 (top panel) is partially due to the decay of [^{125}I]diiodotyrosine. This accounts theoretically for 6%. The remaining 3% are probably due to a weak deiodase activity of the pronase preparation, since 2–4% iodide was repeatedly found after digestion of pure [^{125}I]monoiodoinsulin using the same method as that described for diiodoinsulins (data not shown). The data prove the validity of using Eqs. (1–3) for the back calculations carried out in the following experiments to obtain the distribution of mono- and diiodityrosine at time zero.

Table 1 shows the distribution of mono- and diiodotyrosine residues in mixed diiodoinsulins prepared according to the lactoperoxidase method. Of the radioactivity, 43% was in A14-diiodotyrosine-insulin and 2% was in other diiodotyrosine insulins. Of the monoiodotyrosine, 30% was in residue A14. Most likely, therefore, nearly all monoiodotyrosine diiodoinsulins contained one A14-monoiodotyrosine paired with either one A19, one B16, or one B26 iodine substituted residue. The presence of 30% A14-monoiodotyrosine but only 25% A19 plus B chain monoiodotyrosine remains unexplained.

The average binding affinity of the mixed diiodoinsulins from the lactoperoxidase preparation is shown in Table 2. The relative affinity was at least as high as that of A14-monoiodoinsulin in adipocytes. We have previously shown that the relative affinity of A19, B16, and B26 monoiodoinsulins are about 60%, 100%, and 200% in rat adipocytes.[3,4,13] If one assumes that the impact on the affinity of one monoiodotyrosine residue in the insulin molecule is not affected by the presence of another, then A14-[^{125}I]diiodotyrosine-insulin must possess the same or an even higher affinity than A14-[^{125}I]monoiodoinsulin. On the other hand, the affinity of the mixed preparation was reduced in hepatocytes and IM-9 lymphocytes, perhaps due to a reduced affinity of A14-[^{125}I]diiodotyrosine-insulin (Table 2). The relative biologic potency of this diiodoinsulin preparation in adipocytes was measured as 102 ± 9%, SD, N = 6. It was found in other experiments that freshly isolated [^{125}I]diiodoinsulin was 97–98% precipitable in 13% trichloroacetic acid and behaved as [^{125}I]monoiodoinsulin when precipitated with an excess of insulin antibody,[18] when applied to paper chromatography,[17] or passed through a cellulose column under conditions designed to purify iodination mixtures.[19]

The distribution of iodine in diiodoinsulins prepared according to the iodate method is shown in Table 3. The average binding affinity was markedly attenuated: 23 ± 2% in adipocytes, 27 ± 10% in hepatocytes, and 30 ± 11% in IM-

TABLE 2
Binding affinity of heterogeneous diiodoinsulins prepared using the lactoperoxidase method

	Adipocytes	Hepatocytes	IM-9 lymphocytes
Affinity relative to A14 – [^{125}I]monoiodo-insulin (%)	124 ± 16*	76 ± 5† 82 ± 8†	74 ± 3‡

* Mean ± SD of four independent experiments.
† Two experiments; mean of six replicates ± SD (N = 6). These experiments were carried out on the same days as experiments with adipocytes; the difference between the two cell types was highly significant ($P < 0.001$ in each case).
‡ Mean ± SD of three experiments.

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TABLE 3
Distribution of mono- and diiodotyrosine in diiodoinsulins prepared using the iodate method

	Position	A14	A19	B16 + B26
Distribution (percentage of total radioactivity)	Monoiodotyrosine	13.6	22.4	1.5
	Diiodotyrosine	3.9	58.5	0.1

Distribution was determined at day 14, and numbers represent the distribution calculated back to day zero.

9 lymphocytes (SD, N = 3). It is likely, therefore, that A19-[125I]-diiodotyrosine-insulin has an affinity of only a few percent of that of A14-monoiodoinsulin.

Finally, the distribution in diiodoinsulins prepared using the lactoperoxidase method in the presence of 6M urea is shown in Table 4. The average binding affinity was 150 ± 10% (SD, N = 4) in adipocytes. This is probably due to the high content of B26 monoiodotyrosine.[13] The affinity was not distinguishable from that of A14-monoiodoinsulin in hepatocytes and IM-9 lymphocytes (data not shown).

Properties of the decay products. The decrease in the content of [125I]diiodoinsulin in fraction of the total radioactivity follows Eq. (3) (cf. METHODS) assuming an isotopic abundance of 100%. However, the measured average binding affinity in adipocytes was found to decrease slightly less with time. Thus, by 100 days it had decreased by 58% as compared with an expected value of 68% according to Eq. (3). The same phenomenon has been noted with A14-[125I]-monoiodoinsulin.[4] The reason may be either that the isotopic abundance is less than 100% or that some 125I-monoiodo decay products retain some binding affinity.

