Rapid Publications

Immunodetection of Insulin After Transfer from Gels to Nitrocellulose Filters

A Method of Analysis in Tissue Extracts

KEIJI KAKITA, KEITH O'CONNELL, AND M. ALAN PERMUTT

SUMMARY

This report describes the development of a rapid method for detection of nanogram quantities of insulin in tissue extracts after electrophoresis. Following electrophoresis the proteins are transferred to nitrocellulose filters and treated with a photoreactive crosslinking agent. Filter bound insulin is detected by antiinsulin antibody and ^{125}I-protein A, followed by autoradiography. The photoaffinity crosslinking is simple, rapid, and stable, and does not require reactive binding sites on derivatized paper. Under these conditions insulin maintains its immunoreactivity yet can be washed extensively to reduce nonspecific background; as little as 10 ng can be visualized. The method has proven to be useful for rapid analysis of qualitative as well as quantitative differences in immunoreactive insulin in tissue extracts. DIABETES *31*: 648–652, July 1982.

Filter hybridization of electrophoretically separated DNA, RNA, or protein has become a standard procedure for detection of specific molecules in crude extracts. The method generally involves three steps, including separation by electrophoresis, transfer to filters, and analysis by incubating the filter with a labeled probe. The initial technique of blotting of DNA to nitrocellulose paper as described by Southern[1] in 1975 has become a valuable tool for detecting specific genes representing one part in a million of total DNA.

To detect insulin in pancreatic extracts after electrophoresis, we evaluated previously described methods,[2–5] but encountered a number of problems. In general, the methods were for proteins larger than 20,000 mol wt and not suitable for smaller polypeptide hormones. This report describes a ready method for separation of insulin from crude extracts by gel electrophoresis, electrophoretic transfer to nitrocellulose filters, and covalent attachment of the insulin by photoaffinity crosslinking. With this method, nanogram quantities of insulin in whole pancreatic extracts can be detected.

From the Washington University School of Medicine, Department of Internal Medicine, Division of Metabolism, 660 South Euclid Avenue, St. Louis, Missouri 63110.
Address for reprint requests to M. Alan Permutt, M.D., at the above address.
Received for publication 15 April 1982.

METHODS

Pancreatic tissue was homogenized in 70% acid alcohol and insulin partially purified as described.[6] Electrophoresis was performed in Tris-glycine (pH 8.9) buffer in 12.5% polyacrylamide slab gels according to the method of Davis,[7] as previously described,[6] or Tris-glycine buffer containing SDS or 7 M urea.[6,8]

After electrophoresis transfer of protein from gels to filters was accomplished electrophoretically onto presoaked nitrocellulose filters (0.45 μm, Schleicher and Schuell, Inc., New Hampshire) in 25 mM Tris, 192 mM glycine (pH 8.3) at 4–5 V, 70 mA for 4 h at room temperature (Electroblot, E-C Apparatus Corp., St. Petersburg, Florida). After transfer, the filters were placed in plastic bags (Seal-A-Meal) and soaked in 4% bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) in 10 mM phosphate buffer (pH 7.4) for 1 h at 40°C.

Photolinkage was performed with HSAB (N-hydroxysuccinimidyl-P-azidobenzoate, Pierce Chemical Co., Rockford, Illinois). HSAB (1.7 mg) was dissolved in 250 μl dimethyl sulfoxide (Sigma), then diluted 1:50 in phosphate buffer. Approximately 10 ml was added to the filters followed by incubation for 2 min in a darkened room at 4°C.[9] The filters were photolyzed for 10 min with a long wave ultraviolet light (principle wave length 366 nm) obtained from Black Ray B 100A Ultra-Violet Products, Inc., (California) at a distance of 15 cm from the surface of the filter. The chemical reaction was terminated by the addition of 1 M Tris-HCl (pH 7.5) to give a final concentration of 20 mM. The filter was washed twice at room temperature for 5 min with 10 ml of 10 mM phosphate buffer (pH 7.4) and incubated with 1:10,000 final dilution of guinea pig anti-insulin serum in phosphate buffer for 3 h at 37°C. Photolinkage was performed again as previously after washing with buffer. The filter was again

