Proc. Natl. Acad. Sci. USA
Vol. 73, No. 5, pp. 1427–1431, May 1976
Biochemistry

Binding of insulin to isolated nuclei

(insulin receptors/hormone action/plasma membranes/glucagon)

IRA D. GOLDFINE AND GEORGE J. SMITH

Metabolism Section (111F), Department of Medicine, Veterans Administration Hospital, 4150 Clement St., San Francisco, California 94121; and Department of Medicine, University of California Hospitals, San Francisco, Calif.

Communicated by Rudi Schmid, January 12, 1976

ABSTRACT **Specific binding sites for ^{125}I-labeled insulin were detected in purified nuclei isolated from rat liver. Binding was rapid, reversible, and directly proportional to the number of nuclei employed. Unlabeled native insulin, at concentrations as low as 1 ng/ml, significantly inhibited the binding of labeled hormone, whereas unlabeled proinsulin and desoctapeptide insulin were less potent. In contrast, glucagon, thyrotropin, growth hormone (somatotropin), and prolactin were without effect. Under identical incubation conditions, ^{125}I-labeled glucagon bound to liver plasma membranes 5- to 10-fold more strongly than did insulin; in contrast, glucagon did not bind to liver nuclei.**

These studies demonstrate the presence of specific binding sites for insulin in purified nuclei isolated from rat liver. In addition, they suggest that the nucleus may be an intracellular site of insulin action.

Insulin is a potent hormone which has profound metabolic effects in many tissues. These range from rapid effects on membrane transport to long-term effects on RNA and DNA synthesis (1–3). However, despite over 50 years of research, the primary action(s) of insulin is (are) still unknown. It is the general opinion that the first step in the action of insulin is binding of the hormone to a specific receptor protein on the surface of target cells. After binding, it is presumed that the hormone–receptor complex leads to all of the subsequent actions of the hormone. In concert with this view of insulin action, specific receptors for insulin have been identified on plasma membranes of target cells (4–6).

In tissues such as liver and fat, insulin has long-term effects on the synthesis of certain enzymes regulating the intracellular metabolism of glucose (7–9). These effects are believed to result from an increased production of messenger RNA (7, 10–12). In addition, in liver, insulin has been shown to increase DNA template activity (13) as well as DNA·RNA hybridization (14). In liver and in other tissues such as mammary gland and skin fibroblasts, insulin increases DNA synthesis (15, 16). If insulin acts exclusively at the cell membrane, an indirect mechanism to transmit the insulin signal, such as the production of a second messenger, must be postulated in order to explain how insulin regulates intracellular events. However, if insulin binding sites can be identified on intracellular structures such as nuclei, then it is possible that insulin also has direct effects on nuclei and other subcellular organelles.

Several groups have suggested that insulin can bind to a variety of subcellular fractions including microsomes (17), mitochondria (18), Golgi fractions (19), and nuclei (17). These studies, however, failed to provide definitive evidence that the preparations of subcellular components employed were entirely free of contamination by plasma membranes. Others have reported that insulin binds only to the plasma membrane and not to other subcellular structures (20, 21).

Abbreviations: T_3, triiodothyronine; STM, sucrose–Tris–magnesium buffer; B, F, bound and free insulin.

Techniques are now available for the preparation of purified nuclei from liver and other tissues which are free of subcellular contaminants (22). Such preparations have been employed by Oppenheimer, Surks, and colleagues (23) as well as others (24–26) to demonstrate directly the presence of specific thyroid hormone receptors in nuclei. In the present study we have identified in nuclei purified from rat liver specific binding sites for insulin that cannot be accounted for by contamination with plasma membranes.

MATERIALS AND METHODS

Porcine insulin, 26 units/mg, was purchased from Elanco; glucagon and L-triiodothyronine (T_3) were purchased from Sigma Chemical Co. Bovine growth hormone (somatotropin) and ovine prolactin were generously donated by the Hormone Distribution Office, National Institute of Arthritis, Metabolism and Digestive Diseases. Trypsin was purchased from Worthington Biochemical Corp. Bovine proinsulin was a gift of Dr. R. E. Chance; bovine desoctapeptide insulin (lacking the COOH-terminal octapeptide of the B chain) was a gift of Dr. F. H. Carpenter. Bovine thyrotropin (TSH) was prepared as previously described (27). Radioiodinated T_3 (200 μCi/μg) was purchased from Abbott Laboratories, and neutral-pH, carrier-free Na^{125}I (300–500 mCi/ml) was purchased from New England Nuclear.

