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Photoaffinity Labeling of Insulin Receptor Proteins of Liver Plasma Membrane Preparations†

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ABSTRACT: The photoreactive insulin derivatives $N^{\epsilon B29}$-(azidobenzoyl)insulin (MAB-insulin) and $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azidobenzoyl)insulin (DAB-insulin) were synthesized by reacting bovine insulin with the *N*-hydroxysuccinimide ester of 4-azidobenzoic acid. These derivatives were purified by ion-exchange chromatography on SP-Sephadex, and their identities were established by polyacrylamide gel electrophoresis, amino acid analysis, and end-group determination. Their biological activities were measured by receptor binding assay and fat cell assay. The photoreactivity of these two derivatives was demonstrated by spectral changes and by the formation of covalent polymers of high molecular weight when exposed to light. Radioactive MAB-insulin and DAB-insulin were prepared by iodination with [^{125}I]iodine. These radioactive derivatives were characterized for their photoreactivity, immunoreactivity, and receptor binding to liver plasma membrane. Liver plasma membrane preparations of rat, mouse, and guinea pig were incubated with these radioactive insulin derivatives and irradiated with light. Sodium dodecyl sulfate gel electrophoresis of these plasma membrane preparations after solubilization and reduction showed that two proteins were specifically labeled. The molecular weights of the two radioactive bands were estimated to be about 130 000 and 90 000 in all three species of animals.

The initial event in the action of insulin, like other polypeptide hormones, is its binding to specific receptor on the plasma membrane of the target tissues (Cuatrecasas, 1969; Roth, 1973). Studies on the characterization of the insulin receptor have been indirect in that either the receptor is identified and characterized after disruption of the plasma membrane by solubilization or its characterization is deduced from the effects of enzymic or chemical modifications of the membrane on the binding of insulin (Cuatrecasas, 1971, 1972; Ginsberg et al., 1976; Jacobs et al., 1977). In order to identify the insulin receptor in situ, we have applied the technique of photoaffinity labeling which has been used successfully to label specific functional sites on cell membranes (Haley & Hoffman, 1974; Cabantchik et al., 1976; Ji, 1977; Das et al., 1977; Rosenblit & Levy, 1977; Trosper & Levy, 1977; Bregman & Levy, 1977). Levy (1973) had reported on the preparation of photoreactive aryl azide derivatives of insulin, but the use of these derivatives to label insulin receptor proteins of plasma membrane was not described. In a preliminary communication (Yip et al., 1978), we reported the synthesis and use of a photoreactive insulin derivative, (4-azidobenzoyl)insulin, to label specifically a protein ($M_r \sim 125\,000$) in the plasma membrane of rat adipocytes. The photoreactive insulin preparation used in that preliminary study was a mixture of mono- and di(azidobenzoyl) derivatives of insulin obtained by reacting ^{125}I-labeled insulin with (4-azidobenzoyl)-*N*-hydroxysuccinimide. In the present study, we first synthesized, purified, and characterized two photoreactive insulin analogues: $N^{\epsilon B29}$-(azidobenzoyl)insulin and $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azido-

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benzoyl)insulin. These analogues were then iodinated with [^{125}I]iodine and then used in the labeling of insulin receptor proteins in plasma membranes prepared from the livers of rat, mouse, and guinea pig.

Experimental Procedures

Materials. Crystalline bovine insulin was a gift from the Connaught Laboratories, Ltd., Canada. Carrier-free [^{125}I]-iodine in NaOH and D-[2-^{3}H]glucose were purchased from New England Nuclear Corp., Boston. *N*-Hydroxysuccinimide ester of 4-azidobenzoic acid was prepared according to the method of Galardy et al. (1974). Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Solutions of urea prepared from urea obtained from Aldrich Chemicals were treated with activated charcoal powder before use. All other chemicals were reagent grade.

Preparation of (Azidobenzoyl)insulins. Crystalline bovine insulin, 48 mg, was suspended in 2 mL of redistilled dimethylformamide and 40 μL of redistilled triethylamine in a siliconized 35-mL conical glass centrifuge tube. To this suspension was added 32 mg of (4-azidobenzoyl)-*N*-hydroxysuccinimide. The reaction mixture was stirred magnetically at room temperature in the dark for 60 min. (Azidobenzoyl)insulin was precipitated from the reaction mixture by the sequential addition of 3 mL of ice-cold absolute ethanol and 5 mL of ice-cold diethyl ether. After 60 min in an ice bath, the precipitate was recovered by centrifugation and lyophilized.

Separation and Purification of (Azidobenzoyl)insulins. $N^{\epsilon B29}$-(Azidobenzoyl)insulin and $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azidobenzoyl)insulin were separated and purified from the precipitate obtained above by ion-exchange chromatography on a SP-Sephadex column (2.7 × 50 cm). SP-Sephadex C-25 was equilibrated in 1.5 M acetic acid containing 0.02 M NaCl and 7 M urea. The precipitate was dissolved in 4 mL of urea–acetic acid for chromatography. The column was developed at a flow rate of 60 mL/h with a salt gradient generated by the use of 500 mL of the starting urea–acetic acid and 500 mL of urea–acetic acid containing 0.4 M NaCl. The eluate was collected in fractions of 5 mL. Fractions containing the insulin derivative were pooled, dialyzed, and lyophilized. The lyophilized material was rechromatographed under the same conditions to yield the desired (azidobenzoyl)insulin which was desalted by gel filtration and stored as a lyophilized powder at −20 °C in the dark.

