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Biochemistry

Human insulin prepared by recombinant DNA techniques and native human insulin interact identically with insulin receptors

(genetic engineering/biotechnology/monoiodinated insulin)

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ABSTRACT **Human insulin synthesized from A and B chains separately produced in *Escherichia coli* from cloned synthetic genes (prepared by the Eli Lilly Research Laboratories, Indianapolis, IN) was characterized by examining its interaction with human cultured lymphocytes, human circulating erythrocytes *in vitro*, and isolated rat fat cells. The binding behavior of the biosynthetic insulin with human cells was indistinguishable from that of native human or porcine insulins, with respect to affinity, association and dissociation kinetics, negative cooperativity, and the down-regulation of lymphocyte receptors. Similarly, the biosynthetic insulin was as potent as the native insulins in stimulating lipogenesis in isolated rat fat cells. We also examined the receptor binding characteristics of ^{125}I-labeled human and porcine insulins monoiodinated solely at Tyr-A14, which were obtained by means of high-performance liquid chromatography of the iodination reaction mixture (this material was prepared by B. Frank, Eli Lilly Research Laboratories). In all aspects studied, the pure [*Tyr*^{A14}-^{125}I]iodoinsulins were superior as tracers to the monoiodoinsulin purified by the more conventional method of gel filtration.**

The possibility of a future shortage of insulin for the treatment of diabetics has recently caused considerable disquiet (1). According to the National Diabetes Advisory Board of the U.S. Department of Health, Education and Welfare, insulin supplies in the United States will be adequate only for the next 20 years (2). The need to discover alternative sources of insulin production is obvious. Moreover, because bovine and porcine insulins are immunogenic in a number of diabetic patients (3), the availability of human insulin might offer considerable advantage.

Recent progress in DNA chemical synthesis and in recombinant DNA technology has allowed the cloning and production of the A and B chains of human insulin in *Escherichia coli* from chemically synthesized genes (4, 5). After purification of the A and B chains by high-performance liquid chromatography (HPLC), human insulin has been synthesized by the formation of the correct disulfide bonds (6–8, *) and shown to be radioimmunologically reactive (5).

In the present work, we have studied the binding of this "biosynthetic" human (H^{BIO}) insulin to human insulin receptors and compared its properties to those of native human (H^{NAT}) and porcine (P) insulins. The characteristics of all three insulins were the same with respect to all binding properties studied, as well as to the ability to induce down-regulation of receptors in lymphocytes and to elicit a biological response *in vitro* in isolated rat fat cells.

The iodination of insulin can result in the labeling of Tyr-A14, Tyr-A19, or various B-chain residues (9). Iodination by the stoichiometric chloramine-T (10, 11) or lactoperoxidase (12) methods results in iodination primarily but not exclusively at Tyr-A14 (9). Studies of homogeneous insulins labeled with ^{125}I at Tyr-A14 or Tyr-A19 (A14-^{125}I-insulin and A19-^{125}I-insulin, respectively) have shown the A19 derivative to be only half as active as the A14 derivative (9). We used this study as an opportunity to compare the binding properties of insulin monoiodinated by the stoichiometric method and purified by gel filtration, as done in most laboratories (including ours), with those of a carrier-free A14-^{125}I-insulin obtained by one-step purification with HPLC (13). It is clear that the HPLC method of purification, like the polyacrylamide gel electrophoresis method (9), yields a tracer of much higher quality for receptor studies.

MATERIALS AND METHODS

Insulins. P insulin (lot 121042) was a gift from Novo (Copenhagen, Denmark). H^{BIO} insulin (4, 5, *) was prepared by the Eli Lilly Research Laboratories (Indianapolis, IN) (14). The H^{BIO} and H^{NAT} insulins were gifts from Ronald Chance through the courtesy of Herman Debruyne (Eli Lilly, Benelux). Homogeneous P and H^{BIO}A14-^{125}I-insulins were prepared by lactoperoxidase iodination, purified by means of HPLC using a RP-1/C18 reverse-phase system (13), and kindly provided by Bruce Frank (Eli Lilly Research Laboratories) (exact methods to be published elsewhere). We iodinated P insulin with ^{125}I ($Na^{125}I$, IMS-300, Amersham) as described (11) to a specific activity of 180.9 μCi (1 Ci = 3.7×10^{10} becquerels) per μg (average 0.5 mol of ^{125}I per mol of insulin) and purified it by elution on a superfine Sephadex G-75 column (1.6 × 28 cm). Samples of the two HPLC-purified insulins were run on the same column. All three tracers satisfied the chromatographic criteria of a "good tracer." Aggregated insulin and degraded fragments were undetectable. The 5% trichloroacetic acid precipitability was 97.4%, 98.6%, and 98.4%, respectively, for our labeled P insulin and the Lilly H^{BIO} and P A14-^{125}I-insulin tracers.

