Evidence for Separate Handling In Vivo of Different Regions of the Insulin Molecule Using A14- and B1-labeled Insulin Tracers

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SUMMARY

To compare the metabolic characteristics and degradation of insulin tracers labeled unselectively, selectively at the A14 position (A14-monoiodoinsulin), and selectively at the B1 position (B1-monoiodoinsulin), we have followed the time course of disappearance of intact (immunoprecipitable [IP] and trichloroacetic acid [TCA] precipitable) iodoinsulin after bolus injection into greyhounds. We have used noncompartmental analysis to determine metabolic clearance rate (MCR) and apparent distribution space (DS). We have also measured the appearance of non-IP- and non-TCA-precipitable fragments, and have developed a mathematical model using compartmental analysis to explain the observed differences.

B1-Monoiodoinsulin has a significantly higher MCR (16.3 ml/min/kg) than both A14-monoiodoinsulin (10.6 ml/min/kg) and unfractionated tracers (7.6 ml/min/kg) as determined by immunoprecipitation, and reaches the values observed for native insulin in greyhounds. MCR values obtained by TCA precipitation are approximately one-half of those obtained by IP for all 3 tracers. The concentration of non-IP fragments is significantly lower with B1-monoiodoinsulin than with the other tracers. Compartmental analysis suggests this to be due to greater intracellular retention of the B1 moiety during the experimental period.

We conclude that: (1) by the criterion of MCR, B1-monoiodoinsulin seems to behave more like native insulin than other preparations tested; (2) the reduced MCR of A14-monoiodoinsulin raises doubts about its validity as a tracer for insulin; (3) a high-molecular-weight product of insulin degradation, which includes both the B1 and the A14–A19 regions of the molecule, is released into the circulation; and (4) smaller fragments containing A14–A19 reappear in the circulation more rapidly than fragments containing B1. Thus, the use of specifically labeled insulin tracers allows investigation of the mechanisms of intracellular processing in vivo. **DIABETES *33*:721–727, August 1984.**

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Elucidation of the mechanisms of the actions and metabolism of insulin is heavily dependent on the use of radioactive insulin derivatives as tracers. It is a prerequisite for accurate interpretation of such work that the tracer used should have identical biologic properties to the native hormone. Direct iodination of insulin produces a heterogeneous mixture of iodinated insulins labeled largely at positions 14 and 19 of the A-chain (A14-tyrosine and A19-tyrosine) and it is now well established that these mixed preparations show reduced metabolism in vivo[1–3] and reduced biologic activity in vitro, particularly if heavily iodinated.[4–8]

More recently, advances in fractionation techniques have allowed the preparation, first, of monoiodoinsulin[9,10] and more recently of A14- and A19-monoiodoinsulins.[11–14] Monoiodoinsulin preparations have been found by some authors[9–11] to have biologic properties indistinguishable from native insulin, while others have found slightly reduced biologic potency.[15–17] These differences may perhaps be explained by differences in the proportions of A14- and A19-monoiodoinsulin in the mixture: the A19-tyrosine is involved in the putative active site of insulin and A19-^{125}I-monoiodoinsulin has been shown to have reduced potency both in vivo[3] and in vitro.[12,18] A14-^{125}I-monoiodoinsulin, however, represents a real advance in the development of insulin tracers and is reported to show equipotency with native insulin in isolated adipocytes.[12,18] However, even with this material, some doubts as to its biologic integrity remain. Preliminary reports of in vivo studies have suggested it to have reduced metabolic clearance rates,[3] while preliminary in vitro studies with A14-^{127}I-monoiodoinsulin have shown a higher potency than insulin in isolated rat adipocytes.[19]