Iodination of (Azidobenzoyl)insulins. The (azidobenzoyl)insulin, 0.5 mg, was suspended in 0.25 mL of water and made soluble by the addition of 2 μL of 20% NaOH, followed immediately by the addition of 0.25 mL of 0.4 M phosphate buffer, pH 7.4. Ten microliters of this solution was reacted with 1 mCi of carrier-free [^{125}I]iodine and Chloramine-T (Hunter & Greenwood, 1962). The iodinated material, after purification on a column (0.5 × 6 cm) of cellulose powder, was stored at −20 °C in 0.1-mL lyophilized aliquots. It remained stable for at least 1 month. Specific activity of the iodinated material averaged about 150 μCi/nmol.

Preparation of Liver Plasma Membrane. Plasma membrane was prepared according to the method of Neville (1968) (to step 11) from the livers of 100-g male Sprague–Dawley rats, 500-g male guinea pigs, and 2–3-month-old 15–20-g Swiss mice. 5′-Nucleotidase (Heppel & Hilmoe, 1955) and Na/K-ATPase (Uesugi et al., 1971) were assayed as marker enzymes. The membrane preparations were stored in aliquots kept frozen at −76 °C.

Binding of ^{125}I-Labeled (4-Azidobenzoyl)insulin to Liver Plasma Membrane. The binding of ^{125}I-labeled (4-azidobenzoyl)insulin to liver plasma membrane was carried out as described previously for adipocyte plasma membrane (Yip et al., 1978). Krebs-Ringer bicarbonate buffer, pH 7.4, was added to suspend the plasma membrane and to dissolve the insulin. The volume of the reaction mixture was 0.45 mL, and the amount of membrane protein used was approximately 2.8, 1.0, and 1.0 mg/mL, respectively, for rat, guinea pig, and mouse liver. The concentration of radioactive (azidobenzoyl)insulin was approximately 15 nM. Binding was carried out in plastic microfuge tubes at room temperature for 30 min in the dark. The mixture was then photolyzed for 20 min, by using two 250-W General Electric sun lamps at a distance of approximately 15 cm. During photolysis, the tubes were kept at 4 °C in a circulating cold-water bath constructed of Lucite. After photolysis, the membrane was recovered by centrifugation in a Beckman-Spinco microfuge for 5 min and stored frozen for further processing.

Photolysis of (Azidobenzoyl)insulins. The purified (azidobenzoyl)insulin derivatives (1 mg/mL in phosphate-buffered saline, pH 7.4) were photolyzed in small siliconized glass tubes, kept at 4 °C in the cold-water Lucite bath, by using two sun lamps at a distance of approximately 15 cm. Aliquots were taken at various time intervals and quickly frozen for further analysis. To study the effect of light on spectral changes, we directly photolyzed the insulin derivative in quartz cells using one sun lamp at a distance of approximately 15 cm. Since the irradiation time was short, it was not necessary to keep the sample cool. Spectral changes at 30-s intervals were measured in a Cary-15 recording spectrophotometer.

Polyacrylamide Gel Electrophoresis. The discontinuous buffered $NaDodSO_4$[1]–polyacrylamide slab gel electrophoresis method (Ames, 1974) was used to analyze the plasma membrane pellet after photolysis. The pellet was reduced with 0.2 mL of 0.1 M dithiothreitol in 2% $NaDodSO_4$ by boiling for 5 min. The soluble material obtained after centrifugation in a microfuge for 5 min was analyzed in 10% gel. Approximately equal amounts of radioactivity in 50–100 μL were applied to the gel slots for comparison. After staining with Coomassie blue and destaining, we dried the gel slab for radioautography on Kodak RP Royal X-OMat film using a Du Pont Cronex Lightning-Plus intensifying screen (Swanstrom & Shank, 1978). Purity of the (azidobenzoyl)insulins was determined by polyacrylamide slab gel electrophoresis by the procedure of Poole et al. (1974) modified to obtain 15% gel containing 8 M urea and 0.9 M acetic acid which separates insulin derivatives according to the number of net positive charges. The $NaDodSO_4$ slab gel system described by Busse & Carpenter (1976) was used to demonstrate polymer formation.

Other Analytical Methods. The biological activity of insulin derivatives was determined by using isolated rat adipocytes as described (Moody et al., 1974). Concentrations of the insulin derivatives were determined by amino acid analysis. Receptor binding assay was carried out by using rat liver plasma membrane and radioactive monoiodinated insulin as we have described (Yip & Moule, 1976). The same procedure was used to study the liver plasma membrane binding of iodinated radioactive photoreactive insulin analogues which were used at a concentration of 15 nM. Qualitative end-group

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Boc, *tert*-butoxycarbonyl; FDNB, fluorodinitrobenzene; MAB-insulin, $N^{\epsilon B29}$-(azidobenzoyl)insulin; DAB-insulin, $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azidobenzoyl)-insulin; TAB-insulin, $N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$-tri(azidobenzoyl)insulin; CBM-insulin, $N^{\alpha A1}$,$N^{\epsilon B29}$-carbonylbis(L-methionyl)insulin; DB-insulin, $N^{\alpha A1}$,$N^{\epsilon B29}$-bis(*tert*-butoxycarbonyl)insulin.