Growth of Cultured Lymphocytes. Human lymphocytes (line IM-9) were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml, by feeding three times a week (11, 15).

Lymphocyte Binding Assay. The conditions for the lymphocyte binding assay and measurement of association kinetics and dissociation kinetics have been extensively described elsewhere (11, 15–17). The assay buffer consists of 100 mM Tris·HCl at pH 7.6, 120 mM NaCl, 1.2 mM magnesium sulfate, 1 mM

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Abbreviations: HPLC, high-performance liquid chromatography; P insulin, native porcine insulin; H^{NAT} insulin, native human insulin; H^{BIO} insulin, biosynthetic human insulin.

* Itakura, K. (1979) *Second International Insulin Symposium*, Aachen, Federal Republic of Germany, pp. 71–72 (abstr.).

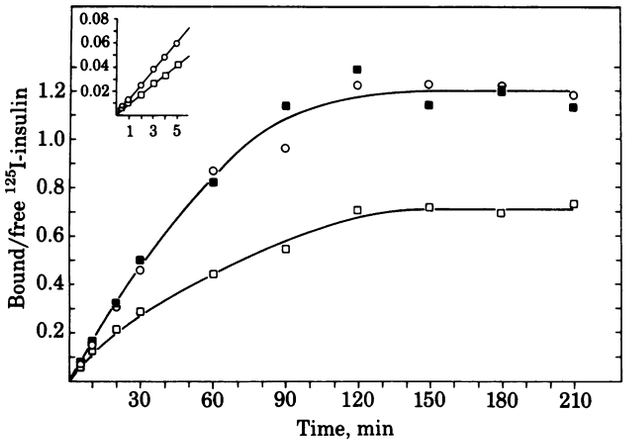

FIG. 1. Kinetics of association of the three radioiodinated insulins to IM-9 lymphocytes. H^{BIO} A14-^{125}I-insulin (○) at 16 pM, P A14-^{125}I-insulin (■) at 11 pM, and P ^{125}I-insulin (□) at 24 pM were incubated at 15°C with IM-9 lymphocytes (2.85×10^6 cells per ml). At the times indicated, the specific bound/free labeled insulin was determined (11). (*Inset*) Initial rates, determined in a separate experiment (expanded scales).

EDTA, 10 mM glucose, 15 mM sodium acetate, and bovine serum albumin at 10 mg/ml. Studies were done at 15°C. Equilibrium binding of the ^{125}I-insulins in the presence of increasing concentrations of unlabeled insulin was determined after 150 min (180 min in some experiments) at 15°C. Binding of the ^{125}I-insulins in the presence of unlabeled insulin at 10 μg/ml was measured and subtracted as "nonspecific binding" (11) to yield specific binding data. These data were then analyzed according to Scatchard (11, 18) or as average affinity profiles (19).

Erythrocyte Binding Assay. Binding of the ^{125}I-insulins to circulating human erythrocytes from a normal donor was determined according to Gambhir *et al.* (20). The specific binding data were calculated and analyzed as described above (11, 18, 19).

Down-Regulation. Cultured human lymphocytes were preincubated for 16 hr at 37°C with concentrations of insulin ranging from 1 to 100 nM, collected by centrifugation, and washed three times in assay buffer. We determined the residual binding capacity as described (15).

Lipogenesis in Rat Adipocytes. The ability of each of the three insulins to stimulate lipogenesis in isolated rat fat cells was determined according to Gliemann and coworkers (21, 22).

RESULTS

Association Kinetics of Labeled Hormones. The time courses (Fig. 1) of association at 15°C of the H^{BIO} and P A14-^{125}I-insulins were identical, reaching a plateau at approximately 120 min. The half-time for reaching equilibrium was 40 min. In contrast, the binding of the porcine tracer purified by conventional methods was lower at all time points. It reached equilibrium at the same time as the other iodinated insulins, and the half-time was similar (44 min). The initial rate (Fig. 1 *Inset*) of association of the conventional porcine tracer was approximately 69% of that of the HPLC-purified tracers.