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These problems have stimulated efforts to produce alternative insulin tracers that do not depend on iodination of the structural tyrosines of the insulin molecule. Insulin with certain modifications at the B1 position has been shown to retain normal biologic and metabolic properties, and manipulation of the B1 position therefore offers serious potential as a source of alternative tracers. Halban and Offord have prepared a tritiated PheB1 preparation that retains full biologic activity but with relatively low specific activity.[20] 3,5-Diiodo-^{127}I-tyrosine B1-insulin has been reported to fulfill the criteria of a valid insulin, since it combines intact biologic activity and kinetics with a potentially high specific activity.[21] This has led to the development of insulin tracers with monoiododesaminotyrosine in position B1 (B1-monoiodoinsulin).[22] This material compares favorably with native insulin when its metabolic parameters are estimated in vivo.[23] Preparation by semisynthesis of (^{125}I-iodotyrosyl^{B1}) insulin has also been recently reported along with studies of its receptor binding and cell-mediated degradation in isolated hepatocytes.[24] We now, therefore, have two potentially valid tracers (A14-monoiodoinsulin and B1-monoiodoinsulin) labeled at different sites of the insulin molecule. We have set out to compare their clearance and distribution characteristics in vivo and have taken advantage of the different sites of labeling, such that, by examining the different rates of appearance of degraded molecular fragments using compartmental analysis after bolus injection, we can begin to elucidate the mechanism of insulin processing in vivo.

MATERIALS AND METHODS

Tracers. B1-^{125}I-monoiododesaminotyrosine insulin was prepared, purified by high-performance liquid chromatography, and kindly donated by Drs. Bahrami and Brandenburg (Aachen, FRG). The method of preparation involved the semisynthetic exchange of B1-phenylalanine for ^{125}I-monoiododesaminotyrosine (Bolton-Hunter Reagent, Amersham-Buchler; specific activity 1400 Ci/mmol). ^{127}I-monoiododesaminotyrosine was also synthesized and used to prepare B1-^{127}I-monoiododesaminotyrosine insulin. This material was characterized by crystallization, cellulose-acetate electrophoresis, oxidative sulphitolysis, enzymatic degradation, UV absorption, qualitative and quantitative end-group determination, amino acid analysis, and iodine assay and was shown to be equipotent with native insulin in the isolated fat cell assay. The identity of the ^{125}I material was established by comparison of the results of radioactive analysis with the chemical analysis of the ^{127}I material in a mixture of the two, and two separate preparations were subsequently used in these experiments.[22]

Specific activity estimations were performed by double-antibody radioimmunoassay using ^{131}I insulin as the radioligand and an insulin antiserum that has been shown in our laboratory to retain full affinity to B1-modified analogues.[21] The structural integrity and homogeneity of the material was checked before use by immunoprecipitation, trichloroacetic acid (TCA) precipitation, and Sephadex G50 chromatography.

The unfractionated tracer preparations were prepared using either chloramine-T or solid-phase lactoperoxidase (Enzymobead Lactoperoxidase Glucose Oxidase) and Sephadex G50 column chromatography. Specific activity, immunoprecipitability, and TCA precipitability were then estimated as above.

A14-^{125}I-monoiodoinsulins were kindly supplied by the Novo Research Institute, Nordisk Insulin Laboratorium, and Eli Lilly and Company. Preparation followed published methods[13,14] and purification was by either chromatography or polyacrylamide gel electrophoresis. In each case, greater than 96% of ^{125}I was shown to be in the A14 position. The long-term stability of A14-monoiodoinsulin has been shown to be good.[14] All experiments with these preparations were performed within 3 wk of receipt.

Experimental design. In vivo experiments were performed using intact, conscious, overnight-fasted greyhounds. A bolus injection of approximately 10 μCi of tracer material was administered by rapid intravenous injection, and the volume given checked by weighing of the syringe. Blood samples were collected at 1-min intervals for 10 min and subsequently at 5-min intervals until 1 h had elapsed. Serum iodoinsulin concentration was estimated by TCA precipitation and total immunoprecipitation, and plasma glucose concentration by a glucose-oxidase method using a YSI Model 23AM Glucose Analyzer (Yellow Springs Instrument Co.).