washed twice with phosphate buffer and incubated in 10 ml of phosphate buffer for 1 h at room temperature to which 5–10 μCi of I^{125} *Staphyloccocus aureus* protein A (Sigma), iodinated as described [10] had been added. The filter was then washed with 1% Triton X-100 in phosphate buffer (10 ml) for 1 h at room temperature, then with (10–20 ml) water, dried, and exposed to Kodak X-omat x-ray film with an intensifying screen (DuPont) for 12–20 h at −80°C. Densitometric analysis of radioautographs was performed with a Joyce-Loebl microdensitometer MKIIIC with a wedge range absorbance of 0.79–3.76. Area under the peak was estimated as (height of peak) × (width at ½ height)/2.

RESULTS

Evaluation of electrophoretic conditions and transfer to filters. Pancreatic extracts were electrophoresed in Tris-glycine buffer at pH 8.9. This nondenaturing buffer system was chosen because proteins are separated according to both size and charge. Rat insulins I and II, which differ by only one basic amino acid, are readily separable.[11] Addition of SDS or urea to the electrophoresis buffer impaired transfer efficiency (see below). Our initial efforts were to transfer proteins to diazotized filters, but this requires removal of glycine from the gel prior to transfer, since glycine inactivates diazo groups. We found that during removal of glycine buffer most of the insulin was lost from the gel. For this reason, we tried borate[12] and phosphate[13] electrophoresis buffers but found poor resolution.

Transfer of insulin from polyacrylamide gels to filters was evaluated with I^{125}-labeled insulin under various conditions. Blot transfer by simple diffusion required 24–48 h and never yielded greater than 20% transfer. Electrophoretic transfer in 0.7% acetic acid to nitrocellulose paper as described by Towbin et al.,[3] resulted in recovery of 12–35% of the insulin initially applied to the gel depending on current and time of transfer. In phosphate buffer (50 mM, pH 7.5), recovery was 24% on nitrocellulose and 35–43% on diazotized paper. The best results were obtained by electrophoretic transfer in Tris-glycine buffer, which yielded quantitative recovery (109.9 ± 10.0%, N = 8) on nitrocellulose filters in 4 h. Addition of SDS or urea lowered recovery to 38–49%.

Immobilization of insulin to nitrocellulose filters. Following electrophoretic transfer to nitrocellulose paper, the paper was dried and treated with anti-insulin antibody and ^{125}I-protein A. Background was often unacceptably high, even when the filters were pretreated with 4% albumin to reduce nonspecific binding. Background could be minimized by washing extensively at room temperature with 1% Triton X-100, but insulin was also progressively washed from the filters (Figure 1A–C). For this reason, a method of crosslinking insulin to nitrocellulose paper was devised. A photoreactive heterobifunctional crosslinking reagent, hydroxysuccinimidyl-P-azidobenzoate (HSAB) was chosen to covalently link insulin to albumin pretreated nitrocellulose filters. HSAB does not require derivatization of the peptide, nor does it have stringent chemical requirements for affinity crosslinking.[9] Nitrocellulose filters were adsorbed with 4% bovine serum albumin and insulin photoaffinity crosslinked. The sensitivity of detection was increased greater than 100-fold (Figure 1D). Insulin appeared to be firmly attached to the nitrocellulose since the antibody-^{125}I-protein A complexes could be removed by washing at 37°C, the filters treated again with antibody and ^{125}I-protein A, and comparable radioautographs obtained.