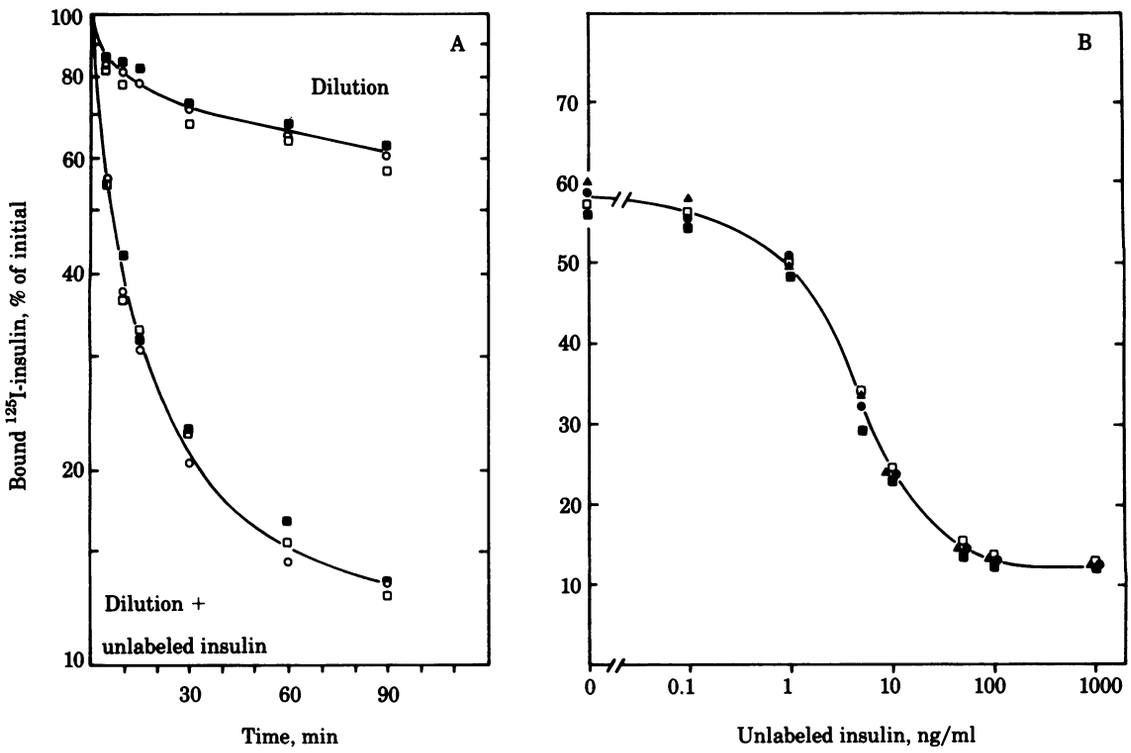

FIG. 2. (*A*) Kinetics of dissociation from IM-9 lymphocytes of the three radioiodinated insulins. H^{BIO} A14-^{125}I-insulin (○), P A14-^{125}I-insulin (■), and P ^{125}I-insulin (□) at 18–20 pM were incubated with 3×10^7 cells per ml for 90 min at 15°C. Dissociation was initiated by 1:40 dilution of 50-μl samples in the presence and absence of 0.17 μM unlabeled insulin as described (16, 17). The radioactivity on the cells, expressed as a percentage of that present at zero time, is plotted as a function of the time elapsed after dilution. (*B*) Effect of insulin concentration in the dilution medium on the dissociation of labeled insulins from cells. The conditions for binding the labeled insulins were the same as in *A*. Samples (50 μl) were transferred to 2 ml of medium in the absence and presence of unlabeled insulin (0.1–1000 ng/ml or 0.017–170 nM). Bound H^{BIO} A14-^{125}I-insulin was diluted in the absence and presence of unlabeled H^{BIO} (●), H^{NAT} (▲), or P (■) insulins, and bound P ^{125}I-insulin in the absence and presence of unlabeled P insulin (□). After 60 min at 15°C, the sedimented cells were counted. The data are expressed as radioactivity bound to cells at 60 min, in percentage of that bound at the time of dilution (zero time of *A*).

Dissociation Kinetics. The dissociations of all three tracers from IM-9 lymphocytes at 15°C, in 1:40 dilutions, were nearly identical (Fig. 2*A*). Each dissociation showed non-first-order kinetics and was accelerated to the same extent by the presence in the dilution medium of the respective unlabeled tracer at 0.17 μM (Fig. 2*A*). Moreover, the dose–response curves for the accelerating effect of the three unlabeled insulins tested were superimposable (Fig. 2*B*). Unlabeled P insulin was equally effective in enhancing the dissociation of either H^{BIO} A14-^{125}I-insulin or our P ^{125}I-insulin.

