Finally, raising the pH of the DEAE-Sepharose column from 5.8 to 6.0 eliminates the peak of inactive material that precedes the elution of functional carrier in the original procedure. When these modifications are used, the amount of lactose carrier recovered from the column, as judged by the recovery of [^{3}H]NPG-labeled tracer, is increased from 40% to 70%. The combination of the three modifications permits purification of *lac* carrier in 3-fold greater yield and on a scale 30-fold greater than that reported in the original procedure.

The rapid filter assay described here should be readily adaptable to the assay of symporters other than the *lac* carrier. Obviously, however, such applications require conditions for solubilization and reconstitution of these proteins in functional form. To date, relatively few prokaryotic carriers have been successfully reconstituted (Hirata et al., 1976; Lee et al., 1979; Newman & Wilson, 1980; Tsuchiya et al., 1982). The availability of a rapid means of surveying a wide variety of detergents and conditions should aid efforts in this direction.

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Modification of the Insulin Receptor by Diethyl Pyrocarbonate: Effect on Insulin Binding and Action†

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ABSTRACT: Insulin binding to rat liver plasma membranes is inhibited in a time- and dose-dependent fashion by prior treatment of membranes with the histidine-specific reagent diethyl pyrocarbonate. If all receptors are occupied by unlabeled hormone during diethyl pyrocarbonate treatment, no inhibition of ^{125}I-labeled insulin binding is observed following washout of unlabeled hormone and unreacted reagent. Scatchard analysis of the binding inhibition due to diethyl pyrocarbonate reveals a loss in receptor number rather than a change in receptor affinity for hormone. Fat cells treated with diethyl pyrocarbonate exhibit a rightward shift in the dose–response relationship for insulin-stimulated glucose oxidation consistent with a loss in receptor number due to the reagent. The pH profile for inhibition of insulin binding by diethyl pyrocarbonate and the partial reversibility of this inhibition by hydroxylamine are consistent with modification of a histidine residue. These results suggest that a histidine residue at or near the receptor binding site is required for formation of the biologically relevant insulin–receptor complex.

Insulin triggers a variety of metabolic responses in target tissue by specifically binding to a cell surface protein, the insulin receptor. A minimal subunit structure for the insulin receptor has recently been identified by several laboratories using techniques such as affinity chromatography (Jacobs et al., 1977, 1980a), photoaffinity labeling (Yip et al., 1978, 1980) affinity cross-linking (Pilch & Czech, 1979, 1980a), and purification on immobilized antireceptor antibody (Harrison & Itin, 1980). Taken together, these data support a structure for the insulin receptor that consists of a disulfide-linked tetramer of two M_r 125 000 subunits (α) and two M_r 90 000 subunits (β) (Massague et al., 1981). Both subunits are glycoproteins (Jacobs et al., 1980b; Hedo et al., 1981), and the β subunit has a site that is particularly sensitive to proteolysis (Massague et al., 1981).

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It has been assumed that insulin binds to this receptor structure and a conformational change takes place that in turn generates an intracellular signal or message. A hormone-induced receptor conformational change has in fact been postulated based on data showing differential proteolytic sensitivity of hormone-occupied vs.-unoccupied receptor (Pilch & Czech, 1980b). Moreover, several groups have presented evidence for the generation of a chemical mediator arising from insulin–receptor interaction that may be the hormone's messenger (Seals & Czech, 1981; Kiechle et al., 1981; Larner et al., 1979). This chemical mediator has not been isolated as yet and the details of signal generation are still undescribed. A variety of protein-modifying reagents are knwon to effect insulin binding and action when added to cells and membranes containing insulin receptors. These include trypsin (Kono & Barham, 1971; Cuatrecasas, 1971a; Pilch et al., 1981), dithiothreitol (Jacobs & Cuatrecasas, 1980; Schweitzer et al., 1980), and various chemical reagents (Cuatrecasas, 1971b). However, it cannot be determined from these previous studies what domain of the insulin receptor was modified that led to the observed changes in insulin binding parameters.