TCA precipitation was measured in duplicate. Serum (250 μl) was added to 1.25 ml of 15% TCA and the mixture was then centrifuged at 2400 rpm. The supernatant was separated from the precipitated pellet by decantation. ^{125}I counts in each fraction were then counted on a gamma counter (Wallac LKB 1280 Ultrogamma). The TCA precipitability of the tracer material used for the bolus was also assessed using 10 μl of this material added to 240 μl of dog serum, and then the procedure outlined above was followed. The total TCA-precipitable radioactivity administered was then calculated and the radioactivity of the serum sample was expressed as percent dose per liter (% dose/L) for both precipitated and soluble fractions.

Immunoprecipitation was also measured in duplicate. Serum (200 μl) was added to 500 μl of CPM buffer (0.05 M phosphate plus 0.1% human serum albumin, pH.7.4) and 100 μl of anti-insulin antiserum at a 1:3000 dilution in CPM buffer. After a first incubation period of 24 h at 22°C, 100 μl of 1:50 rabbit anti-guinea pig serum and 1:1600 normal guinea pig serum were added and the mixture was then incubated for a further 48 h at 4°C. The antibody-bound radioactivity was then separated by centrifugation and decantation. The immunoprecipitability of the initial bolus mixture was also measured in the same assay using 10 μl of mixture at 3 dilutions, 690 μl of CPM, and antisera as above. Again, sample radioactivity was expressed as percentage immunoprecipitable dose per liter for both antibody-precipitable and nonantibody-precipitable fractions.

MATHEMATICAL ANALYSIS AND MODEL DEVELOPMENT

Two methodologic approaches were adopted. For estimation of metabolic clearance rate (MCR) and apparent distribution space (DS), a standard noncompartmental analysis was undertaken. For explanation of the time course of appearance of molecular fragments, compartmental analysis was required.

Noncompartmental analysis. Bolus decay curves for insulin and insulin tracers are not adequately described by a single exponential function. However, the sum of two ex-

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ponentials was found to describe our data adequately, and no statistically significant improvement to the fit was achieved by the addition of a third exponential, as assessed by residual error using the sum of least squares (F test) and also by checking the distribution of the error.[25]

MCR and DS estimations were therefore derived from the fitting of a double exponential equation to each individual set of data (equations 1–3):

$$y = Ae^{-\alpha t} + Be^{-\beta t} \quad (1),$$

$$MCR = \frac{\alpha \cdot \beta \cdot D^*}{A\beta + B} \quad (2),$$

and

$$DS = \frac{D^*}{A + B} \quad (3),$$

in which y = derived insulin concentration (% D^*/L), A,α and B,β = the coefficient and exponent of the fitted exponents, t = time (min), MCR = metabolic clearance rate (ml/min), DS = distribution space (ml), and D^* = total dose administered.

Compartmental analysis. The decay curves of intact iodoinsulin were further analyzed by compartmental analysis using a two-compartment model (Figure 1). The first compartment includes the accessible (i.e., plasma) pool and the second comprises sites other than plasma where iodoinsulin is inaccessible to sampling and will include insulin bound to receptor sites. The appearance of radiolabeled molecular fragments in the circulation could be adequately described by the use of a separate single compartment model for "fragment" distribution and removal (f in Figure 1). The input of fragments to this model could theoretically come either from the first or the second compartment of the insulin model. Both of these possibilities were tested and it was found that the data for fragments with each tracer method could only be explained on the basis of input from compartment two. Since compartment two includes receptors, this supports the overwhelming body of evidence that receptor-bound insulin is the major substrate for insulin degradation.[26-29]