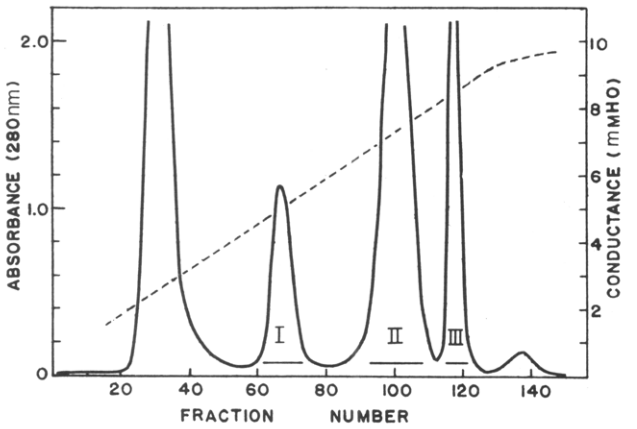

FIGURE 1: Ion-exchange chromatography of (azidobenzoyl)insulins on SP-Sephadex C-25. Experimental details are described in the text.

analysis using dansyl chloride in the presence of $NaDodSO_4$ and two-dimensional thin-layer chromatography was carried out according to the method of Weiner et al. (1972). Quantitative determination of end group was performed by using fluorodinitrobenzene as described by Africa & Carpenter (1970). Protein was determined by the Lowry method modified for membrane proteins (Markwell et al., 1978). Tryptic digestion was carried out by using 0.3 mg of the sample dissolved in 0.2 mL of 0.001 N HCl. An aliquot (0.1 mL) was used for concentration determination on the amino acid analyzer after acid hydrolysis. The remaining portion (0.1 mL) was lyophilized and redissolved in 0.1 mL of 0.05 M Tris at pH 8.0 and digested with 10 μL of trypsin (TPCK–trypsin at 1.5 mg/mL in 0.001 N HCl and 0.001 N $CaCl_2$) at 24 °C for 4 h. Digestion was terminated by adding 0.1 mL of 0.2 M sodium citrate with 2% (v/v) of thiodiglycol (25% w/w in water) at pH 2.2. Release of alanine was quantitated by amino acid analysis using 75 μL of the digest.

Results and Discussion

Purification and Characterization of (Azidobenzoyl)-insulins. Insulin contains three primary amino groups (glycine A-1, phenylalanine B-1, and lysine B-29) that can react with the activated ester of 4-azidobenzoic acid. Under our experimental conditions, when insulin was reacted with the activated ester, a mixture of mono-, di-, and trisubstituted insulins was obtained with a negligible amount of unreacted insulin. As shown in Figure 1, ion-exchange chromatography of the mixture on SP-Sephadex in the presence of urea resolved the mixture into three protein peaks, I, II, and III, which were eluted after an unretarded ultraviolet absorbing component, presumably a hydrolyzed product of the ester. Polyacrylamide gel electrophoresis of the three protein peaks after rechromatography established that I, II, and III were tri-, di-, and monosubstituted, respectively, when their electrophoretic mobilities were compared to those of standard insulin derivatives with known substitutions (Figure 2). Amino-terminal determination using dansyl chloride showed no detectable terminus in I, only phenylalanine in II, and glycine and phenylalanine in III, suggesting that all three amino groups were modified in I, the amino groups of glycine A-1 and lysine B-29 were modified in II, and the amino group of lysine B-29 was modified in III. The sites of modification were further determined by the use of fluorodinitrobenzene. The three amino groups of insulin react quantitatively with this reagent at pH 8.0. The recovery of glycine, phenylalanine, and lysine from the FDNB-treated insulin after acid hydrolysis is decreased by one residue each when compared with insulin not treated