The present study was undertaken to more rigorously define the receptor functional group chemistry responsible for recognition of insulin. Liver and fat cell plasma membranes were treated with the histidine-reactive reagent diethyl pyrocarbonate (Miles, 1977). Insulin binding is shown to be rapidly diminished by this reagent. Importantly, the presence of unlabeled insulin during the diethyl pyrocarbonate treatment protects against binding inhibition. Diethyl pyrocarbonate reacts with proteins to preferentially give ethoxycarbonyl derivatives of histidine (Miles, 1977). The inhibition of binding due to this reagent exhibits a pH dependency consistent with histidine modification. These data support the notion that diethyl pyrocarbonate inhibits insulin binding by reacting with a histidine residue at the insulin binding domain of the receptor.

Materials and Methods

Preparation of Cells and Membranes. Fat cells were prepared from the epididymal fat pads of 150–200-g male Sprague-Dawley rats by collagenase digestion according to the procedure of Rodbell (1964). Minced fat pads were digested for 1 h in Krebs-Ringer phosphate buffer, pH 7.4, containing 1 mg/mL collagenase and 30 mg/mL bovine serum albumin. The digested fat pads were filtered through nylon mesh and washed twice in Krebs–Ringer phosphate buffer, pH 7.4, containing 30 mg/mL albumin. The cells were then used for bioassay or membrane preparation as described below.

Adipocyte plasma membranes were prepared from isolated fat cells according to the following protocol, which is a modification of the McKeel & Jarett (1970) procedure. Fat cells prepared as described above were washed an additional time with 10 mM Tris buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. The cells were suspended in 2–3 volumes of the Tris buffer and disrupted with six strokes of a motor-driven Potter-Elvehjem homogenizer. This mixture was centrifuged at 3000*g* for 10 min. The supernatant was separated from the pellet and fat cake and then layered onto 32% w/w sucrose in a Sorvall SV-288 vertical rotor tube. The homogenate was centrifuged for 1 h at 19 000 rpm in the vertical rotor, and the plasma membranes were collected from the top of the 32% sucrose layer. The plasma membranes were diluted with 10 mM Tris, pH 7.4, and pelleted at 30000*g* for 20 min. The membrane pellet was resuspended in 10 mM Tris, pH 7.4, at 2–4 mg of membrane protein/mL and stored at −20 °C prior to use.

Rat liver plasma membranes were prepared essentially as described by Carey & Hirschberg (1980). Livers were excised from 150–200-g male Sprague-Dawley rats and washed with ice-cold 5 mM Hepes buffer, pH 7.6, containing 0.5 mM benzamidine and 0.25 M sucrose. The livers were minced and homogenized in 1–4 volumes of Hepes buffer with four strokes of a mechanically driven Potter-Elvehjem tissue grinder. The homogenate was filtered through cheesecloth and spun 10 min at 2000*g*. The pellet was collected, mixed with a minimum of Hepes buffer, and resuspended with a loose-fitting Dounce homogenizer. This suspension was adjusted to 47% w/w sucrose by adding 66% w/w sucrose and monitoring the refractive index. The 47% sucrose suspension was added to a Beckman SW 27.1 centrifuge tube and overlayed with 41% w/w sucrose. Following centrifugation of the membranes for 90 min at 25 000 rpm, the plasma membrane button was collected from the top of the 41% sucrose layer. The plasma membranes were suspended in 50 mM Hepes, pH 7.6, with a Dounce homogenizer and were spun for 10 min at 30000*g*. The final pellet was resuspended in 50 mM Hepes to 4–6 mg/mL membrane protein and stored at −20 °C until use.