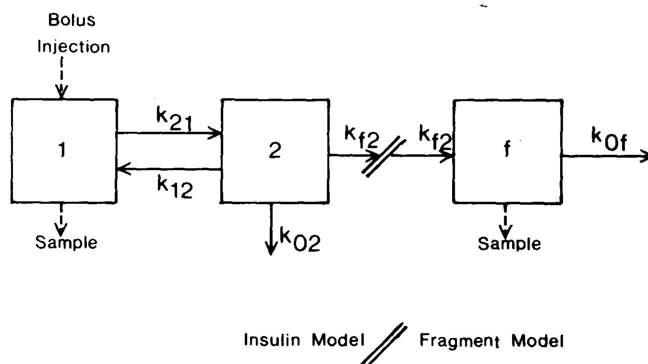

FIGURE 1. Compartmental model used for analysis of insulin tracer boluses. Compartments 1 and 2 were used to analyze the insulin disappearance curves. Compartment f was added to describe the data for insulin fragments. Arrows represent transition pathways governed by the rate constants k_{ij}. The differential equations describing the system are as follows:

$$\dot{x}_1 = k_{12}x_2 - k_{21}x_1,$$

$$\dot{x}_2 = k_{21}x_1 - k_{12}x_2 - k_{02}x_2 - k_{f2}x_2,$$

$$\dot{x}_3 = k_{f2}x_2 - k_{0f}x_f,$$

$$y_1 = \frac{x_1}{V_1},$$

$$y_f = \frac{x_f}{V_f}, \text{ and}$$

$$x_1(0) = D^*,$$

in which x_1 and x_2 are the mass of tracer in compartments 1 and 2 (cpm), x_f is the mass of labeled fragments in compartment f (cpm), k_{ij} are the fractional transfer rate constants to compartment i from j (min^{-1}), y_1 and y_f are the simulated concentrations of intact insulin and fragments respectively (cpm/ml), and V_1 and V_f are the volumes of compartments 1 and f, respectively (ml).

Some previous models of insulin metabolism, such as those of Sherwin et al.[30] and McGuire et al.[31] have proposed a three-compartment model with parallel losses from the sampling compartment into rapidly and slowly equilibrating pools. These are linear models developed with the use of native insulin in man rather than with insulin tracers. Our model, which is also linear but based on tracer methodology, differs from these earlier models in several respects. First, there is no improvement to the fit of our data by the addition of an intermediate or parallel third compartment. Second, our data on insulin "fragments" can only be explained on the basis of input from compartment two, whereas in Sherwin's model, insulin loss and degradation is via the plasma compartment. Thus, although a variant of these earlier models can be made to fit our data for insulin alone, the data for fragments require the different approach described above.

The fragments data available do not justify the use of more than one compartment, since direct measurement of the input is not possible and a more complex model would require further unjustifiable assumptions. However, since the use of a single compartment adequately described the data available, the final model was formulated as in Figure 1.

It was initially assumed that the molecular fragments occupied the same apparent volume of distribution as insulin. This allowed an initial estimate of rate constants k_{0f} and k_{f2}. With this configuration, it was found that k_{f2} was in fact a fraction of the total loss from compartment two, i.e., that not all of the insulin lost from the system could be accounted for by the appearance of fragments. The mathematical solution to this problem can be expressed in modeling terms in two ways: (1) either a fraction of the insulin lost from the system can be assumed to be sequestered in a compartment from which there is no return of fragments into compartment f during the time course of the experiment. This is shown in Figure 1 by the presence of the rate constant k_{02}; or (2) the entire loss of insulin from the model can be accommodated by increasing the volume of fragment distribution (Vf), in which case k_{02} can be ascribed the value zero. The results of both interpretations will be shown and their relative merits discussed. Parameter estimation was performed using a minimization algorithm[32] with the minimized function being the sum of least-square error.

RESULTS

Table 1 shows the initial characteristics of the tracer preparations and the results of noncompartmental analysis.

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TABLE 1
Chemical and biologic characteristics of the tracer preparations, and results of noncompartmental analysis

Tracer preparation	Percentage TCA precipitability	Percentage immuno-precipitability	Specific activity (μci/g)	MCR by TCA (ml/min/kg)	MCR by immuno-precipitability (ml/min/kg)	DS by TCA (ml/kg)	DS by immuno-precipitability (ml/kg)
B1-MII*	99	96.3–96.5	26–60	9.3 ± 1.55† (4)	16.34 ± 0.44 (3)	79.21 ± 10.1 (4)	61.0 ± 5.1 (3)
A14-MII	92–99	93–95	200–324	5.72 ± 1.63 (6)	10.6 ± 0.56 (6)	46.1 ± 4.5 (6)	59.8 ± 5.0 (6)
Unfractionated	90–98	75–94	50–220	4.32 ± 0.33 (4)	7.56 ± 0.28 (4)	62.8 ± 4.9 (4)	62.3 ± 4.3 (4)

*MII, monoiodoinsulin. †Mean ± SEM.
For methodologic details and statistical analysis see text.
Figures in parentheses indicate number of experiments.