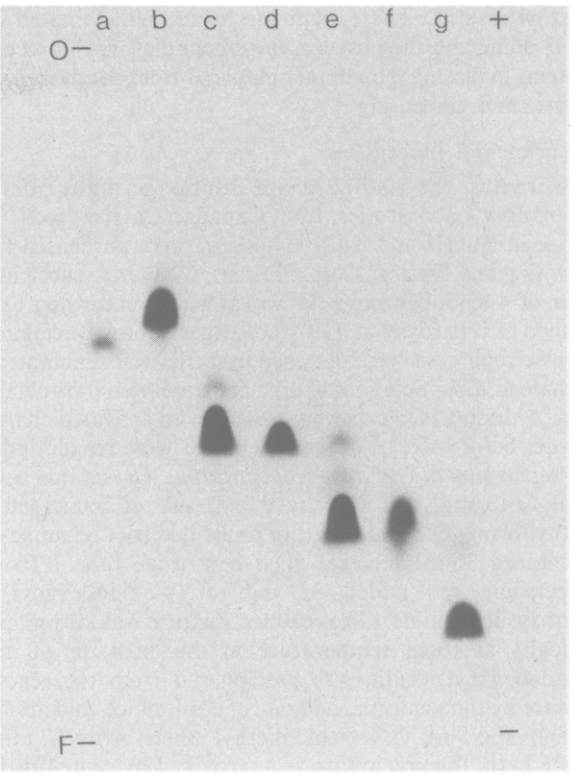

FIGURE 2: Urea–acetic acid polyacrylamide gel electrophoresis of rechromatographed materials of peaks I, II, and III. (a) Peak I; (b) $N^{\alpha A1}$,$N^{\alpha B1}$,$N^{\epsilon B29}$-tris(*N*-mesyl-L-methionyl)insulin (Yeung, 1976); (c) peak II; (d) CBM-insulin (Busse & Carpenter, 1976); (e) peak III; (f) $N^{\alpha B1}$-(*N*-mesyl-L-methionyl)insulin (Yeung, 1976); (g) bovine insulin. O, origin; F, front.

Table I: Amino Acid Analyses of Photoreactive Bovine Insulin Derivatives with (+) and without (–) FDNB Treatment

amino acid	no. of residues[a] MABI (–)	MABI (+)	DABI (–)	DABI (+)	theoretical[b] for insulin (–)	theoretical[b] for insulin (+)
Asp	2.94	2.80	2.99	2.73	2	3
Thr	0.78	1.06	0.78	0.90	1	1
Ser	2.53	2.48	2.55	2.53	3	3
Glu	7.05	7.20	7.01	7.27	7	7
Pro	1.08	0.98	1.01	0.82	1	1
Gly[c]	4.20	3.01	4.16	3.98	4	3
Ala	3.12	3.03	3.25	3.00	3	3
$^1/_2$-Cys	N.D.	5.58	N.D.[d]	5.54	6	6
Val	4.72	4.82	4.78	4.45	5	5
Met						
Ile	0.55	0.56	0.62	0.63	1	1
Leu	6.20	6.21	6.15	6.22	6	6
Tyr	3.50		3.78		4	
Phe[c]	3.10	2.05	2.91	2.31	3	2
Lys[c]	0.95	1.19	0.96	0.98	1	
His	2.10		2.06		2	
Arg	1.05	1.16	1.03	1.12	1	1

[a] Based on Asp plus Glu = 10. [b] Theoretical values according to Ryle et al. (1955). [c] Amino acid residues of interest. [d] N.D., not determined.

with FDNB. Therefore, by comparing the amino acid composition of the insulin derivative with and without FDNB treatment, one can determine the exact location of the amino group where chemical modification has occurred. Amino acid analyses (Table I) of these materials before and after reaction with FDNB confirmed that II was $N^{\alpha A1}$,$N^{\epsilon B29}$-di(azidobenzoyl)insulin (DAB-insulin) and III was $N^{\epsilon B29}$-(azidobenzoyl)insulin (MAB-insulin). These two insulin derivatives

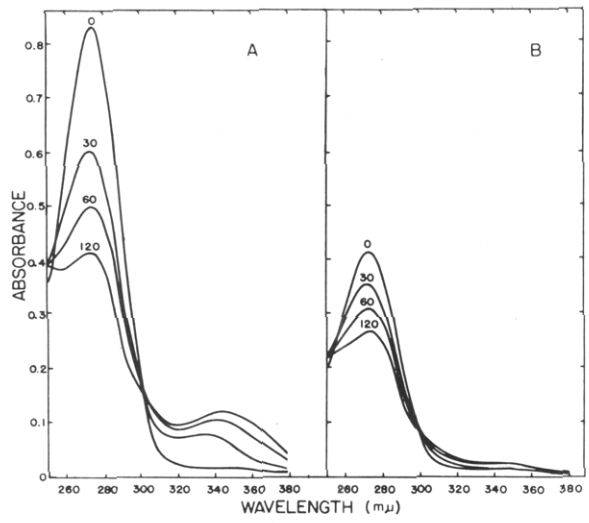

FIGURE 3: Ultraviolet spectral changes of DAB-insulin (A) and MAB-insulin (B) during photolysis. The derivative was dissolved in phosphate buffer, pH 7.4, at a concentration of 150 and 100 μg/mL, respectively, for DAB-insulin and MAB-insulin and photolyzed in the quartz cell for the total time (in seconds) indicated.

were further characterized by trypsin digestion for the quantitative release of the carboxyl-terminal B-30 alanine. In the case of DAB-insulin, no alanine was detected after tryptic digestion, confirming that the B-29 lysine residue was quantitatively modified. Tryptic digestion of MAB-insulin released 9% of the B-30 alanine, indicating that 9% of the B-29 lysine was not modified. Since the electrophoretic mobility of this derivative showed the loss of one positive charge, the result of the tryptic digestion suggests that MAB-insulin contained 91% $N^{\epsilon B29}$-(azidobenzoyl)insulin and the remainder represents monosubstitution at the A-1 glycine or the B-1 phenylalanine. The formation of the tri-, di-, and mono(azidobenzoyl) derivatives of insulin was in keeping with the known reactivity of the amino groups of glycine A-1, phenylalanine B-1, and lysine B-29 of insulin (Geiger et al., 1971). Although the characterization of these insulin derivatives could not rule out completely the possibility of other amino acid residues being modified, the mild reaction conditions used (60 min at room temperature) would tend to minimize this possibility.