Insulin Binding Assay. The binding assay was initiated by adding 20 μL of plasma membrane suspension (80–120 μg of protein) in 50 mM Hepes, pH 7.6, to 180 μL of 50 mM Hepes, pH 7.6, containing 5 mg/mL bovine serum albumin and (3–6) $\times 10^{-10}$ M ^{125}I-labeled insulin. Unlabeled insulin was present at 1.75×10^{-6} M in some tubes to determine nonspecific binding. The data shown represent specific ^{125}I-labeled insulin binding where binding in presence of 1.75×10^{-6} M unlabeled insulin has been subtracted from the total binding. The nonspecific binding was never more than 20% of the total binding. Membranes were incubated with ^{125}I-labeled insulin for 30 min at 24 °C and then binding was terminated by the addition of 2.0 mL of ice-cold 50 mM Hepes, pH 7.6, containing 1 mg/mL albumin. The membranes were separated from the media by rapid filtration on 0.45–0.50-μm cellulose acetate filters (Amicon, Millipore EH, and Nucleopore "Membra-Fil" were all satisfactory). The membranes were rapidly washed with 4.0 mL of ice-cold Hepes buffer, pH 7.6, containing 1 mg/mL albumin, and bound insulin was determined with a γ counter. Experimental points are the average of triplicate determinations.

Treatment of Membranes with Group-Specific Reagents. Membranes were suspended in 50 mM Hepes buffer, pH 7.0 (but see Figure 3), at 4–5 mg/mL membrane protein. Diethyl pyrocarbonate, *N*-acetylimidazole, and tetranitromethane were freshly made up in ethanol or water just before use at 100-fold the desired final concentration. The reagent solution was then added to the membranes at a 1:100 dilution, and the reaction was allowed to proceed for the desired time. The reaction was quenched by the addition of a 5-fold excess of ice-cold 50 mM Hepes buffer, pH 7.6, and immediate centrifugation for 10 min at 30000*g*. The membrane pellet was resuspended at approximately 2 mg/mL of protein and centrifuged again for 10 min at 30000*g*. This pellet was then resuspended in 50 mM Hepes, pH 7.6, for addition to the binding medium. When membranes were pretreated with unlabeled insulin (7×10^{-7} M) prior to reagent addition, the first resuspension was followed by a 10-min incubation at room temperature before centrifugation. This incubation was repeated a second time before the final resuspension of the membrane pellet for addition to the binding assay.

Other Assays. Conversion of D-[1-^{14}C]glucose to $^{14}CO_2$ by isolated fat cells was performed as in Fain et al. (1967), exactly as described previously (Pilch et al., 1981). Protein was de-

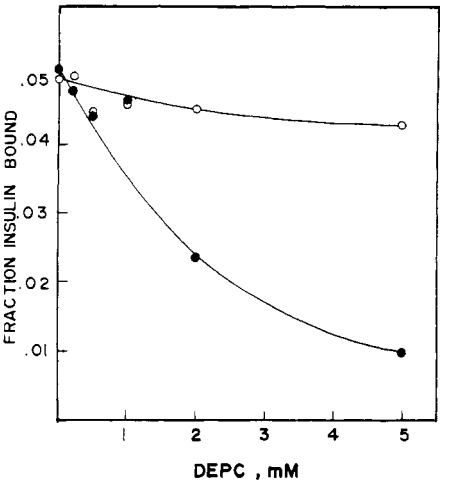

FIGURE 1: Effect of diethyl pyrocarbonate (DEPC) on insulin binding to rat liver plasma membrane. Liver plasma membranes (4–5 mg/mL in 50 mM Hepes, pH 7.0, 24 °C) were treated with diethyl pyrocarbonate for 5 min at the concentrations indicated in the presence (open circles) or absence (closed circles) of 7×10^{-7} M unlabeled insulin. The unlabeled insulin was added 15 min prior to the diethyl pyrocarbonate. The reaction was terminated by addition 5 volumes of ice-cold 50 mM Hepes, pH 7.6, and the membranes were rapidly centrifuged (10 min, 30000*g*). The membranes were resuspended in this buffer and incubated 10 min at 24 °C. An additional wash, centrifugation, and incubation were performed prior to determination of specific ^{125}I-labeled insulin binding as described under Materials and Methods.

termined by the method of Lowry et al. (1951).