Iodination of Insulin and Glucagon. Insulin was radioiodinated by the stoichiometric chloramine-T technique under the conditions described for bovine thyrotropin (27). The initial concentrations of reactants were 15 μM insulin, 30 μM Na^{125}I, and 30 μM chloramine-T. After the preliminary purification step with Sephadex G-25 (27), the hormone was purified further on Sephadex G-50 (regular). This radioiodinated insulin (80–190 μCi/μg) was 95–97% precipitable by 10% trichloroacetic acid and greater than 90% precipitable by excess insulin antibodies.

Glucagon was radioiodinated as previously described (28).

Preparation of Nuclei and Plasma Membranes. Nuclei were prepared by a combination of the sucrose-density centrifugation (29, 30) and the Triton X-100 techniques (31). Female Sprague–Dawley rats, 80–120 g, fed ad lib, were anesthetized with ether and decapitated. After exsanguination the liver was rapidly removed, freed of connective tissue, placed in a chilled beaker, and minced. The following steps were performed at 4°. Ten grams of liver was added to 100 ml of buffer containing 0.25 M sucrose, 10 mM $MgCl_2$, and 20 mM Tris·HCl, pH 7.85 (STM buffer). The tissue was then homogenized with 10 strokes with a Potter-Elvehjem homogenizer (2000 rpm), filtered through four layers of cheesecloth, and centrifuged at 800 × *g* for 10 min. The pellet was then suspended in 60 ml of 2.2 M sucrose plus 1 mM $MgCl_2$, divided into six portions, and centrifuged at 53,000 × *g* for 45 min. The combined pellets were then suspended in STM buffer plus 0.5% Triton X-100 (10 ml/g wet weight of liver) and centrifuged at

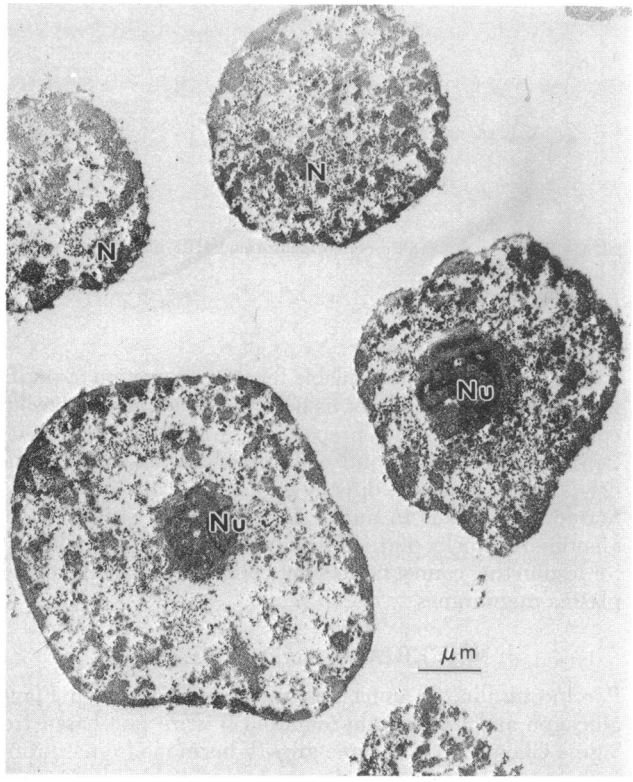

FIG. 1. Electron micrograph of the sucrose-Triton nuclear preparation. Nuclei were fixed in 1% glutaraldehyde–0.1 M cocadylate, post-fixed with 1% osmium tetroxide, and embedded in Epon-Araldite. Magnification is ×8400. (N = nucleus; Nu = nucleolus.)

800 × *g* for 10 min; this step was repeated once. The pellet was then suspended in 100 ml of STM buffer and centrifuged, and then suspended in assay buffer (see below). This nuclear preparation (sucrose-Triton nuclei) had a protein/DNA ratio of 2.2 and a RNA/DNA ratio of 0.22.

Electron microscopy, kindly performed by Dr. Joseph Goodman, revealed intact and broken nuclei (Fig. 1). No intact cells, plasma membranes, vesicles, or other cellular organelles were seen.