The photoreactivity of these purified derivatives was established by their rapid spectral changes when irradiated with light (Figure 3). Their ability to cross-link intermolecularly during photolysis was demonstrated by the formation of polymers of high molecular weight when exposed to light (Figure 4). Polymer formation was a light-dependent process. The apparent discrepancy between reactivity measured by spectral changes and that measured by polymer formation is due to the use of quartz cells in the spectral study and the use of plastic tubes in the polymerization experiment.

TAB-insulin was devoid of biological activity in the rat adipocyte assay whereas DAB-insulin and MAB-insulin had approximately 21 and 67% of biological activity, respectively, when compared with insulin. Dose–response analysis of the biological activity of DAB-insulin and MAB-insulin showed no decrease in their intrinsic activity while their apparent affinity of binding was decreased (Figure 5A). This decrease in receptor binding was confirmed by the results of the receptor binding assay (Figure 5B). The apparent binding affinity, estimated from the bioassay, was 1.4×10^{-10} and 3.8×10^{-11} M for DAB-insulin and MAB-insulin, respectively, compared to 2.7×10^{-11} M for bovine insulin. The loss of biological activity in TAB-insulin and DAB-insulin where the α-amino group of Gly A-1 was modified is consistent with the important

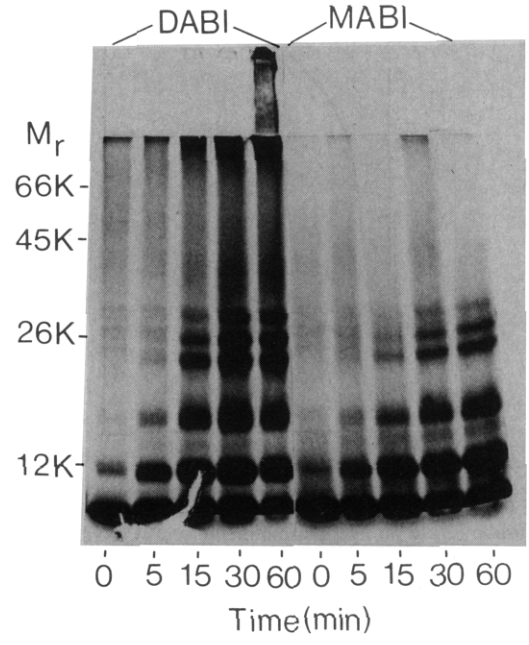

FIGURE 4: Time-dependent photolysis induced polymerization of DAB-insulin and MAB-insulin. Electrophoresis of the aliquots taken at different time intervals was carried out by using $NaDodSO_4$ slab gel according to the method of Busse & Carpenter (1976).

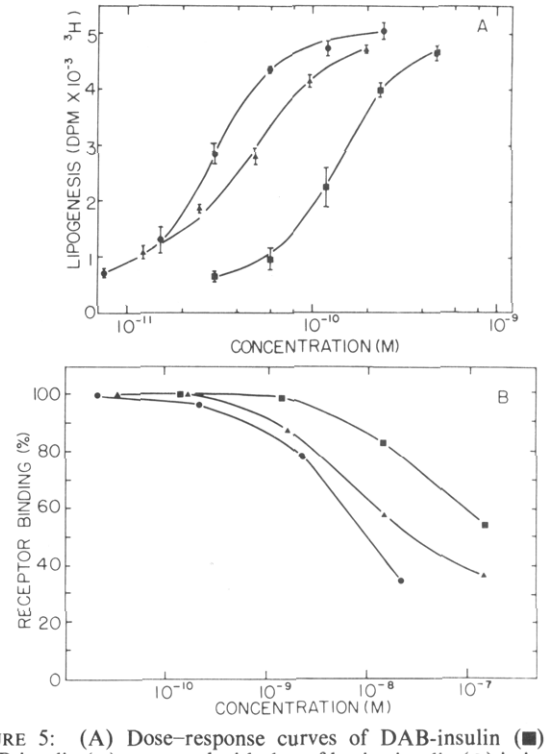

FIGURE 5: (A) Dose–response curves of DAB-insulin (■) and MAB-insulin (▲) compared with that of bovine insulin (●) in isolated fat cell assay. Vertical bars represent SE. (B) Receptor binding assay of DAB-insulin (■) and MAB-insulin (▲) compared with that of bovine insulin (●). Values are the average of triplicate determination.

role of this amino terminus in the action of insulin (Brandenburg & Ooms, 1969; Katsoyannis & Zaluts, 1972; Friesen et al., 1977). However, the decrease of activity in MAB-insulin where only the amino group of Lys B-29 was substituted was slightly higher than that obtained when this amino group was acetylated (Brandenburg & Ooms, 1968; Katsoyannis & Zaluts, 1972; Friesen et al., 1977). This might be the result of the neutralization of charge and the addition of the bulky and nonpolar azidobenzoyl group as well as the possibility that