Reagents. Monocomponent insulin was obtained from Dr. Ron Chance of the Eli Lilly Co. ^{125}I-Labeled insulin was prepared from insulin and Na^{125}I by using an immobilized glucose oxidase–lactoperoxidase enzymic method (Enzymobeads, Bio-Rad Laboratories) to a specific activity of 60–100 μCi/μg. All other reagents were purchased from Sigma Chemical Co.

Results

The concentration dependency for diethyl pyrocarbonate inhibition of insulin binding is shown in Figure 1. After 5 min of reaction, half-maximal inhibition of tracer ^{125}I-labeled insulin binding is observed at approximately 2 mM diethyl pyrocarbonate, and 80% inhibition of binding is achieved at 5 mM reagent. Significantly, the addition to membrane of 7×10^{-7} M unlabeled insulin 15 min prior to diethyl pyrocarbonate addition provides nearly complete protection from tracer binding inhibition. In these protocols, the membranes are thoroughly washed to remove unreacted reagent and unlabeled insulin prior to measurement of tracer ^{125}I-labeled insulin binding. The washes are completely effective in removing the unlabeled insulin since the control values (no diethyl pyrocarbonate) are the same for membranes pretreated with insulin or not. Thus, it appears that the unlabeled insulin is protecting a domain of the receptor from reaction with diethyl pyrocarbonate and the subsequent inhibition of tracer insulin binding.

The results shown in Figure 2 further support this hypothesis. This figure shows the time course of diethyl pyrocarbonate mediated inhibition of insulin binding in protocols identical with those employed for Figure 1. After 30 min of diethyl pyrocarbonate treatment, 10% of control binding remains. The presence of 7×10^{-7} M insulin during the 30-min incubation with diethyl pyrocarbonate affords significant protection against receptor inactivation. As expected from the dynamics of insulin–receptor interaction, the protective effect of insulin diminishes with increasing time of diethyl pyrocarbonate treatment. That is, over the time course of the experiment, an increasing number of receptors will have undergone a dissociation–reassociation event during which diethyl pyrocarbonate could inactivate the unoccupied receptor.

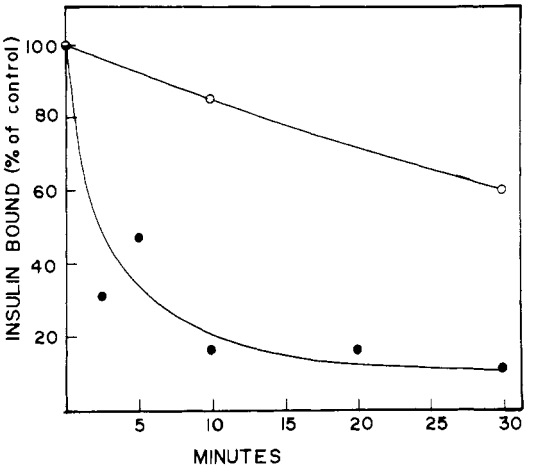

FIGURE 2: Time course of diethyl pyrocarbonate inhibition of insulin binding. Unlabeled insulin (7×10^{-7} M final concentration) was added (open circles) or not added (closed circles) to rat liver membranes suspended at 4–5 mg/mL in 50 mM Hepes, pH 7.0. After 15 min at 24 °C, a 1:100 dilution of 0.5 M diethyl pyrocarbonate in ethanol was added. Reaction was terminated at the times indicated by addition of ice-cold buffer (5 volumes of 50 mM Hepes, pH 7.0) and rapid centrifugation (30000*g*, 10 min). Subsequent washing and assessment of specific ^{125}I-labeled insulin binding capacity were exactly as described in the legend to Figure 1 and under Materials and Methods.