Subsequently, it was found that nuclei of similar high purity (as determined by electron microscopy) could also be obtained even if centrifugation through 2.2 M sucrose was omitted. These nuclei (Triton nuclei) had a protein/DNA ratio of 2.4 and a RNA/DNA ratio of 0.31. In addition, these nuclei had undetectable levels of Na^+-K^+ ATPase, a marker enzyme for the plasma membrane; succinate dehydrogenase, a marker enzyme for mitochondria; and 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an enzyme bound to microsomal membranes (34) (Table 1). The characteristics of the binding of insulin to both types of nuclear preparations were very similar.

Liver plasma membranes were prepared by the method of Lesko *et al.* (35).

DNA, RNA, and protein were measured by standard techniques (36–38).

Hormone Binding. Purified nuclei or plasma membranes were suspended in assay buffer (STM buffer plus 2 mM EDTA and 5 mg/ml of bovine serum albumin, pH 7.85).* Typically,

* Albumin was added to eliminate nonspecific binding of the hormone to nonbiological surfaces (4, 5). Increasing the albumin concentration from 2.5 to 20 mg/ml did not influence the binding of insulin to nuclei. No significant binding was seen in the absence of nuclei (Table 2).

Table 1. Enzyme studies in isolated nuclei

Enzyme	Organelle	Activity (μmol/mg of protein per min)
Na^+-K^+ ATPase	Nuclei	Undetectable
	Plasma membranes	0.065
Succinate dehydrogenase	Nuclei	<9
	Mitochondria	580
HMG-CoA reductase	Nuclei	Undetectable
	Microsomes	222

Enzymes were measured by published techniques (32–34). HMG-CoA is hydroxymethylglutaryl-CoA.

1–2 mg/ml of nuclear protein or 0.1–0.2 mg/ml of membrane protein were incubated with labeled and unlabeled hormones at 24°. At appropriate times, 200 μl aliquots were obtained, layered over 200 μl of assay buffer chilled to 4°, and centrifuged at 10,000 × *g* in a Beckman 152 Microfuge (1 min for nuclei and 3 min for membranes). The supernatant radioactivity was aspirated, the tips of the tube containing the insulin or glucagon bound to the pellets were cut off, and the radioactivity was measured. In studies of T_3 binding, the pellet was washed twice with 200 μl of buffer to reduce nonspecific binding (26). Nuclear binding of insulin was expressed as the ratio of bound over free (B/F) hormone/mg of protein per ml.

RESULTS

Incubation Time and Protein Concentration. Insulin bound rapidly to isolated rat liver nuclei. Binding was one-half maximal at 30 min, maximal at 90 min (Fig. 2), and remained at a plateau for up to 4 hr (data not shown). Nonspecific binding of insulin (binding in the presence of excess unlabeled insulin) ranged from 40 to 50% of total binding. Similar high ratios of nonspecific binding to total binding have been reported in studies of T_3 binding to nuclei prepared under similar conditions (25, 26), and in studies of the binding of insulin to isolated fat cells (39). In the present investigation nonspecific binding was due in part to the trapping of insulin in the cell pellet, since either modifying the method of centrifugation or employing filtration reduced nonspecific binding to 30% of total (Table 2).

Binding of insulin was linear up to 2.5 mg/ml of nuclear protein. Preincubation of nuclei with low concentrations of trypsin (5 μg/ml) for 30 min at 24° abolished the ability of the

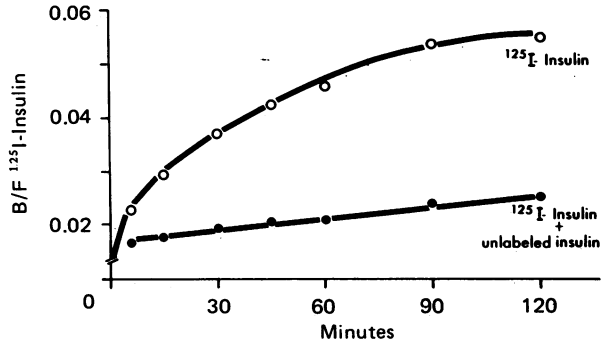

FIG. 2. Time course of insulin binding. [^{125}I]Insulin (0.16 ng/ml) was incubated in the presence and absence of unlabeled insulin (200 μg/ml) with nuclei (sucrose-Triton) at 1.3 mg of protein per ml. Each point is the mean of duplicate determinations.