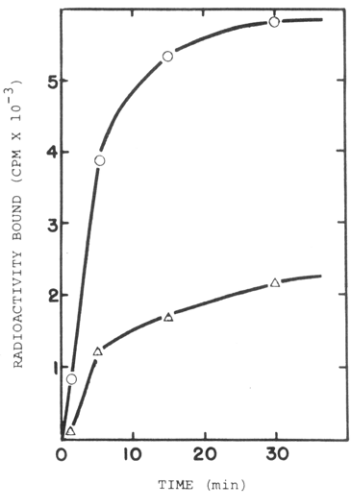

FIGURE 6: Specific binding of ^{125}I-labeled DAB-insulin (△) and MAB-insulin (○) to rat liver plasma membrane at room temperature. Concentration of the derivative was 15 nM. Nonspecific binding was approximately 30% of the total binding. Values are the average of duplicate determination.

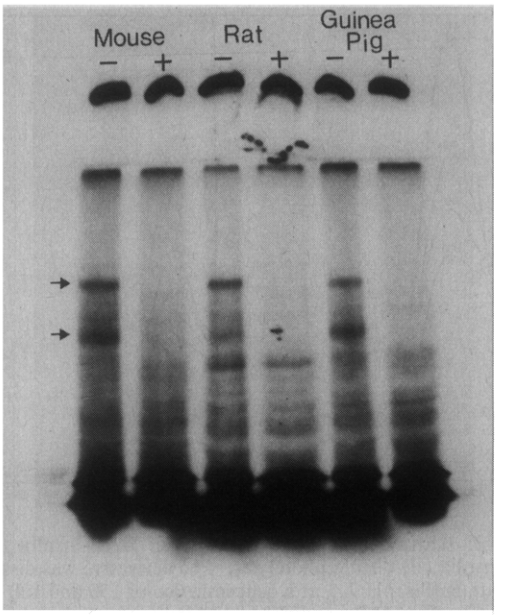

FIGURE 7: Radioautograph of liver plasma membrane proteins labeled with ^{125}I-labeled MAB-insulin, in the absence (−) and presence (+) of 50 μg of bovine insulin. The arrows indicate the two radioactive bands specifically displaced by excess insulin.

the MAB-insulin preparation might contain as much as 9% $N^{\alpha A1}$-(azidobenzoyl)insulin.

Radioiodination of (Azidobenzoyl)insulins and Photoaffinity Labeling of Liver Plasma Membrane Proteins. In our preliminary study (Yip et al., 1978) we prepared the photoreactive insulin from radioiodinated insulin obtained by iodination using lactoperoxidase. The radioactive photoreactive insulin so obtained was a mixture of unreacted radioactive insulin and derivatives. The proportion of these components in the preparation cannot be adequately controlled and the preparation as such is not easily amenable to separation, purification, and characterization. In this study, we have been able to obtain three photoreactive insulin derivatives TAB-, DAB-, and MAB-insulin in pure form for characterization with respect to their chemical nature and biological activity. Since TAB-insulin was found to be biologically inactive, it was not used for further study. To obtain radioactive photoreactive insulins, we labeled DAB-insulin and MAB-insulin with ^{125}I by using Chloramine-T. Sulfitolysis (Dixon & Wardlaw, 1960) of the radioactive DAB-insulin and MAB-insulin showed that approximately 20% of the ^{125}I label was on the B chain. Tryptic digestion showed that 65% of the ^{125}I label on the B chain was on tyrosine B-16 and the remainder was on the azidobenzoyl group.

More than 90% of the radioactivity in both derivatives was precipitable by antiinsulin serum. Both iodinated DAB-insulin and MAB-insulin were bound by rat liver plasma membrane (Figure 6). ^{125}I-Labeled DAB-insulin was less actively bound as expected from its lower apparent binding affinity. Binding of these derivatives approached equilibrium after 30 min at room temperature. Using the procedure we described in our previous communication (Yip et al., 1978), the radioactive derivatives when photolyzed cross-linked covalently to bovine serum albumin nonspecifically and to the heavy and light chains of antiinsulin immunoglobulin specifically (data not shown). This covalent cross-linking reaction was completely dependent on light. Binding of the radioactive MAB-insulin to liver plasma membrane of rat, mouse, and guinea pig following exposure to light resulted in the covalent labeling of a great number of membrane proteins as revealed by radioautography (Figure 7). The labeling of these membrane proteins was totally dependent on photolysis, since no labeling was observed in the absence of light. Two major protein bands were specifically and intensely labeled since their labeling was totally abolished by an excess of native insulin. In the three species of animals studies, the molecular weight of these two radioactive bands was estimated to be 130000 and 90000. The detection of these two radioactive bands required the reduction of the solubilized membrane with dithiothreitol. In our previous communication (Yip et al., 1978) we considered and ruled out the possibility of cross-linking of proteins of lower molecular weight by the photoreactive insulins. In this study and specifically in the case of radioactive MAB-insulin, this possibility is definitely eliminated because there is only one photoreactive group. Thus, the two membrane proteins that were labeled specifically may have a molecular mass of about 130000 and 90000 daltons, respectively, less the molecular mass of the B chain of insulin since the A chain is cleaved off by reduction with dithiothreitol. This estimation may be qualified because of the anomalous electrophoretic behavior of membrane proteins in $NaDodSO_4$. A similar pattern of labeling was obtained when radioactive DAB-insulin was used. This similarity in labeling pattern was expected since the A-1 glycine and the B-29 lysine are in close proximity to each other (Adams et al., 1969). The labeling of these two proteins by the photoreactive insulin derivatives was affected by insulin analogues with partial activity in a manner reflecting the biological activity of the analogue (Figure 8). Inactive analogues gave rise to increased labeling of the two bands, possibly because they decreased the loss of the tracer ^{125}I-labeled MAB-insulin to nonspecific adsorption or degradation. In addition, the intensity of labeling showed a dose-dependent response to the addition of native insulin in nanogram per milliliter concentration (Figure 9). The labeling of two protein components in the liver plasma membrane preparations by MAB-insulin may be contrasted to our previous finding of only one labeled protein component having a M_r of about 130000 by using plasma membrane of rat adipocytes (Yip et al., 1978). This discrepancy may be due to the use of purified photoreactive insulin in the present study whereas in our previous experiments (Yip et al., 1978) the ^{125}I-labeled photoreactive insulin preparation contained a significant amount of unmodified radioactive insulin. It is conceivable that in those