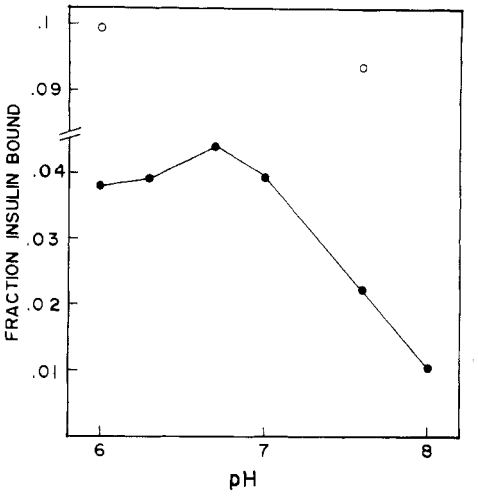

FIGURE 3: pH dependency of diethyl pyrocarbonate mediated insulin binding inhibition. Liver plasma membranes (4–5 mg/mL) were equilibrated with 50 mM Hepes buffer at the pHs indicted. Diethyl pyrocarbonate in ethanol was added to the membranes to a final concentration of 5 mM reagent and 1% ethanol. Reaction was allowed to proceed for 5 min at 24 °C and was quenched by the addition of 5 volumes of ice-cold Hepes buffer at the various pHs used followed by immediate centrifugation (10 min, 30000*g*). The membranes were washed an additional time in 50 mM Hepes, pH 7.6, prior to determination of specific ^{125}I-labeled insulin binding. The open circles represent insulin binding to membranes treated with 1% ethanol but not diethyl pyrocarbonate at the pH indicated.

Diethyl pyrocarbonate preferentially reacts with the imidazole moiety of histidine residues in proteins (Miles, 1977). The protonation of this imidazole usually exhibits a pK of about 7.0 and diethyl pyrocarbonate reacts with the unprotonated form. Figure 3 depicts the pH profile for inhibition of insulin binding by diethyl pyrocarbonate. Membranes treated with diethyl pyrocarbonate above pH 7.0 show substantially less binding than those treated at pH 7.0 or lower.

Table I: Effect of Hydroxylamine on Diethyl Pyrocarbonate Inhibited Insulin Binding[a]

addition 1	addition 2	insulin bound (pmol/mg of protein)	% of control
		0.069	100
	NH_2OH	0.063	90
DEPC		0.027	39
DEPC	NH_2OH	0.038	55

[a] Liver plasma membranes were treated with 5 mM diethyl pyrocarbonate (DEPC) for 5 min at 24 °C (addition 1). The reaction was quenched and membranes were washed as described in the legends to Figures 1–3. The membranes were then incubated for 1 h at 24 °C with 0.5 M hydroxylamine (NH_2OH), pH 7.0 (addition 2). The hydroxylamine was removed by addition of 5 volumes of 50 mM Hepes, pH 7.6, and centrifugation. This step was performed twice, then ^{125}I-labeled insulin binding was measured, and specific binding was determined as described under Materials and Methods.

The pH change alone does not effect binding (open circles). These results are consistent with modification of a receptor histidine residue by diethyl pyrocarbonate.

Histidine modification by diethyl pyrocarbonate can be reversed by hydroxylamine under the appropriate conditions (Miles, 1977). Accordingly, after diethyl pyrocarbonate exposure (5 mM, 5 min) membranes were incubated in 0.5 M hydroxylamine, pH 7.0, for 1 h at room temperature and were then thoroughly washed prior to assaying binding capacity (see Materials and Methods). Table I shows the results of a representative protocol employing hydroxylamine. Significant recovery from diethyl pyrocarbonate mediated binding inhibition is evident although this reversal is not complete. In all such experiments performed (five), hydroxylamine itself inhibited binding from 10 to 30% and recovery ranged from undetectable to nearly complete (90% of the value for hydroxylamine alone). It is known that diethyl pyrocarbonate can react twice with the same histidine residue to form a doubly derivatized adduct (Miles, 1977). The formation of this bis(ethoxycarbonyl)imidazole derivative is highly concentration dependent and is not reversible upon hydroxylamine treatment (Miles, 1977). It is likely that partial formation of this adduct accounts for the variable degree of reversibility observed in the present studies. Others have reported recoveries of activity from zero (Cromartie, 1981) to 80% (Fujioka et al., 1980) for hydroxylamine treatment of diethyl pyrocarbonate inactivated enzymes known to have essential hisitidine residues.