The Sephadex profile of 125I-diiodoinsulins prepared by the lactoperoxidase method, at day 2 (top) and day 115 (bottom) is shown in Figure 3. The binding affinity (adipocytes) of the material eluting as diiodoinsulin at day 115 was within 10% of that of A14-monoiodoinsulin. The peak in the fraction corresponding to the total column volume was identified as iodide since it was extracted by chloroform after oxidation with H_2O_2. It constituted about 38% of the total activity. This agrees reasonably well with the assumption that the [125I]-diiodotyrosine-insulin (43% at time zero, Table 1) decays to iodide. The peak corresponding to the void volume representing polymers constitutes about 19% of the total radioactivity. It is likely, therefore, that some [125I]monoiodotyrosine-diiodoinsulin molecules (57% at time zero) form polymers upon decay. The "binding" of iodide was negligible in adipocytes, hepatocytes, and IM-9 lymphocytes. The polymers were precipitable in trichloroacetic acid (Figure 3), about 50% stayed at the origin when applied to wick chromatography,[17] and they were 80% precipitable in 82% ethanol[18] even in the absence of insulin antibodies (data not shown). They also largely (like insulin and diiodoinsulins) passed a cellulose column.[19] The specific binding (cf. METHODS) of the polymers was small, about 5% of that of A14-monoiodoinsulin in adipocytes and not different from zero in hepatocytes and IM-9 lymphocytes (data not shown). However, as shown in Table 5, the nonspecific binding, although variable, was much higher than that of A14-[125I]monoiodoinsulin, particularly in hepatocytes. In addition, this material had a marked tendency to bind to cellulose acetate filters (Table 5).

DISCUSSION

The present work shows that about one-third of the radioactivity in heterogeneous 125I-insulin prepared by the lactoperoxidase method with 0.3–0.4 mol I/mol insulin is in diiodoinsulins. In addition, such preparations will contain minor amounts of triiodoinsulins, which are separated from diiodoinsulins by polyacrylamide gel electrophoresis.[11] We have previously shown[4] that the content of diiodoinsulins is roughly the same when chloramin-T is used as the oxidizing agent instead of H_2O_2/lactoperoxidase. In addition, Schneider et al.[19] have found that the binding properties as well as the content of diiodotyrosine, and therefore probably of diiodoinsulin, was the same whether a large excess or stepwise stoichiometric addition of chloramin-T was employed. It is likely, therefore, that the content of diiodoinsulins in 125I-insulin preparations is in general primarily related to the degree of iodination (i.e., mol I/mol insulin).

Calculations based on the assumption that iodine is incorporated at random into the four reactive sites on the two A chain tyrosines, and only in these sites, show that 25% of the radioactivity will be in diiodoinsulins (about 29% in diiodoinsulins plus triiodoinsulins) when the degree of iodination is 0.4.[19] We now know that these assumptions are not correct. The incorporation of iodine occurs more rapidly in position A14 than in A19 (lactoperoxidase and chloramin-T methods), and incorporation in the B chain tyrosines is not negligible. The content of diiodoinsulin is, however, only slightly higher than that calculated previously using the simplifying assumptions.

Diiodotyrosine insulins account for roughly half of the diiodoinsulins prepared in aqueous buffer in the absence of urea (45% using lactoperoxidase, Table 1; 61% using io-

TABLE 4
Distribution of mono- and diiodotyrosine in diiodoinsulins prepared using the lactoperoxidase method in the presence of 6M urea

	Position	A14	A19	B16	B26
Distribution (percentage of total radioactivity)	Monoiodotyrosine	40.6	10.2	13.6	23.0
	Diiodotyrosine	6.0	6.1	0.4	0

Numbers represent the distribution calculated back to day zero. Note: The content of diiodotyrosine in the isolated B chain was determined as 8% in separate experiments. It is likely, therefore, that some deiodination occurred during the tryptic digestion of the B chain.