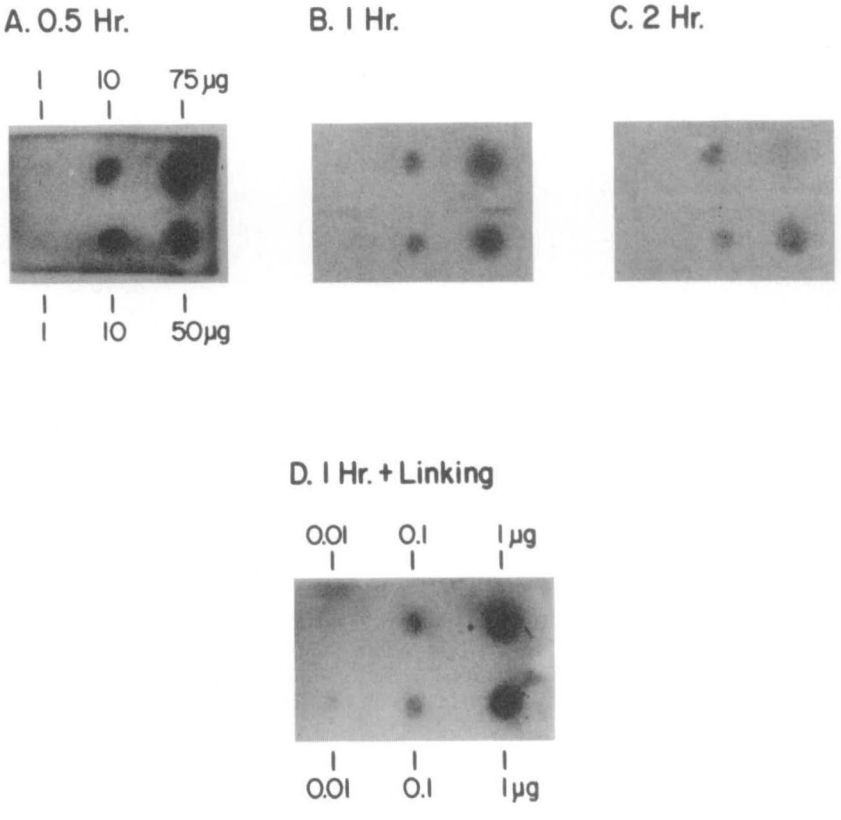


FIGURE 1. The effect of washing on removal of insulin from nitrocellulose paper in the presence and absence of photoreactive linkage. Bovine insulin in the concentrations indicated was spotted on nitrocellulose filters, soaked in albumin, and immunodetected with anti-insulin antibody and ^{125}I-protein A as described in METHODS. The filters were then washed for the times indicated. (A–C) Auto-radiographs of the same filter washed for progressively longer times. (D) Autoradiograph of a separate filter with less insulin, photoaffinity crosslinked, and washed for 1 h.

Immunodetection of filter bound insulin.. The effects of antibody concentration, time, and temperature of incubation were evaluated to optimize detection of immobilized insulin. Anti-insulin serum at a dilution of less than 1:1,000 produced a large amount of nonspecific binding to filters and unacceptable background. Incubation with 1:10,000 dilution for 3 h at 37°C gave optimal binding. Even at this dilution of antibody, however, with short washing time there was a very large amount of background. As the washing time was increased above 3 h, progressively less insulin was detected, suggesting that the antibody was being removed from the insulin. HSAB was therefore also added following antibody treatment, to crosslink antibody to the insulin. Using this method a stable immune complex was formed which could be extensively washed until background was satisfactory.

The limits of detection of insulin could be enhanced by treating the filter-bound insulin-antibody complex a second and third time with anti-insulin antibody and ^{125}I-protein A. For example, insulin samples from 100–800 ng were electrophoresed in triplicate, electroblotted, and crosslinked to a nitrocellulose filter. Following treatment with anti-insulin serum and ^{125}I-protein A, 800 ng of insulin was barely detected (Figure 2A, lanes 1–3). When antibody and ^{125}I-protein A treatment was repeated a second (Figure 2B) and third time (Figure 2C), less than 100 nanograms of insulin (Figure 2C, lanes 10–12) was easily detected. As sensitivity was increased, other more acidic desamidoinsulins were also visualized.

To determine whether the immunodetection of filter-bound insulin could be used to quantitate the relative amount of insulin in extracts, densitometric analysis of the radioautographs in Figure 2 is shown in Figure 3A. After two antibody treatments (Figure 2B) the ^{125}I-protein A bound was proportional to the insulin electrophoresed, from 100–400 ng (Figure 3A) but this relationship did not hold beyond 400 ng insulin, since the linear response range of the film was exceeded. The linear relationship between the amount of insulin electrophoresed and the radioautographic signals could be optimized by altering the number of repeated antibody treatments, and/or exposure time according to the amount of insulin analyzed. For example, the ^{125}I-protein A detected was proportional to the insulin electrophoresed over a fourfold range in the experiment illustrated in Figure 3B.