Plasma glucose values are not shown, but were unchanged after bolus injection of tracer in every case. Figure 2 shows representative decay curves obtained by total immunoprecipitation for each of the three types of tracer, while Figure 3 shows the appearance curves for molecular "fragments" for the same three experiments.

MCR and DS. The MCR values by immunoprecipitation show significant differences among the three types of tracer. The values for B1-monoiodoinsulin are significantly higher ($P < 0.001$) than the values for both A14-monoiodoinsulin and unfractionated tracers, and at 16.3 ml/min/kg reach the values seen with native insulin in our laboratory after bolus injection or infusion in both greyhounds and man[1,3,21,33] (see DISCUSSION). A14-monoiodoinsulin does, nevertheless, show significantly higher values ($P < 0.001$) than unfractionated tracer preparations, although not reaching our observed range for native insulin.

Similar differences in MCR values among the three groups, but at a significantly lower level, are seen when TCA precipitation is used, the values for each tracer preparation being approximately one-half those seen with immunoprecipitation.

By contrast, the apparent distribution space estimations by immunoprecipitation are similar for all three materials and do not differ from the values observed with native insulin. The distribution space value for B1-monoiodoinsulin when measured by TCA is significantly greater than that for A14-monoiodoinsulin ($P < 0.01$), but the true significance of this finding is doubtful since it is not seen with immunoprecipitation.

Handling of fragments. From Figure 3, it can be seen that there is an initial fall as injected non-IP material is distributed and cleared from the circulation. After 4–5 min, there is a rise as metabolism of intact insulin proceeds. However, it can be seen that the concentration reached for B1-monoiodoinsulin is appreciably lower than for the other two materials and falls more rapidly by the end of the experiment. This difference could be explained either by a slower rate of appearance of fragments, a faster rate of disappearance, a greater volume of distribution for fragments derived from the B1 label, or a combination of one or more of these possibilities. The data were therefore analyzed using the model described above to further elucidate the reasons for this difference. The results derived from the model are shown in

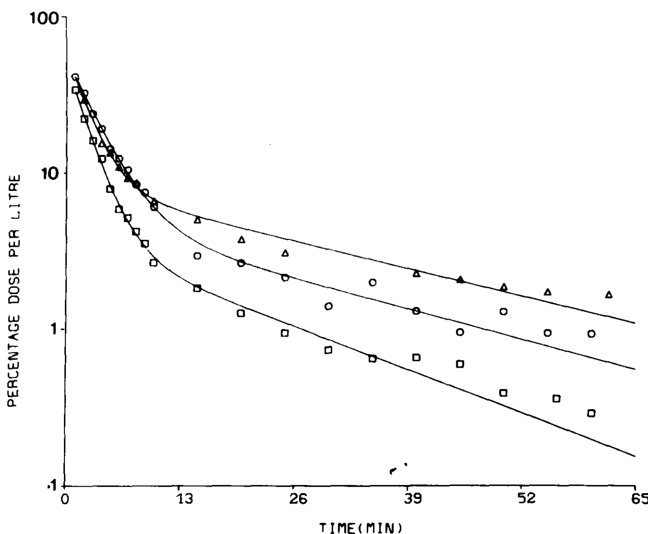

FIGURE 2. Plasma disappearance curves for immunoprecipitable insulin tracer in three individual experiments. The y-axis is tracer concentration normalized to percentage of dose per liter plotted on a logarithmic scale. The x-axis is time in minutes from injection of bolus. △, unfractionated tracer; ○, A14-monoiodoinsulin; and □, B1-monoiodoinsulin. The lines represent the computer-simulated best fit to the data.