Inhibition of insulin binding by diethyl pyrocarbonate could be a result of a loss in receptor affinity for hormone or a loss in receptor number. The latter situation would arise if reaction of an individual receptor with diethyl pyrocarbonate led to a complete blockage of insulin binding to that receptor. Figure 4 shows the results of a Scatchard plot of insulin binding to untreated or diethyl pyrocarbonate treated liver plasma membranes. It is evident that the receptor retains its affinity for insulin (4×10^{-9} M) but a loss in high-affinity receptor number occurs. Thus, it appears that diethyl pyrocarbonate inhibits insulin binding in an all or none fashion.

Since diethyl pyrocarbonate effectively reduces insulin receptor number without altering its affinity for hormone, a rightward shift in the dose–response relationship between insulin and biological action of the hormone should be observable. Diethyl pyrocarbonate inhibits the binding of insulin to fat cell plasma membranes in exactly the same manner as it does to liver plasma membranes (data not shown). Therefore,

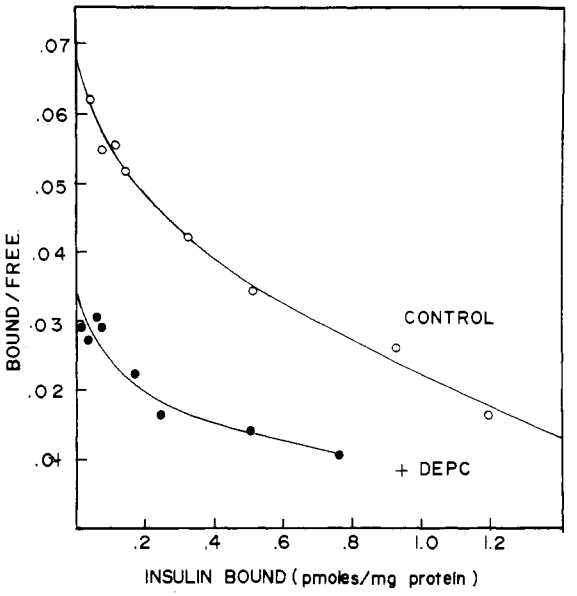

FIGURE 4: Scatchard plot of insulin binding to diethyl pyrocarbonate (DEPC) treated membranes. Rat liver plasma membranes were treated with 2 mM diethyl pyrocarbonate for 5 min at 24 °C (closed circles) or not treated (open circles). The reaction was terminated and membranes were washed exactly as described in the legends to Figures 1–3. ^{125}I-Labeled insulin binding was measured on treated and untreated membranes in the presence and absence of increasing amounts of unlabeled insulin. Bound ^{125}I-labeled insulin was separated from free insulin as described under Materials and Methods.