Competition Curves. Each of the three labeled insulins was incubated to equilibrium at 15°C (with lymphocytes or erythrocytes) in the absence or presence of increasing concentrations of each of the three unlabeled species. The competition curves (Fig. 3) determined for the three unlabeled insulins were superimposable with all three tracers and with both types of human cells, despite the lower initial binding of the conventional porcine tracer.

Scatchard Plots. The experiment described in Fig. 3 was conducted for each of the three tracers with its homologous insulin, using identical concentrations of lymphocytes from the same pool of cells. When these binding data or the erythrocyte binding data of Fig. 3 were analyzed according to Scatchard (18), curvilinear plots were obtained (Fig. 4). The horizontal intercept indicates the maximal binding capacity or receptor concentration (R_o) and was the same for the three labeled and three unlabeled insulins. However, the bound/free ratio was lower at every point when the conventional porcine tracer was used, corresponding to a lower apparent affinity measured with this tracer. This was true with both types of human cells. The average affinity profiles of these data (not shown), when normalized to the initial binding, were superimposable, indicating that the relative falls in apparent affinity occurring with progressive saturation are comparable and independent of the tracer used.

Down-Regulation. The abilities of three different concentrations of the three unlabeled insulins to down-regulate the receptors in IM-9 lymphocytes during a 16-hr preincubation at 37°C were compared. All three insulins decreased the binding to comparable extents (Fig. 5) at equivalent doses.

Stimulation of Lipogenesis in Rat Fat Cells. Because no such biological effect of insulin has been described in the human cells used in this study, we tested the ability of the three unlabeled insulins to stimulate the incorporation of [3-^{3}H]glucose into the lipids of isolated rat fat cells (21, 22). The dose–response curves of the three insulins were superimposable (Fig. 6).

DISCUSSION

The H^{BIO} insulin recombined from isolated A and B chains produced by *E. coli* after cloning of synthetic genes (4, 5) was identical to native pancreatic human (H^{NAT}) and porcine (P) insulins