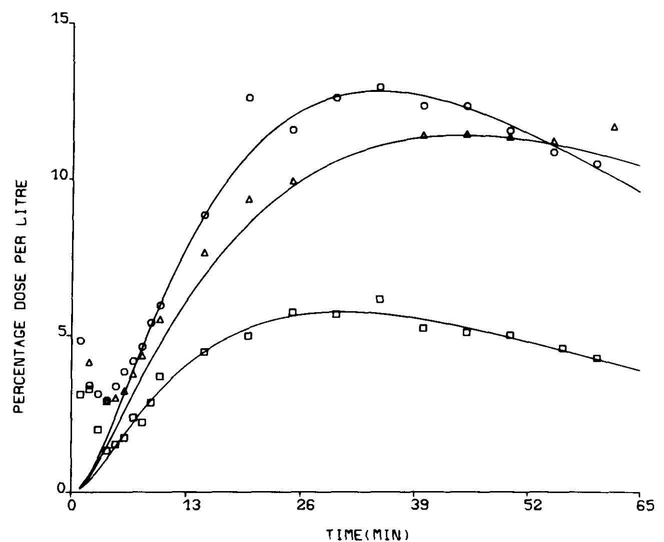

FIGURE 3. The plasma concentration of nonimmunoprecipitable fragments in three individual experiments. The y-axis is fragment concentration normalized to percentage of dose per liter and plotted on a linear scale. The x-axis is time in minutes from injection of bolus. Lines are computer-simulated best fit to data. Symbols used are as in Figure 2.

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TABLE 2
Compartmental analysis: calculated parameter values obtained with V_f fixed (immunoprecipitation data)

Tracer preparation	k_{21} (min^{-1} × 10^2)	k_{12} (min^{-1} × 10^2)	k_{f2} (min^{-1} × 10^2)	k_{02} (min^{-1} × 10^2)	k_{0f} (min^{-1} × 10^2)	Percentage entering V_f
B1-MII	45.1 ± 4.27	4.2 ± 1.82	1.2 ± 0.09	5.0 ± 1.4	2.1 ± 0.75	20.55 ± 4.93
A14-MII	40.2 ± 11.14	5.4 ± 3.42	2.7 ± 0.35§	1.72 ± 1.2§	3.0 ± 0.77	63.76 ± 14.95†
Unfractionated	34.1 ± 2.75‡	5.4 ± 0.2§	1.9 ± 0.35*	1.8 ± 0.31§	1.9 ± 0.53	51.37 ± 9.12†

Results obtained by compartmental analysis using the mathematical approach, whereby V_f is fixed and equal to that of insulin (approximate plasma space).
Values shown are mean ± 1SD.
Symbols represent significant differences from B1-monoiodoinsulin (unpaired *t* test): *P < 0.05, †P < 0.01, ‡P < 0.005, and §P < 0.001.

Tables 2 and 3 using both methodologic approaches described above. Table 2 shows the parameter estimations obtained from the model with V_f fixed and k_{02} present and Table 3 shows the V_f estimation obtained when k_{02} is absent and V_f varying. It should again be stressed that both approaches produce identical fits to the data. Choosing between them would require access to unobtainable information concerning the rate of input of fragments to the fragments model.

From Table 2, it can be seen that significant differences in the parameters are identifiable at several levels. Values for k_{21} and k_{12} are significantly higher and lower, respectively, for B1-monoiodoinsulin than for unfractionated material, but the values for A14-monoiodoinsulin that lie between the values for the two other tracers do not differ significantly from either of them. The value for k_{f2} is lower for B1-monoiodoinsulin and is matched by a much higher value for k_{02}, indicating that the rate of return of fragments from B1 tracer to the circulation is much lower and that a greater proportion is sequestrated in an inaccessible (presumably intracellular) pool during the experiment. From these rate constants, one can calculate a figure for the percentage of the initial bolus that enters the accessible pool of the fragments model and it can be seen to be much lower for B1-monoiodoinsulin than for the other materials during the experiment. During the time course of the experiment, 80% of the fragments generated with B1 tracer are sequestered in an inaccessible site compared with only 36% with A14-monoiodoinsulin. The fact that k_{0f} is similar for all three materials indicates that the differences in observed serum concentrations of the fragments are not due to different rates of fragment clearance.