Table 2. Binding of insulin to nuclei as determined by centrifugation and filtration

Method	[^{125}I]Insulin bound (cpm/ml)	
	Total binding (unlabeled insulin absent)	Nonspecific binding (unlabeled insulin present)
A. Nuclei absent		
Centrifugation	311 ± 13	281 ± 15
Filtration	764 ± 15	709 ± 72
B. Nuclei present		
Centrifugation	5064 ± 301	1786 ± 91
Filtration	5524 ± 84	2197 ± 157

[^{125}I]Insulin (0.21 ng/ml) was incubated with Triton nuclei (2 mg of protein per ml) in the absence and presence of 200 μg/ml of unlabeled insulin for 90 min, and bound and free hormone were separated. Centrifugation: 100 μl of suspension was layered over 200 μl of chilled 0.8 M sucrose and centrifuged for 3 min, and the supernatant was aspirated. Filtration: 100 μl of suspension was added to 5 ml of chilled assay buffer and filtered through Oxoid filters (Amersham) under suction. Each value is the mean ± SD for triplicate determinations. The ratio of nonspecific nuclear binding to total nuclear binding was 0.32 with the centrifugation technique and 0.31 with the filtration technique. In the analyses of these ratios, the blanks (nuclei absent) were subtracted.

nuclei to subsequently bind insulin, suggesting that the binding sites on nuclei were protein in nature.

Plasma Membrane Enrichment Studies. The addition of plasma membranes to tissue homogenates (2 or 4 mg of plasma membrane protein per g of liver) failed to increase the binding of insulin to nuclei prepared subsequently (Table 3). Further, washing the nuclei with plasma membranes solubilized in Triton X-100 did not increase the ability of nuclei to bind insulin (Table 4), indicating that nuclei did not absorb insulin receptors from plasma membranes during the Triton X-100 washings.

Dissociation. The addition of an excess of unlabeled insulin to the reaction at steady state demonstrated that binding was reversible (Fig. 3). Dissociation was not rapid; approximately 40% of the nuclear receptor–insulin complex dissociated in 2 hr. As has been reported in studies of the insulin receptor in liver plasma membranes (40), dissociation of the insulin–nuclear receptor complex was not a simple, first-order process.

Effects of Unlabeled Hormones. The binding of labeled insulin was inhibited by unlabeled insulin at concentrations as low as 1 ng/ml (25 μunits/ml, 160 pM) (Fig. 4). One-half maximal inhibition of binding occurred at 50–100 ng/ml, and maximal inhibition was seen at 10^5 ng/ml. Proinsulin had approximately 5% the potency of native insulin, and desoctapeptide insulin was even less active. Glucagon, thyrotropin, growth hormone, and prolactin were without effect.

Table 3. Lack of effect of enriching liver homogenates with plasma membranes prior to preparing nuclei

	B/F [^{125}I]Insulin	
	Exp. A	Exp. B
Nuclei from control homogenates	0.040 ± 0.002	0.041 ± 0.004
Nuclei from enriched homogenates	0.030 ± 0.002	0.033 ± 0.003

Six grams of liver were homogenized in 60 ml of STM (see *Materials and Methods*). To one portion ("enriched"), purified plasma membranes were added (2 mg of protein per g of liver, Exp. A; or 4 mg of protein per g of liver, Exp. B), and nuclei were prepared (Triton). Nuclei (0.8 mg of protein per ml) were then incubated with 0.3 ng/ml of [^{125}I]insulin and sampled after 120 min of incubation. Each point is the mean ± SD for triplicate determinations.

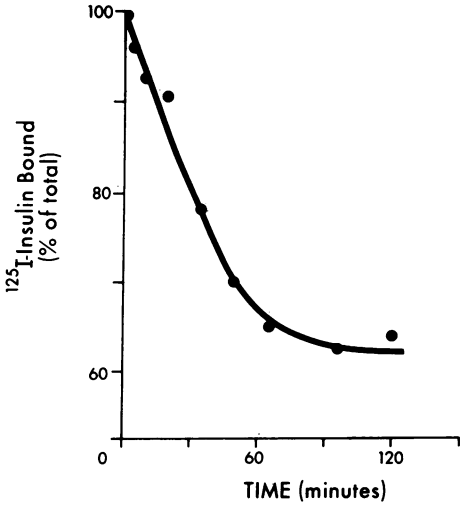

FIG. 3. Dissociation of the insulin–receptor complex. [^{125}I]Insulin (0.25 ng/ml) was incubated with 2.2 mg of protein per ml of nuclei (Triton) for 90 min. Unlabeled insulin (200 μg/ml) was then added and dissociation was followed for 120 min. Nonspecific binding (in the presence of 200 μg/ml of insulin) has been subtracted. Each point is the mean of triplicate determinations.