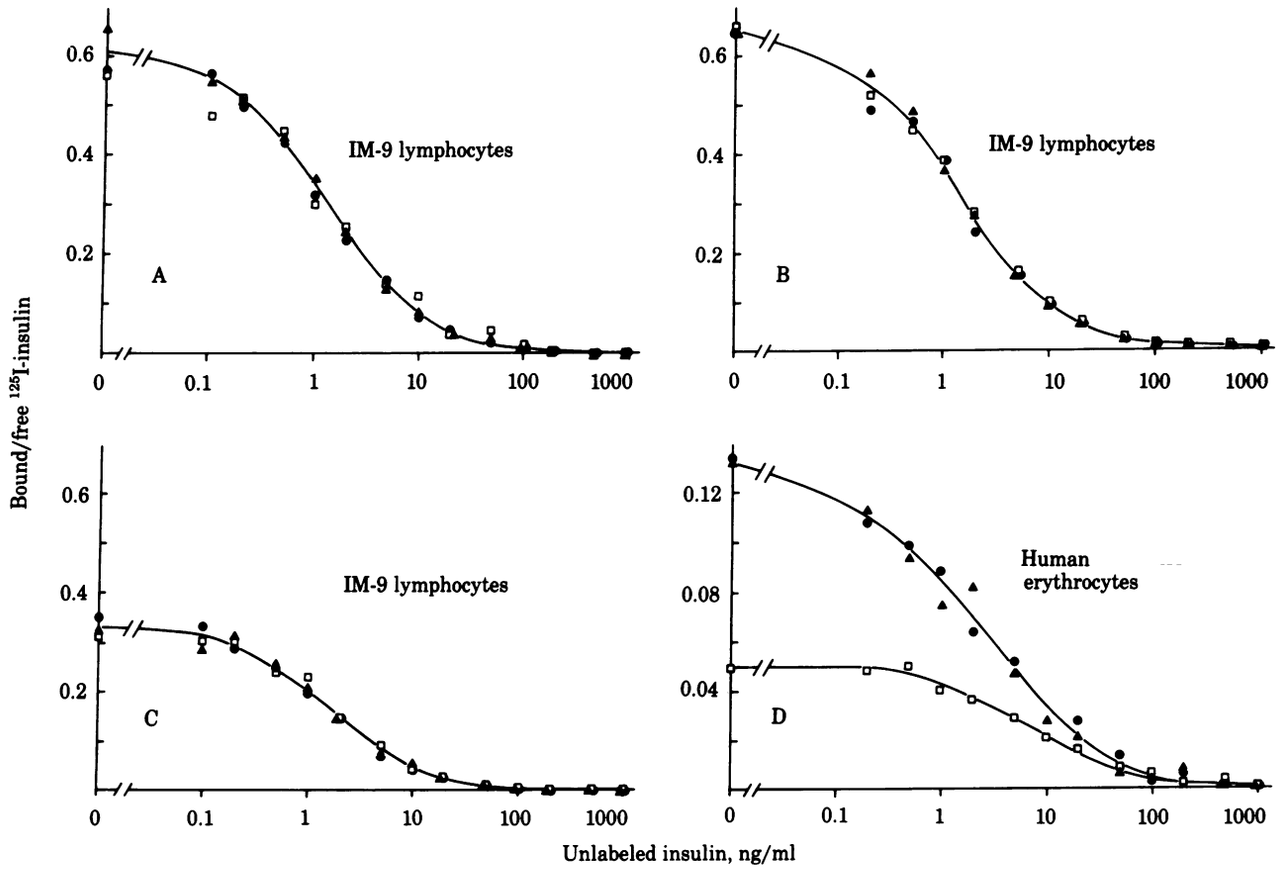

FIG. 3. (*A*, *B*, and *C*). Competition of the three unlabeled insulins with each of the tracers for binding to IM-9 lymphocytes. Tracers are: (*A*) P A14-^{125}I-insulin (13 pM), (*B*) H^{BIO} A14-^{125}I-insulin (18 pM), and (*C*) P ^{125}-I-insulin (18 pM). The unlabeled insulins are: H^{BIO} (●), H^{NAT} (▲), and P (□). IM-9 lymphocytes (2.85×10^6 per ml) were incubated at 15°C for 180 min with labeled insulin at the above concentrations, plus increasing concentrations of unlabeled insulin (0.1–1000 ng/ml or 0.017–170 nM), and the specific bound/free radioactivity was determined (11). (*D*) Competition curves for human erythrocytes. Upper curve: competition of unlabeled H^{BIO} (●) or H^{NAT} (▲) insulin with H^{BIO} A14-^{125}I-insulin (16 pM). Lower curve: competition of unlabeled P insulin (□) with P ^{125}I-insulin (15 pM). Fifty milliliters of blood was used to prepare erythrocytes (20) at a final concentration of 4.4×10^9 cells per ml. Only 15 reticulocytes per 1000 cells were present. The cells were incubated for 90 min at 15°C and the bound/free ratio was determined as in *A–C*.

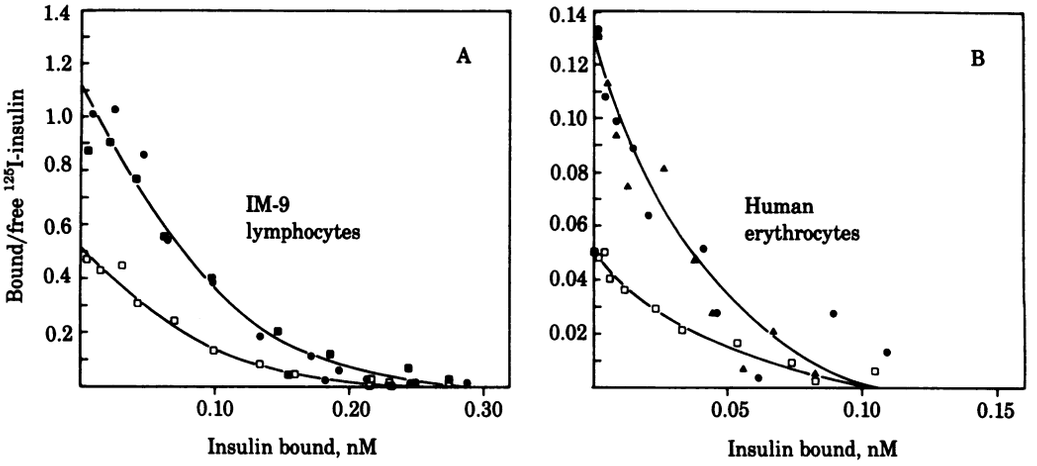

FIG. 4. Scatchard plots of insulin binding to IM-9 lymphocytes (*A*) and human erythrocytes (*B*). (*A*) Binding experiments similar to those of Fig. 3 *A–C* were performed with cells from a single batch (2.6×10^6 cells per ml). H^{BIO} A14-^{125}I- (●), P A14-^{125}I- (■), or P ^{125}I- (□) insulin was equilibrated with increasing concentrations (as in Fig. 3) of the homologous unlabeled insulin. The bound/free radioactivity at equilibrium is plotted as a function of bound hormone. (*B*) Binding data of Fig. 3*D* were replotted as the bound/free radioactivity as a function of bound insulin. Symbols are the same as in Fig. 3*D*.