The alternative approach, in which 100% of the fragments are assumed to enter the accessible pool, requires V_f to be varied to allow k_{02} to be zero. The required value for V_f is shown in Table 3, and, at 305.6 ml/kg for B1-monoiodoinsulin, greatly exceeds the value for the other two materials. To accommodate distribution exceeding 300 ml/kg, one again has to assume a large intracellular component. It can be seen therefore that both approaches indicate greater intracellular retention of material derived from B1-monoiodoinsulin than material derived from the other two tracers. This implies either differences in the handling of these materials after receptor binding or differences in the biologic characteristics of the molecular fragments released.

DISCUSSION

These experiments show that B1-monoiodoinsulin has a significantly higher metabolic clearance rate than A14-monoiodoinsulin and unfractionated tracer preparations. As stated earlier, the observed MCR values with B1-monoiodoinsulin, when measured by immunoprecipitability after injection of tracer amounts in fasted greyhounds, reach the observed range for native insulin when measured by radioimmunoassay at low-steady-state plasma concentrations in our laboratory in both greyhounds[3,21] and man.[2,33] Possible differences in antibody-binding properties between radioimmunoassay and total immunoprecipitation inevitably hamper direct comparison, but the results suggest that B1-monoiodoinsulin is more "insulin-like" than alternative iodoinsulin preparations and this conclusion is supported by the previously published data on B1-3,5-^{127}I-diiodotyrosine insulin,[21] in which radioimmunoassay measurement of steady-state plasma concentrations of the analogue give results indistinguishable from those of native insulin.

By contrast, the MCR values for A14-monoiodoinsulin, although significantly higher than those of the unfractionated tracer preparations, fall below the values with cold insulin (15–30 ml/min/kg) as observed and below the values for B1-monoiodoinsulin previously reported,[3,21] suggesting some detectable difference compared with the native hormone in vivo. These findings raise a note of caution, since they conflict with the published in vitro data that suggest A14-monoiodoinsulin to be fully active in stimulating lipogenesis by isolated adipocytes.[12,18] The reason for this discrepancy remains unclear, particularly since with a large series of insulin analogues studied in our laboratory in recent years a strong correlation has been found between MCR estimations and in vitro lipogenesis and binding potencies; this correlation provides strong evidence that, at physiologic concentrations, the majority of insulin clearance is mediated directly via the insulin receptor.[34]

Alternative explanations for the higher MCR of B1-monoiodoinsulin, such as removal of the B1 moiety by amino-

TABLE 3
Compartmental analysis: values for V_f assuming 100% transfer of degraded insulin to the fragments model (immunoprecipitation data)

	B1-MII	A14-MII	Unfractionated
V_f (ml/kg)	305.6 ± 32.4 (3)	85.6 ± 7.1* (4)	108.9 ± 11.2* (4)

Values shown are mean ± 1SD.
Number of estimations in parentheses. For A14–MII, N = 4 for compartmental analysis and modeling of fragments as in two earlier experiments; no data available for nonimmunoprecipitable radioactivity.
Asterisks represent significant differences from B1-monoiodoinsulin, unpaired *t* test: *P < 0.001.

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peptidases before receptor binding, are not tenable in view of the finding that low-molecular-weight fragments containing the B1 moiety appear more slowly in the sampling compartment than fragments containing the A14–A19 region.