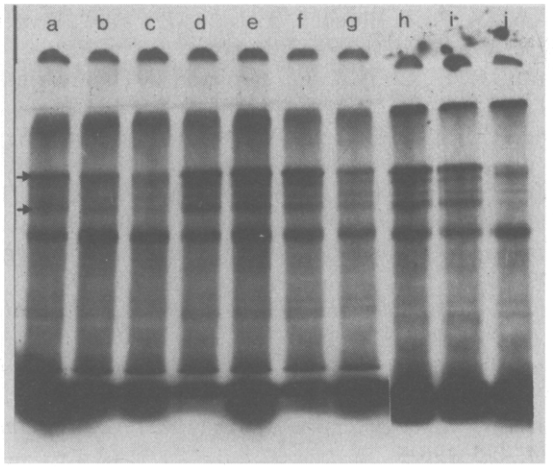

FIGURE 8: Radioautograph of rat liver plasma membrane labeled with ^{125}I-labeled MAB-insulin in the presence of 150 ng of natural or modified insulins. (a) No addition; (b) $N^{\alpha B1}$-arginylinsulin; (c) $N^{\alpha B1}$-methionylinsulin; (d) sulfonated A chain of insulin; (e) sulfonated B chain of insulin; (f) CBM-insulin; (g) DB-insulin; (h) bovine proinsulin; (i) guinea pig insulin; (j) bovine insulin. This figure is a composite of two radioautograms from two separate gel slabs, slots a–g from one and slots h–j from another. The biological activities of these insulin derivatives compared to those of native insulin are as follows: $N^{\alpha B1}$-arginylinsulin and $N^{\alpha B1}$-methionylinsulin, 38 and 64%, respectively (Yeung et al., 1979); sulfonated A and B chains, inactive (Dixon & Wardlaw, 1960); CBM-insulin, 6.5% (Busse & Carpenter, 1976); DB-insulin, 75% (Geiger et al., 1971); bovine proinsulin, 5% (Gliemann & Sorensen, 1970); guinea pig insulin, 10% (Zimmerman et al., 1974). The M_r 130 000 and 90 000 bands are indicated by the two arrows.

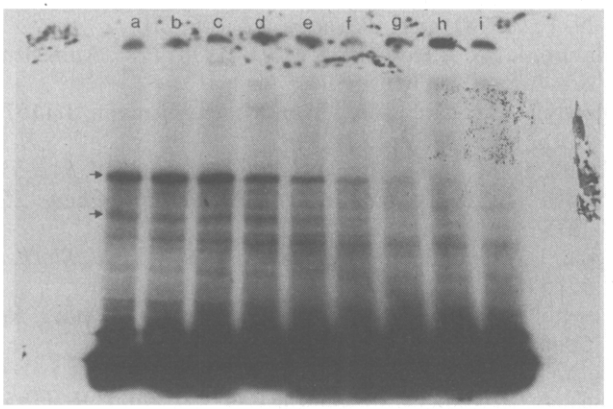

FIGURE 9: Radioautograph of rat liver plasma membrane proteins labeled with ^{125}I-labeled MAB-insulin in the presence of increasing amounts of native insulin. (a) 0 ng; (b) 8 ng; (c) 16 ng; (d) 32 ng; (e) 64 ng; (f) 125 ng; (g) 250 ng; (h) 500 ng; (i) 50 μg. The arrows indicate the two radioactive bands specifically displaced.

experiments the labeling of the proteins by the photoreactive insulins was considerably decreased because of the presence of the unmodified radioactive insulin which would compete more effectively for binding to the proteins. The net result of this competition would be a lower degree of photolabeling by the photoreactive insulins, making it difficult to detect the labeling of the second band. Indeed, when MAB-insulin was used in this study to label rat adipocyte plasma membrane prepared as described previously (Yip et al., 1978), two bands similar to the two in the liver plasma membrane preparations were now detected (Figure 10).