with respect to all properties examined: binding affinity, kinetics of association and dissociation, down-regulation of receptors, and stimulation of lipogenesis.

The binding of insulin to its receptors is crucially dependent on the integrity of the tertiary structure of the insulin molecule (23) as well as on the conservation of a number of invariant surface residues involved in the receptor-binding region (24, 25). A single amino-acid substitution in this region, as recently found in a rare case of human diabetes caused by the secretion of an abnormal insulin, can cause marked impairment in the receptor binding and biological effect of insulin (26).

Therefore, the superimposable receptor-binding competition curves of the unlabeled H^{NAT} and H^{BIO} insulins, as well as the superimposable kinetics of the H^{BIO} and P A14-^{125}I-insulins, suggest that the tertiary as well as the primary structures of the biosynthetic molecule have been integrally reconstituted. This agrees well with its intact biological potency *in vitro*. Thus, the integrity of a human hormone prepared by genetic engineering, with respect to binding to a human receptor, has now been demonstrated.

We have also examined the ability of the unlabeled H^{BIO} insulin to accelerate the dissociation rate of labeled insulins from the receptor, as well as the ability of H^{BIO} A14-^{125}I-insulin to have its dissociation rate accelerated by an unlabeled species. We have previously interpreted this property as the ability of the hormone to induce receptor-mediated affinity changes (negative cooperativity) (11, 16, 17, 19, 25). Although it has been challenged (27–29), we still believe that our original interpretation is the most plausible, both on theoretical grounds (30) and on the basis of recent experimental data (31, †, ‡), as discussed elsewhere (32, 33). In this study, we have simply used the ob-

† De Meyts, P., Michiels-Place, M., Schüttler, A. & Brandenburg, D. (1980) *The Endocrine Society*, 62nd Annual Meeting, p. 112 (abstr.)

‡ Keefer, L. M., Michiels-Place, M. & De Meyts, P. (1980) *The Endocrine Society*, 62nd Annual Meeting, p. 112 (abstr.)

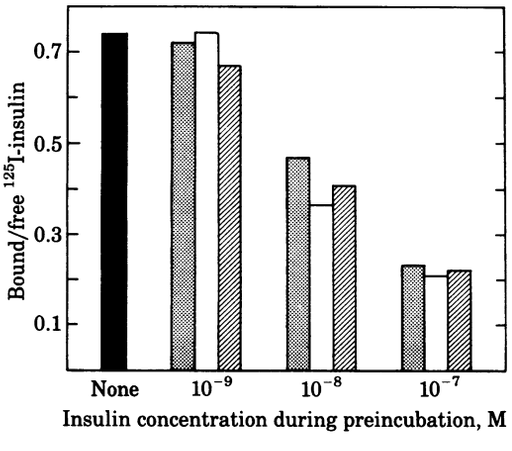

FIG. 5. Down-regulation of insulin receptors of IM-9 lymphocytes by the three unlabeled insulins. Cells were preincubated with insulin at the indicated concentrations for 16 hr, washed, resuspended (4.7×10^6 per ml), and incubated with H^{BIO} A14-^{125}I-insulin (13 pM) for 150 min at 15°C. Solid bar, no insulin in preincubation; stippled bars, P insulin; open bars, H^{NAT} insulin; hatched bars, H^{BIO} insulin.