Detection of insulin in crude pancreatic extracts. Use of the immunoelectrophoretic method for detecting insulin is illustrated in Figure 4. Extracts of pancreas from dog and humans were electrophoresed, transferred to a nitrocellulose filter, and stained with Coomassie blue (Figure 4A). Dog pancreas is in lane 1, and human pancreas is in lanes 2 and 3. The pancreas in lane 2 was from a maturity-onset diabetic who was insulin requiring 10 yr prior to death, while in lane 3 is a normal pancreas. A prominent band (arrow) migrating with the same mobility as bovine insulin was seen in dog (lane 1) and normal human pancreas (lane 3). No band comparable to insulin was seen in the diabetic pancreas, while most of the other pancreatic proteins appeared comparable. A radioautograph of this gel after immunodetection (Figure 4B) shows dog and human insulin as discrete bands while no immunoreactive insulin was detected in the diabetic pancreas.

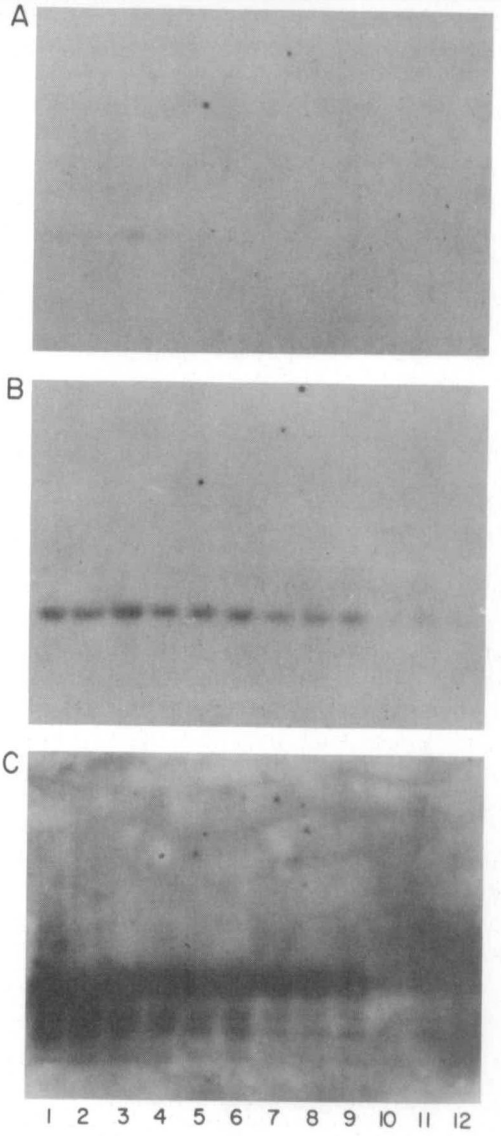


FIGURE 2. Enhanced sensitivity of detection of insulin by repeated antibody treatment. Various amounts of insulin were electrophoresed, electroblotted to nitrocellulose filters, and treated with anti-insulin serum and ^{125}I-protein A. Autoradiographs of filters following (A) single antibody treatment, (B) antibody treatment twice, and (C) antibody treatment three times are shown. Bovine insulin present in lanes 1–3, 800 ng; lanes 4–6, 400 ng; lanes 7–9, 200 ng; and lanes 10–12, 100 ng.

DISCUSSION

The present report combines use of electrophoretic separation with the sensitivity of radioimmunoassay to resolve insulin in highly complex mixtures of proteins such as crude pancreatic extracts. Replicas of the proteins separated on polyacrylamide gels were produced with high resolution. The photoaffinity crosslinking agent HSAB was found to be very effective in crosslinking insulin to albumin on nitrocellulose filters. Photoaffinity crosslinking with HSAB requires an accessible amino group on either insulin or albumin to initiate the labeling process.[9] Once the chemical reaction of HSAB occurs, the aryl azide is activated by uv light to aryl nitrene, which requires little or no chemical specificity for further reaction. The covalent linkage of hormone and albumin can occur by reaction of the nitrene with both peptide and carbohydrate components of proteins. Preliminary ex-