Comparison of Insulin, Glucagon, and T_3 Binding. The specific binding of insulin, glucagon, and T_3 was studied in purified rat liver nuclei and in purified rat liver plasma membranes (Fig. 5); insulin bound to both cellular fractions. Under identical incubation conditions, ^{125}I-glucagon bound 5- to 10-fold more strongly to plasma membranes than did ^{125}I-insulin. In contrast to the significant binding of insulin to nuclei, glucagon did not bind at all to these structures. Under these same incubation conditions, T_3 bound to nuclei but not to plasma membranes. Other studies revealed that ^{125}I-thyrotropin (27) did not bind to isolated nuclei.

DISCUSSION

In the present study, specific binding sites for insulin were identified in rat liver nuclei. This binding fulfilled the re-

Table 4. Effect of washing nuclei with solubilized plasma membranes

	B/F [^{125}I] Insulin	
	60 min	120 min
Control wash	0.011 ± 0.001	0.019 ± 0.001
Solubilized plasma membrane wash	0.009 ± 0.001	0.021 ± 0.002

Nuclei (Triton) were purified from 4 g of liver up until the last wash with Triton X-100. One half of the preparation was then washed in 20 ml of STM–Triton X-100 in which 1 mg of plasma membrane protein had been solubilized. The other half was washed in STM–Triton X-100 in which 1 mg of bovine serum albumin had been solubilized. Both portions were then washed once in STM–Triton X-100 and once in STM. Purified nuclei (0.8 mg of protein per ml) were then incubated with 0.6 ng/ml of [^{125}I]insulin and sampled after 60 and 120 min. Each point is the mean ± SD for triplicate determinations.

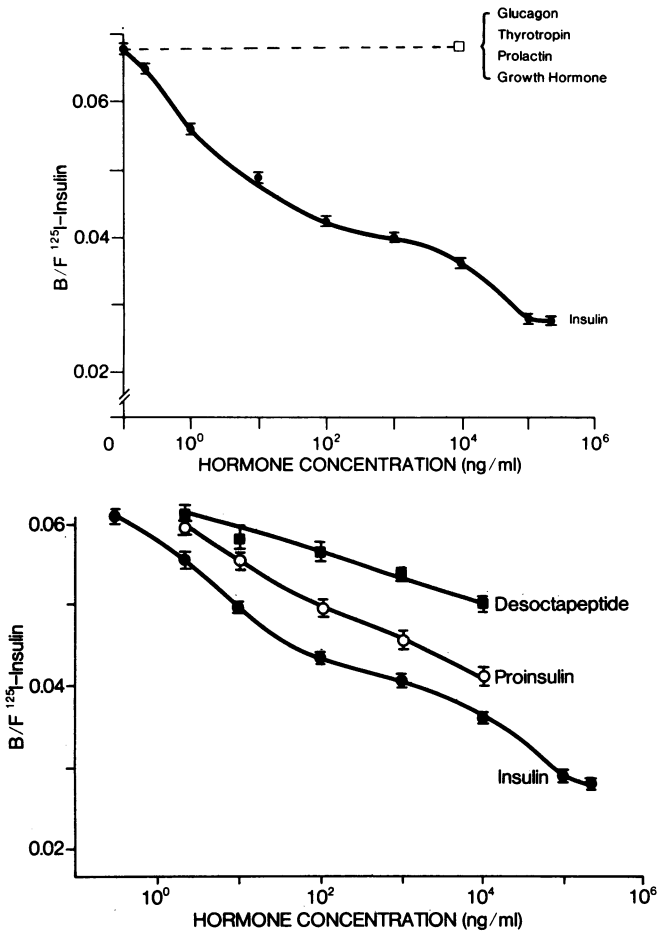

FIG. 4. *Top.* Effect of insulin and other hormones on the binding of [^{125}I]insulin. *Bottom.* Effect of insulin, proinsulin, and desoctapeptide insulin on the binding of [^{125}I]insulin. In both studies [^{125}I]insulin (0.18 ng/ml) was incubated with the above concentrations of labeled hormone and 1.5 mg of protein per ml nuclei (Triton) for 120 min. Each point is the mean ± SD of quadruplicate determinations.