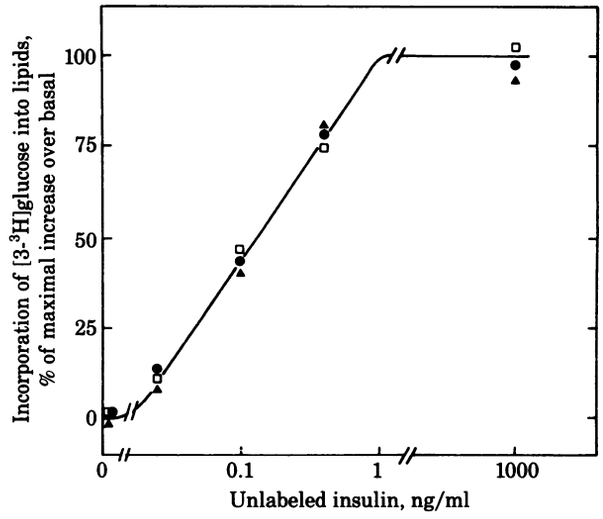

FIG. 6. Stimulation of lipogenesis in isolated rat fat cells by unlabeled insulins. Incorporation of [3-^{3}H]glucose into lipids was measured after a 2-hr incubation at 37°C by the method of Gliemann *et al.* (21, 22). ○, H^{BIO}; ▲, H^{NAT}; and □, P.

served acceleration in the dissociation rate as another indication of the maintained integrity of the structure of the H^{BIO} insulin, because it is well established that this property, whatever its physical basis, can be altered by specific chemical changes in the insulin molecule (25, 34). The preservation of the ability of H^{BIO} insulin to induce the acceleration in dissociation is another index of its identity to H^{NAT} insulin.

As previously shown for H^{NAT} insulin (35), the affinity of H^{BIO} insulin for the receptor as well as its biological activity *in vitro* (including down-regulation of the receptor) are identical to those of P insulin. Hence, its potential advantage in treating diabetics does not reside in an increased efficacy at the receptor level, but in its easier availability and, importantly, in a potentially decreased immunogenicity. A preliminary report on the hypoglycemic potency of the H^{BIO} insulin in healthy men stressed minor differences from P insulin (36). There is clearly no indication of such differences in our results.

In this same study, we have confirmed and extended the observations of Gliemann and his coworkers (9) that an insulin tracer iodinated exclusively on Tyr-A14 binds much better to the insulin receptor than does the A19-^{125}I isomer or the mixture of isomers obtained with conventional purification. Both A14-labeled tracers appear to have a remarkably longer lifetime and have been used for more than 2 months without a loss of integrity as determined by binding studies, trichloroacetic acid precipitability, and gel filtration. Because purification by HPLC yields the desired product in a single step, this technique (13, 37) appears to be the method of choice for those with access to such equipment. The use of A14-^{125}I-insulin should be of major advantage in clinical studies using circulating human cells, to which the binding is normally low, as is evident in Fig. 3*D*.

The higher affinity of the A14 isomer was shown here to be primarily due to a faster initial rate of association and not to a difference in dissociation kinetics, which remained multiexponential. The use of the conventional tracer thus results only in an underestimation of the affinity of the receptor, affecting neither the curvilinearity of the Scatchard plot nor the measurement of receptor concentration or down-regulation. The conclusions of our previous studies of the dissociation kinetics are similarly not affected.

We wish to recognize Prof. Helmut Zahn on the occasion of his 65th birthday. Prof. M. De Visscher is gratefully acknowledged for his support and Mrs. T. Lambert and P. Lahy for expert secretarial help. L.M.K. is supported by an International Institute of Cellular and Molecular Pathology Fellowship. P.D.M. is Chercheur Qualifié of the Belgian Fonds National de la Recherche Scientifique and supported by Fonds de la Recherche Scientifique Médicale (Belgium) Grant 3.4519.78.