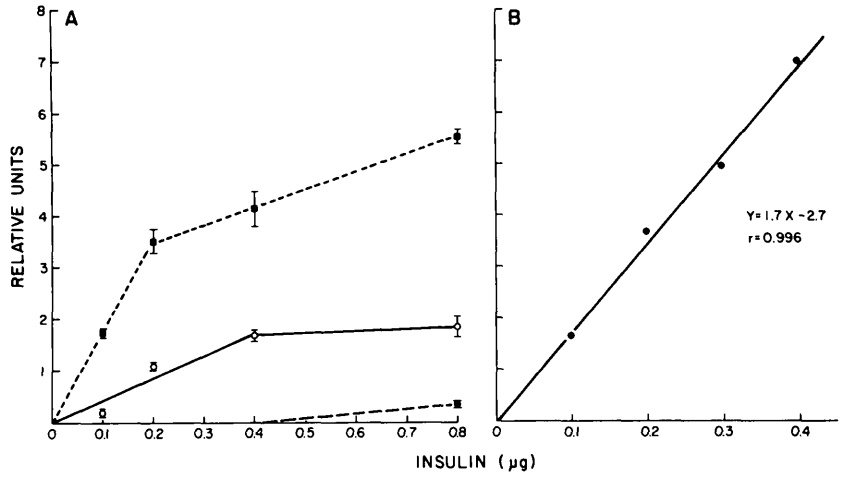


FIGURE 3. Densitometric analysis of *radioautographs in Figure 2*. A. Area under the densitometric tracing of the insulin peak in relative units after 1 antibody treatment (closed circles), 2 treatments (open circles), or 3 treatments (closed squares). B. Densitometric analysis of a separate radioautograph of duplicate insulin samples from 0.1 to 0.4 μg of insulin.

periments with disuccinyl suberate [14] indicated that it works at least as well as HSAB. New chemical crosslinkers are rapidly being developed which may improve sensitivity.

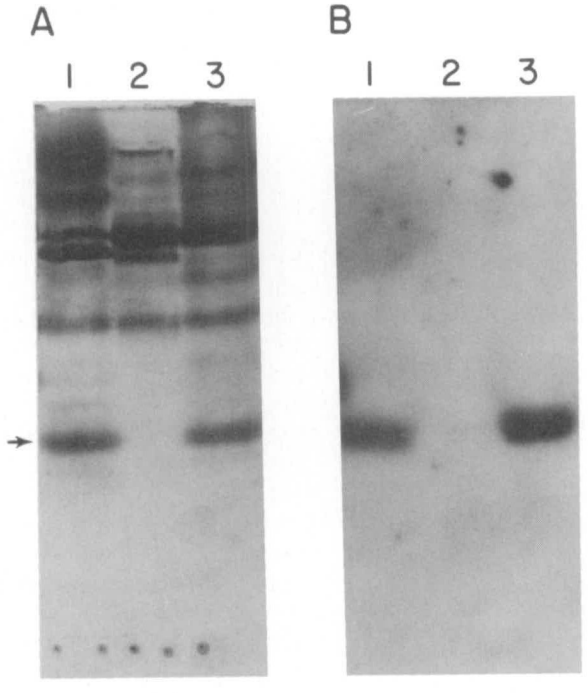
After immunodetection with anti-insulin antibody and ^{125}I-protein A, the radioautographic signal increased linearly with the amount of insulin applied to the gel over at least a fourfold range. This method therefore lends itself to rapid analysis of relative differences in immunoreactive insulin in various biologic preparations. This method has been used to the quantitate the relative abundance of rodent insulins I and II under various experimental conditions.[11] An increase in pancreatic content of insulin I relative to II compared with that in control animals was noted in growth hormone tumor bearing hyperinsulinemic rats,[11] pregnant rats, obese hyperglycemic mice, and glucose-injected mice (manuscript in preparation).

We have attempted to use the immunoelectrophoretic method to measure insulin in plasma at 1–10 ng/ml, which represents less than one part per 10^7 total serum proteins. With affinity chromatography we can achieve a rapid 1000-fold purification of insulin, such that 10 ml of stimulated plasma can be evaluated. The sensitivity is such that insulin in plasma is at the limits of detection, and needs to be increased 10–100-fold before this will be useful.