The radioautograms revealed much radioactivity at the gel front. Most of this radioactivity was due to the A chain which contained 80% of the ^{125}I label of the ^{125}I-labeled MAB-insulin and was split off the membrane-bound ^{125}I-labeled MAB-insulin after reduction with dithiothreitol for electrophoresis. In addition, ^{125}I-labeled MAB-insulin bound but not covalently

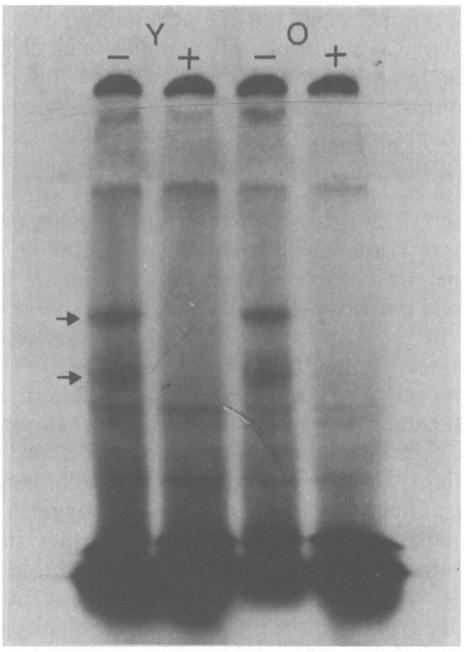

FIGURE 10: Radioautograph of rat adipocytes plasma membrane proteins labeled with ^{125}I-labeled MAB-insulin in the absence (–) and presence (+) of excess insulin. O and Y were two separate membrane preparations. The arrows indicate the two radioactive bands specifically displaced by excess insulin.

cross-linked to the membrane will migrate to the gel front after reduction and electrophoresis, thus adding to the radioactivity detected at the gel front. Therefore, the labeling of the two major protein bands was the net result of binding to the proteins and covalent cross-linking of ^{125}I-labeled MAB-insulin to these proteins through the photoreactive group on its B chain. The efficiency of covalent bond formation in photoaffinity labeling is known to be low because of side reactions during photolysis (Knowles, 1972; Baley & Knowles, 1977). In this study, covalent bond formation was further decreased due to the use of slightly opaque plastic tubes and water cooling which reduced light penetration.

Our observations as discussed above suggest strongly that the two membrane proteins labeled by the photoreactive insulin derivatives are involved in the specific recognition of insulin by the plasma membrane. Indeed, the photoreactive aryl azide groups at A-1 and B-29 are located within the activity domain of the insulin molecule (Wood et al., 1975). It is worth noting that in the liver plasma membrane of the guinea pig the two proteins labeled are similar, if not identical, to those of rat and mouse. Guinea pigs respond to pork or beef insulin better than to its own insulin, suggesting that their insulin receptors are similar to those of other mammals (Zimmerman et al., 1974). Ginsberg et al. (1976) demonstrated, in detergent-solubilized plasma membrane of turkey erythrocytes, the reversible insulin-induced dissociation of insulin receptor with a Stokes radius of 40 Å. Solubilized and purified insulin receptor of rat liver plasma membrane was estimated to have a M_r of about 135 000 in $NaDodSO_4$ electrophoresis as reported by Jacobs et al. (1977), who also postulated that the native insulin receptor with a Stokes radius of 72 Å might be composed of at least two or possibly more such subunits. In a recent communication, Maturo & Hollenberg (1978) reported on the isolation of a nonreceptor glycoprotein from solubilized rat liver plasma membrane, which, when added to an affinity-purified insulin receptor, caused an increase in the receptor's apparent Stokes radius from 38 to 72 Å. They concluded that the membrane glycoprotein might be a nonrecognition subunit

or an effector molecule. In our study, the specific labeling of two membrane proteins with different molecular weights could be interpreted in different ways. It may be that insulin receptors on the plasma membrane are heterogeneous or that they are homogeneous but composed of nonidentical subunits with M_r values of approximately 130000 and 90000, respectively, less the M_r of the B chain. Indeed, Katzen & Soderman (1972) originally reported preliminary evidence of two forms of Triton-solubilized insulin receptor of rat adipocyte membrane. Results reported recently by Krupp & Livingston (1978) were indicative of the presence of two species of insulin receptor in rat adipocyte membrane. Using bifunctional chemical cross-linking agents, Pilch & Czech (1979) also showed recently the covalent linkage of insulin to a fat cell plasma membrane protein of M_r 125000. In view of the available experimental evidence as discussed above, we favor the possibility that the receptor is composed of nonidentical subunits. Our data, however, do not distinguish between recognition and nonrecognition subunits. Since, in the case of MAB-insulin, more than 90% of the aryl azide is on the ϵ-amino group of B-29 lysine, there could be sufficient flexibility in the lysine side chain to allow for cross-linking to either one of the two dissimilar subunits if the association of these subunits is close enough. It is therefore possible that one of the two proteins labeled could be a nonrecognition moiety. Confirmation of this postulation would require the synthesis of photoreactive insulin analogues with the photoreactive group at different sites of the insulin molecule. This is currently being carried out in this laboratory.

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