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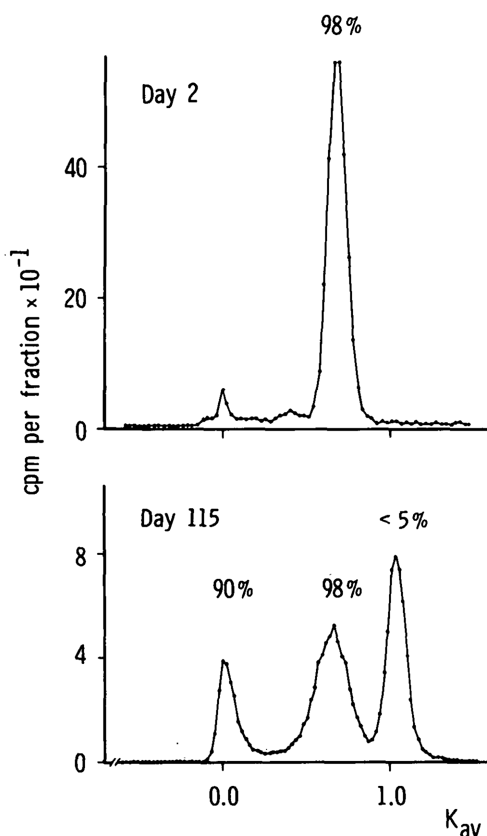

FIGURE 3. Distribution of diiodoinsulin, iodide, and polymers in heterogeneous diiodoinsulins prepared using the lactoperoxidase method. The samples were chromatographed on a Sephadex G-50, fine, column at 4°C using Hepes buffer with 1% albumin (pH 7.4) as the eluent. Top panel: 2 days after preparation of the diiodoinsulin. Bottom panel: 115 days. The numbers above the peaks indicate precipitability in 13% trichloroacetic acid. Radioactivity in fractions beyond K_{av} 1.5 was negligible.

date as the oxidizing agent, Table 3). This fraction decreases markedly when urea is added to the buffer, probably because the reactivity of the four tyrosine residues become more equal under these conditions.

Schneider et al.[19] studied the binding of fractions from starch gel electrophoresis presumed to contain largely diiodoinsulins, and the results were variable. In one preparation specific binding was decreased by about 40%, whereas the binding of the presumed mono- and diiodoinsulins (zones 1 and 2) was indistinguishable in another ^{125}I-insulin preparation. This variability is probably due to varying distributions of the iodine in the tyrosines of both the monoiodoinsulins and the diiodoinsulins. In addition, Schneider et al.[19] found up to a 10-fold variation in the nonspecific binding, a phenomenon that seems best explained by the presence of varying concentrations of polymers (see below).

It appears from the present work that A14-diiodotyrosine-insulin has at least the same binding affinity and biologic activity as A14-monoiodoinsulin in adipocytes (Table 2), whereas A19-diiodotyrosine-insulin has a very low affinity. Interestingly, the affinities of mixed diiodoinsulins relative to that of A14-monoiodoinsulin were different in different cell types (Table 2). This is probably a reflection of the fact that the relative affinities of A19- and particularly B26-monoiodoinsulins vary among cell types.[13]

Due to the high affinity of the heterogeneous diiodoinsulins from a lactoperoxidase iodination, their presence is not a severe problem at time zero. However, radioactive decay products are formed as a function of time—Eq. (4)—and they consist of iodide and polymers approximately in the ratio 2:1 (Figure 3). The iodide seems to be formed mainly from the decay of diiodotyrosine-insulin (Figure 2). Iodide does not contribute significantly to "binding" in the systems tested, and it will therefore cause a decrease in the apparent bound/free ratio. The polymers are precipitable in trichloroacetic acid, and their presence in a ^{125}I-insulin preparation may therefore not be recognized. This is likely to cause problems since their nonspecific binding both to some cells (hepatocytes) and to some inert materials (cellulose acetate membranes) is 10–20 times higher than that of A14-monoiodoinsulin.

TABLE 5
Nonspecific binding of ^{125}I-polymers

	Adipocytes	Hepatocytes	IM-9 lymphocytes	Millipore filters
Binding	5.1 ± 0.2 N = 4	16 ± 3.9 N = 12	3.8 ± 0.4 N = 3	17 ± 4.2 N = 4

Binding was measured using 2 × 10^3 cpm/ml polymers plus 1 μM insulin. The cells were incubated for 45 min at 37°C, transferred to 500 μl Beckman microfuge tubes containing 75 μl silicone oil (D = 0.99 for adipocytes, 1.04 for hepatocytes and IM-9 lymphocytes) and centrifuged. The tubes were cut through the oil phase and the percentage of bound radioactivity determined. The nonspecific binding of A-14-[^{125}I]-monoiodoinsulin was measured in each experiment; numbers represent % bound polymer/% bound A14-monoiodoinsulin. Binding to cellulose acetate filters was determined by adding 20 μl buffered isotope with suction applied followed by a wash with 1 ml Hepes buffer containing 1% albumin, pH 7.4.

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