1. WHO Expert Committee on Diabetes Mellitus (1980) *Second Report*, Technical Report Series, 646 (World Health Organization, Geneva), p. 62.
2. National Diabetes Advisory Board (1978) *A Study of Insulin Supply and Demand* (NIH-78-1588) (U.S. Department of Health, Education and Welfare, Washington, DC).
3. Schlichtkrull, J., Brange, J., Christiansen, A. H., Hallund. O., Heding, C. G. & Jorgensen, K. H. (1972) *Diabetes* **21,** 649–656.
4. Crea, R., Kraszewski, A., Hirose, T. & Itakura, K. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5765–5769.
5. Goeddel, D. V., Kleid, D. G., Bolivar, F., Heyneker, H. L., Yansura, D. G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K. & Riggs, A. D. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 106–110.
6. Meienhofer, J. E., Schnabel, E., Bremer, H., Brinkhoff, O., Zabel, R., Sroka, W., Klostermeyer, D., Brandenburg, D., Okuda, T. & Zahn, H. (1963) *Z. Naturforsch.* B.18, 1120–1128.
7. Du, Y.-C., Zhang, Y.-C., Lu, Z.-X. & Tsou, C.-E. (1961) *Scientia Sinica* **10,** 84–95.
8. Sieber, P., Kamber, B., Hartmann, A., Johl, A., Riniker, B. & Rittel, W. (1977) *Helv. Chim. Acta* **60,** 27–37.
9. Gliemann, J., Sonne, O., Linde, S. & Hansen, B (1979) *Biochem. Biophys. Res. Commun.* **87,** 1183–1190.
10. Roth, J. (1975) *Methods Enzymol.* **37,** 223–233.
11. De Meyts, P. (1976) in *Methods in Receptor Research*, ed. Blecher, M. (Dekker, New York), Vol. 1, pp. 301–383.
12. Sodoyez, J. C., Sodoyez-Goffaux, F., Goff, M. M., Zimmerman, A. E. & Arquilla, E. R. (1975) *J. Biol. Chem.* **250,** 4268–4277.
13. Frank, B. H. (1980) *Diabetes* **29,** Suppl. 2, 106A (abstr.).
14. Chance, R. E., Kroeff, E. P. & Hoffman, J. A. (1981) in *Insulins, Growth Hormone, and Recombinant DNA Technology*, eds. Gueriguian, J. L., Miller, H. I., Schaffenburg, C. A., Gregoire, A. T. & Sobel, S. (Raven, New York), in press.
15. Gavin, J. R., III, Roth, J., Neville, D. M. Jr., De Meyts, P. & Buell, D. N. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 84–88.
16. De Meyts, P., Roth, J., Neville, D. M., Jr., Gavin, J. R., III & Lesniak, M. A. (1973) *Biochem. Biophys. Res. Commun.* **55,** 154–161.
17. De Meyts, P., Bianco, A. R. & Roth, J. (1976) *J. Biol. Chem.* **251,** 1877–1888.
18. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51,** 660–672.
19. De Meyts, P. & Roth, J. (1975) *Biochim. Biophys. Res. Commun.* **66,** 1118–1126.
20. Gambhir, K. K., Archer, J. A. & Carter, L. (1977) *Clin. Chem.* **23,** 1590–1595.
21. Gliemann, J. (1967) *Diabetologia* **3,** 382–388.
22. Moody, A. J., Stan, M. A., Stan, M. & Gliemann, J. (1974) *Horm. Metab. Res.* **6,** 12–16.
23. Blundell, T. L., Dodson, G. G., Hodgkin, D. C. & Mercola, D. A. (1972) *Adv. Protein Chem.* **26,** 279–402.
24. Wood, S. P., Blundell, T. L., Wollmer, A., Lazarus, N. R. & Neville R. W. J. (1975) *Eur. J. Biochem.* **55,** 531–542.
25. De Meyts, P., Van Obberghen, E., Roth, J., Wollmer, A. & Brandenburg, D. (1978) *Nature (London)* **273,** 504–509.
26. Tager, H., Given, B., Baldwin, D., Mako, M., Markise, J., Rubenstein, A., Olefsky, J., Kobayashi, M., Kolterman, O. & Poucher, R. (1979) *Nature (London)* **281,** 122–125.
27. Pollet, R. J., Standaert, M. L. & Haase, B. A. (1977) *J. Biol. Chem.* **252,** 5828–5834.
28. Donner, D. B. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3176–3180.
29. Pollet, R. J., Standaert, M. L. & Haase, B. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4340–4344.
30. De Lean, A. & Rodbard, D. (1979) in *The Receptors, a Comprehensive Treatise*, ed. O'Brien, R. D. (Plenum, New York), Vol. 1, pp. 143–192.
31. De Meyts, P. & Michiels-Place, M. (1978) *Diabetologia* **15,** p227 (abstr.).
32. De Meyts, P. (1981) *Physiol. Rev.*, in press.
33. De Meyts, P. (1980) in *Hormones and Cell Regulation*, eds. Dumont, J. & Nunez, J. (Elsevier/North-Holland, Amsterdam), Vol. 4, pp. 107–121.
34. Olefsky, J. M. & Chang, H. (1978) *Diabetes* **27,** 946–958.
35. Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 1833–1837.
36. Keen, H., Pickup, J. C., Bilous, R. W., Glynne, A., Viberti, G. C., Jarrett, R. J. & Marsden, R. (1980) *Lancet* **ii,** 398–401.
37. Markussen, J. & Larsen, U. D. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Hormones*, eds, Brandenburg, D. & Wollmer, A. (W. de Gruyter, Berlin), pp. 161–168.