While as little as 10 ng of bovine insulin could be detected, we routinely evaluated 50–200 μg of total protein in pancreas extracts. Using this amount even insulin is visible on the Coomassie blue stained gel (see Figure 4A). But the dye is not specific for insulin, while the antibody detects only immunoreactive protein as seen in Figure 4B. No insulin precursors were detected under these conditions. In separate experiments we found that as little at 500 ng of proinsulin could be visualized, and with proinsulin specific antibody this could be improved 10-fold. Therefore evaluation of insulin precursors in tissue extracts may be possible if proper conditions are chosen.

Since differences in both size and net charge can be detected the method can be used to screen for variant insulins in diabetic and nondiabetic pancreas. Analysis of large numbers of samples can be performed on one gel, while older methods required either column chromatography or electrophoresis of each individual sample, followed by slicing the gels into multiple pieces which are extracted and immunoassayed. With the current method resolution is far better than column chromatographic separation of insulin. The procedure takes two days for electrophoresis, transfer, and immunodetection, and 12–20 h for autoradiography. Preliminary results suggest that this method can be used to detect nanogram quantities of growth hormone in pituitary extracts and may be useful for other small polypeptide hormones.

FIGURE 4. Immunoelectrophoretic analysis of dog and human pancreas. Pancreatic extracts were electrophoresed, electroblotted, and immunodetected with anti-immune insulin serum and ^{125}I-protein A as described in METHODS. Lane 1, dog pancreas, lane 2, human diabetic pancreas, and lane 3, normal human pancreas. A. Coomassie blue stained gel, B. Radioautograph of the gel in A after treatment with anti-insulin serum and ^{125}I-protein A. The arrow indicates the mobility of bovine insulin standard.



ACKNOWLEDGMENTS

The authors would like to thank Drs. Stephen Giddings and Peter Rotwein for reading the manuscript and for their helpful discussions, Pamela Helms for assistance in preparing the manuscript, and Drs. C. Kilo and Marie Greider for providing the human pancreas.

This study was supported by NIH research grant AM 25940 and Research Career Development Award AM 00333.

REFERENCES

[1] Southern, E. M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*:503–17, 1975.

[2] Vaessen, R. T. M. J., Kreike, J., and Groot, G. S. P.: Protein transfer to nitrocellulose filters. FEBS Lett. *124*:193–96, 1981.

[3] Towbin, H., Staehelin, T., and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. *76*:4350–54, 1979.

[4] Renart, J., Reiser, J., and Stark, G. R.: Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc. Natl. Acad. Sci. *76*:3116–20, 1979.

[5] Symington, J., Green, M., and Brackmann, K.: Immunoautoradiographic detection of proteins after electrophoretic transfer from gels to diazopaper: analysis of adenovirus encoded proteins. Proc. Natl. Acad. Sci. *78*:177–81, 1981.

[6] Albert, S. G., and Permutt, M. A.: Proinsulin precursors in catfish pancreatic islets. J. Biol. Chem. *254*:2483–92, 1979.

[7] Davis, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. *121*:404–27, 1964.

[8] Laemli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) *227*:680–85, 1970.

[9] Johnson, G. L., MacAndrew, V. I., and Pilch, P. F.: Identification of the glucagon receptor in rat liver membranes by photoaffinity crosslinking. Proc. Natl. Acad. Sci. *78*:875–78, 1981.

[10] Greenwood, F. C., Hunter, W. M., and Glover, J. S.: The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. *89*:114–23, 1963.

[11] Kakita, K., Giddings, S., and Permutt, M. A.: Biosynthesis of rat insulins I and II: Evidence for differential expression of the two genes. Proc. Natl. Acad. Sci. In press.

[12] Ramshaw, J. A. M., Coyne, J. A., and Lewontin, R. C.: The sensitivity of gel electrophoresis as a detector of genetic variation. Genetics *93*:1019–37, 1979.

[13] Weber, K., and Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. *244*:4406–12, 1969.

[14] Pilch, P. F., and Czech, M. P.: Interaction of cross-linking agents with the insulin effector system of isolated fat cells. Covalent linkage of ^{125}I-insulin to a plasma membrane receptor protein of 140,000 daltons. J. Biol. Chem. *254*:3375–81, 1979.