quirements generally accepted to define a biologically important hormone receptor (4–6). The binding of labeled insulin was rapid, reversible, and saturated by physiologic concentrations of unlabeled insulin. Further, the insulin analogues, proinsulin and desoctapeptide insulin, which have diminished activities both *in vitro* and *in vivo* (5, 6), had a proportionately diminished ability to inhibit the binding of labeled insulin. Finally, other hormones unrelated to insulin, such as thyrotropin, growth hormone, glucagon, and prolactin, had no effect on insulin binding.

It was considered possible that the binding observed with nuclei was due to contamination of these structures by plasma membranes; five lines of evidence suggested that this was not the case. First, electron microscopy revealed that the preparations employed did not contain plasma membranes. Since nuclei bound insulin approximately 10% as well as did plasma membranes, it is unlikely that a 10% contamination of the nuclei with plasma membranes would have gone unnoticed. Second, enrichment of homogenates with plasma membranes did not increase the binding of insulin to nuclei. Third, the nuclei were prepared with Triton X-100, a detergent known to solubilize the insulin receptor of plasma membranes (41); also, washing nuclei with solubilized plasma membranes did not increase the binding of insulin. Fourth, the nuclei did not contain the plasma

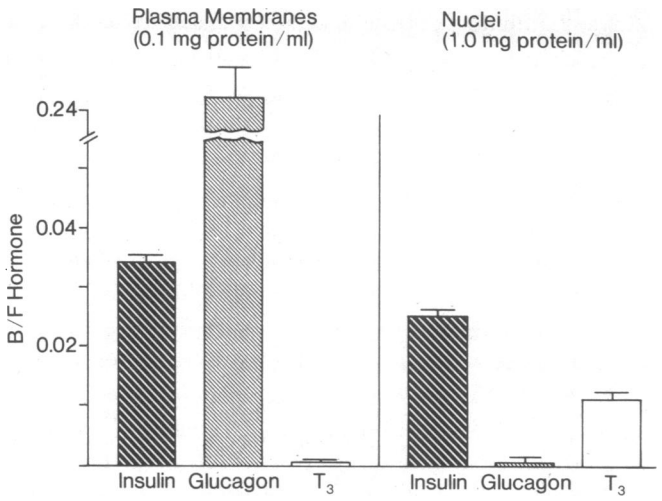

FIG. 5. Comparison of insulin, glucagon, and triiodothyronine (T_3) binding to liver plasma membranes and nuclei. Membranes and nuclei (sucrose-Triton) were incubated for 120 min with [^{125}I]insulin (0.2 ng/ml), [^{125}I]glucagon (0.6 ng/ml), and [^{125}I]triiodothyronine (0.06 ng/ml) in the presence and absence of unlabeled hormones: insulin, 200 μg/ml; glucagon, 50 μg/ml; and triiodothyronine, 1.0 μg/ml. Nonspecific binding has been subtracted. Each point is the mean ± SD of quadruplicate determinations.

membrane enzyme Na^+-K^+ ATPase. Finally, these nuclei did not bind glucagon, a hormone which binds to plasma membranes more strongly than does insulin.

Both electron microscopic and enzymatic studies indicated that contamination with other cellular fractions such as microsomal membranes and mitochondria was not present in the preparations of nuclei employed.

Preliminary studies indicate that insulin also binds to nuclei prepared from human cultured lymphocytes.† This finding suggests that nuclear binding sites for insulin are neither cell nor species specific.

At present it is not known whether insulin enters the intact cell. Several years ago, studies with insulin covalently coupled to large beads of agarose appeared to indicate that insulin acted exclusively at the cell surface (42); however, these data have been questioned (43, 44), and it is now known that the insulin–agarose complex is unstable in the presence of biological fluids (45–47). The solubilized insulin released from such preparations of insulin–agarose is sufficient to account for all of the activity detected (46, 47). Since molecules as large as albumin can penetrate certain cell membranes (48) and the intracellular uptake of insulin into isolated hepatocytes has been reported (49), it is possible that insulin also enters the cell and then binds to intracellular structures.