It is of interest to note that the position of the ^{125}I atom within the molecule does not influence the fact that MCR estimation by TCA precipitation gives values of approximately one-half those obtained by immunoprecipitation. It has been known since 1958[35] that TCA precipitation gives lower values than other methods when used for assessing insulin degradation in vivo, the implication being that a proportion of the radioactivity that is not immunoprecipitable remains TCA precipitable. This suggests the presence of molecular fragments that have been sufficiently degraded to lose immunoreactivity with specific insulin antiserum but that are of sufficient molecular size to retain TCA precipitability. The fact that we have found all three tracers to display this phenomenon to a similar degree implies that a similar proportion of circulating large-molecular-weight fragments contain the B1 moiety as includes the region of the A-chain encompassing the A14 and A19 moieties. Loss of immunoreactivity in these fragments may well result, therefore, from cleavage of a part of the molecule remote from both of these sites.

An elegant and detailed analysis of these differences using unfractionated tracers and mathematical modeling has been reported by Berman et al.,[36] and they conclude that the TCA-precipitable material includes partially degraded insulin that can no longer bind to receptors. Since it is known that the B1 region of the molecule is not essential for the expression of full biologic activity,[21] our findings remain compatible with those of Berman et al.

In addition to direct advantages in terms of biologic activity, insulin tracers labeled selectively at the B1 position provide an opportunity to study the fate of the N-terminus of the B-chain compared with conventional tracer preparations in which the radioactive iodine atom is incorporated within the A-chain. The results of the studies into the appearance of nonimmunoprecipitable material or "fragments" are therefore of interest. The lower concentration of labeled fragments after bolus injection of B1-monoiodoinsulin implies that the B1 region of the insulin molecule is handled differently from the region of the A-chain in which the A14 and A19 tyrosine residues occur. Modeling of these data, by both mathematical approaches used, suggests that the lower observed concentration of B1-containing nonimmunoprecipitable fragments results from greater intracellular retention during the time course of the experiment. This may reflect differences in cell-associated handling of the whole molecule due to the presence, in different positions, of the radioactive iodine. Alternatively, and of greater interest, it may indicate that fragments derived from different parts of the molecule have different metabolic fates within the cell.

The results of these studies when taken together therefore indicate that, during the experiment, a large-molecular-weight fragment is generated that contains both the B1 region and the A14–A19 region of the A-chain (TCA precipitable but not immunoprecipitable). Smaller (nonimmunoprecipitable, non-TCA-precipitable) fragments containing the A14 and A19 tyrosine are released into the circulation to a greater extent than fragments containing the B1 region alone. That the A-chain fragments seen contain both the A14 and the A19 tyrosine is suggested by the similar concentrations seen after injection of both A14-monoiodoinsulin and unfractionated tracer preparations.

Several recent reports of in vitro data lend support to the above findings. Duckworth et al.[28] have identified the production of a B-chain-cleaved intermediate in hepatocytes that contains the B1 moiety and is not demonstrable with A-chain labels. Misbin et al.[37] have reported that at least 10 amino acids are cleaved from the C-terminus of the B-chain after 2-h incubation of hepatocytes at 37°C. Assoian and Tager[24] have presented evidence for the generation of a fragment extending from the N-terminus of the B-chain at B1 to a residue between B7 and B13. All of these reports suggest that cleavage of the B-chain is an important event in the metabolism of insulin after receptor binding.

A more recent report by Assoian and Tager[38] formulates a more detailed model of insulin degradation after receptor binding. Their model postulates the formation of intermediates containing both the A-chain tyrosines and the B1 region, of sufficient molecular size to retain TCA precipitability. Smaller peptide fragments are generated containing the B1 residue attached to a small portion of the A-chain, but not including the A-chain tyrosines. A fragment appearing *earlier* contains the A14 and A19 tyrosines, but does not include the B1 region. This model therefore accords closely with our results both in terms of the absolute findings and in terms of the timing of events.

The use of differential tracer labeling is providing clarification of the complex processes of insulin degradation. The increasing number of available tracer preparations, for example B16- and B26-iodotyrosine insulins,[39] should further facilitate this work. Recent advances in recombinant DNA technology raise the further possibility of the manufacture of a wide variety of "custom-built" insulin tracers of high specific activity via the incorporation of tyrosine residues at defined sites within the molecule.

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