The data of the present study suggest that there may be at least two cellular mechanisms of insulin action. Undoubtedly, insulin binds to receptors on the plasma membrane and this interaction leads directly to changes in various membrane functions such as transport, electrical activity, and the activity of membrane-bound enzymes (1–3). In addition, however, it is well established that insulin regulates various intracellular functions, including the synthesis of DNA, RNA, and protein, as well as the activity of several critical enzymes, but these intracellular effects cannot be ascribed directly to binding at the plasma membrane. It is possible that insulin, like glucagon and certain other hormones, mediates such functions by generating a second messenger at the plasma membrane. A simple and

† I. D. Goldfine and G. J. Smith, unpublished observations.

more straightforward explanation of how insulin regulates intracellular events is that insulin itself enters the cell and then mediates its own actions. The data presented herein demonstrate that insulin in fact binds directly to the cell nucleus *in vitro*. This finding suggests that direct interactions with intracellular structures *in vivo* may constitute a second mechanism of insulin action.

We thank Dr. M. D. Siperstein for his invaluable advice on various aspects of this project, Mr. D. Cohen for performing the assays of hydroxymethylglutaryl-CoA reductase, Dr. D. Vessey for performing the assays of succinate dehydrogenase, Dr. H. Epstein for his provocative dialogues on cellular regulation, Mrs. V. Vaughn for her editorial assistance, and the referees of this manuscript for several valuable suggestions. This work was performed under Veterans Administration Project no. MRIS 3194-01, and was supported in part by Grant 5-ROI-CA15979 (a relocation of 2-RO1-CA08501) from the National Cancer Institute, National Institutes of Health.

1. Pilkis, S. J. & Park, C. R. (1974) *Annu. Rev. Pharmacol.* **14,** 368–385.
2. Fain, J. N. (1974) "Biochemistry of hormones," in *Biochemistry Series One, MTP International Review of Science,* ed. Rickenberg, H. V. (University Park Press, Baltimore, Md.), Vol. 8, pp. 1–23.
3. Krahl, M. E. (1974) *Ann. Rev. Physiol.* **36,** 331–360.
4. Roth, J. (1973) *Metabolism* **22,** 1059–1073.
5. Kahn, C. R. (1975) in *Methods in Membrane Biology,* ed. Korn, E. D. (Plenum Press, New York), Vol. 3, pp. 81–146.
6. Goldfine, I. D. (1976) in *Receptors in Pharmacology,* ed. Smythies, J. R. (Marcel Dekker, Inc., New York), in press.
7. Steiner, D. F. (1966) *Vitam. Horm.* **24,** 1–60.
8. Weber, G., Singhal, R. L., Stamm, N. B., Lea, M. A. & Fisher, E. A. (1966) *Adv. Enzyme Regul.* **4,** 59–81.
9. Krahl, M. E. (1972) in *Insulin Action,* ed. Fritz, I. B. (Academic Press, New York), pp. 461–485.
10. Wool, I. G. (1964) in *Actions of Hormones on Molecular Processes,* eds. Litwack, G. & Kritchevsky, D. (J. Wiley & Sons, Inc., New York), pp. 422–469.
11. Pilkis, S. J. & Salaman, D. F. (1972) *Biochim. Biophys. Acta* **272,** 327–339.
12. Steiner, D. F. & King, J. (1966) *biochim. Biophys. Acta* **119,** 510–516.
13. Morgan, C. R. & Bonner, J. (1970) *Proc. Natl. Acad. Sci. USA* **65,** 1077–1080.
14. Drake, R. L. & Morgan, C. R. (1975) *Biochem. Biophys. Res. Commun.* **62,** 342–348.
15. Stockdale, F. E. & Topper, Y. J. (1966) *Proc. Natl. Acad. Sci. USA* **56,** 1283–1289.
16. Rechler, M. M., Podskalny, J. M., Goldfine, I. D. & Wells, C. A. (1974) *J. Clin. Endocrinol. Metab.* **39,** 512–521.
17. Horvat, A., Li, E. & Katsoyannis, P. G. (1975) *Biochim. Biophys. Acta* **382,** 609–620.
18. Forgue, M.-E. & Freychet, P. (1975) *Diabetes* **24,** 715–723.
19. Bergeron, J. J. M., Evans, W. H. & Geschwind, I. I. (1973) *J. Cell Biol.* **59,** 771–775.
20. Chang, K., Bennett, V. & Cuatrecasas, P. (1975) *J. Biol. Chem.* **250,** 488–500.
21. Crofford, O. B. & Okayama, T. (1970) *Diabetes (Suppl. 1),* **19,** 369 (abstr.).
22. Roodyn, D. B. (1970) in *Subcellular Components,* ed. Birnie, G. D. (University Park Press, Baltimore, Md.), pp. 15–51.
23. Oppenheimer, J. H., Schwartz, H. L., Koerner, D. & Surks, M. I. (1974) *J. Clin. Invest.* **53,** 768–777.
24. Samuels, H. S. & Tsai, J. S. (1974) *J. Clin. Invest.* **53,** 656–659.
25. DeGroot, L. J. & Torresani, J. (1975) *Endocrinology* **96,** 357–369.
26. Spindler, B. J., MacLeod, K. M., Ring, J. & Baxter, J. D. (1975) *J. Biol. Chem.* **250,** 4113–4119.
27. Goldfine, I. D., Amir, S. M., Petersen, A. W. & Ingbar, S. H. (1974) *Endocrinology* **95,** 1228–1233.
28. Goldfine, I. D., Roth, J. & Birnbaumer, L. (1972) *J. Biol. Chem.* **247,** 1211–1218.
29. Chauveau, J., Moule, Y. & Rouiller, C.-H. (1956) *Exp. Cell Res.* **11,** 317–321.
30. Widnell, C. C. & Tata, J. R. (1964) *Biochem. J.* **92,** 313–317.
31. Hymer, W. C. & Kuff, E. L. (1964) *J. Histochem. Cytochem.* **12,** 359–363.
32. Wallach, D. F. H. & Kamat, V. B. (1955) in *Methods in Enzymology,* eds. Neufeld, E. F. & Ginsburg, V. (Academic Press, New York), Vol. VIII, pp. 164–172.
33. Arrigoni, O. & Singer, T. P. (1962) *Nature* **193,** 1256–1258.
34. Brown, M. S., Dana, S. E., Dietschy, J. M. & Siperstein, M. D. (1973) *J. Biol. Chem.* **248,** 4731–4738.
35. Lesko, L., Donlon, M., Marinetti, G. V. & Hare, J. D. (1973) *Biochim. Biophys. Acta* **311,** 173–179.
36. Burton, K. (1956) *Biochemistry* **62,** 315–323.
37. Kerr, S. E. & Seraidarian, K. (1945) *J. Biol. Chem.* **159,** 211–217.
38. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
39. Gammeltoft, S. & Gliemann, J. (1973) *Biochim. Biophys. Acta* **320,** 16–32.
40. Kahn, C. R., Freychet, P. & Roth, J. (1974) *J. Biol. Chem.* **249,** 2249–2257.
41. Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 318–322.
42. Cuatrecasas, P. (1969) *Proc. Natl. Acad. Sci. USA* **63,** 450–457.
43. Katzen, H. M. & Vlahakes, G. J. (1973) *Science* **179,** 1142–1143.
44. Butcher, R. W., Crofford, O. B., Gammeltoft, S., Gliemann, J., Gavin, J. R., Goldfine, I. D., Kahn, C. R., Rodbeil, M., Roth, J., Jarett, L., Larner, J., Lefkowitz, R. J., Levine, R. & Marinetti, G. V. (1973) *Science* **182,** 396–397.
45. Davidson, M. B., Van Herle, A. J. & Gerschenson, L. E. (1973) *Endocrinology* **92,** 1442–1446.
46. Kolb, H. J., Renner, R., Hepp, K. D., Weiss, L. & Wieland, O. H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 248–252.
47. Garwin, J. L. & Gelehrter, T. D. (1974) *Arch. Biochem. Biophys.* **164,** 52–59.
48. Loewenstein, W. R. (1966) *Ann. N.Y. Acad. Sci.* **137,** 441–472.
49. Olefsky, J. M., Johnson, J., Liu, F., Edwards, P. & Baur, S. (1975) *Diabetes* **24,** 801–810.