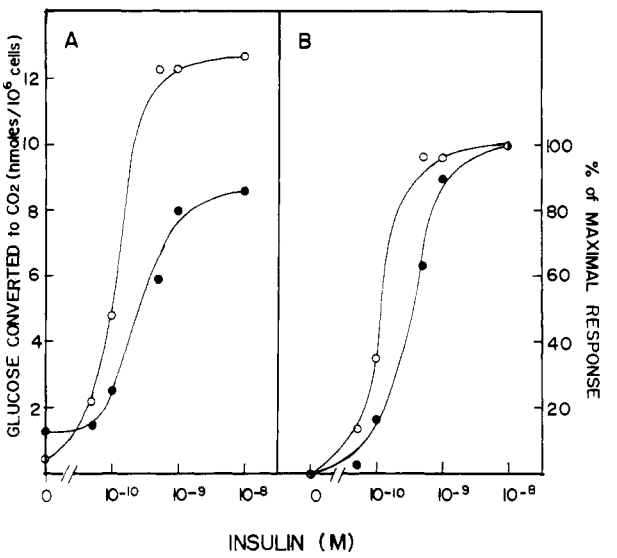

FIGURE 5: Effect of diethyl pyrocarbonate on fat cell glucose oxidation. Isolated fat cells were suspended in an equal volume of Krebs–Ringer phosphate buffer, pH 7.4, containing 30 mg/mL bovine serum albumin and were treated for 5 min at 24 °C with 5 mM diethyl pyrocarbonate and 1% ethanol (closed circles) or 1% ethanol alone (open circles). The reaction was quenched by adding 3–4 volumes of Krebs–Ringer phosphate buffer, pH 7.4, with 30 mg/mL albumin and rapidly centrifuging the cells. This wash was repeated once and the cells were distributed into individual tubes [(2–3) $\times 10^5$ cells/tube] for measurement of [1-^{14}C]glucose oxidation in the presence and absence of the insulin concentrations indicated. The incubation was for 30 min. The plot in panel B was obtained from the data in panel A. 100% equals the glucose converted to CO_2 at 10^{-8} M insulin minus the glucose oxidation at zero insulin.

the ability of fat cells to elevate glucose oxidation rates in response to insulin was measured in diethyl pyrocarbonate treated adipocytes as compared to untreated cells. As shown in Figure 5, cells treated with diethyl pyrocarbonate exhibit slightly elevated basal oxidation rates compared to control

Table II: Effect of Tetranitromethane and *N*-Acetylimidazole on Insulin Binding to Rat Liver Plasma Membranes[a]

reagent	insulin pre-treatment	insulin bound (pmol/mg of protein)	% of control
Experiment 1			
none		0.064	100
tetranitromethane		0.038	59
tetranitromethane	+	0.046	72
none	+	0.059	92
Experiment 2			
none		0.052	100
N-acetylimidazole		0.047	90

[a] Tetranitromethane was made up to 1 M in ethanol, and *N*-acetylimidazole was made up to 0.5 M in 50 mM Hepes, pH 7.6. The reagents were added at a 1:100 dilution to liver plasma membrane suspended (4–5 mg/mL) in 50 mM Hepes, pH 7.6, and reaction was allowed to proceed for 10 min at 24 °C. 7×10^{-7} M unlabeled insulin was added to some membranes 15 min prior to reagent addition. The reaction was terminated by the addition of 5 volumes of ice-cold Hepes, pH 7.6, and immediate centrifugation (10 min, 30000*g*). Unlabeled insulin and unreacted reagent were removed as described in the legend to Figure 1 prior to measurement of specific ^{125}I-labeled insulin binding capacity (see Materials and Methods).

values. In addition, the maximal response due to insulin is blunted by about 30% due to reagent addition. However, a rightward shift in the insulin dose–response curve is apparent as a result of diethyl pyrocarbonate exposure. This is more evident when the data are plotted as a percent of the maximal response due to insulin (Figure 5, panel B). Thus, the effect of diethyl pyrocarbonate on insulin binding (Figure 4) roughly correlates with the expected biological result of such an effect (Figure 5). It is quite probable that diethyl pyrocarbonate has postreceptor effects on fat cells since both basal and maximal glucose oxidation rates are altered by the reagent. An exact correlation between changes in insulin binding due to diethyl pyrocarbonate treatment and changes in insulin action would be unlikely in this circumstance. Nevertheless, the data of Figure 4 showing that diethyl pyrocarbonate treatment reduces apparent high-affinity insulin receptor numbers are qualitatively confirmed by the data shown in Figure 5 documenting a rightward shift in the dose–response curve to insulin following reagent addition.

It has previously been suggested that tyrosine residues are involved in insulin binding based on the ability of tetranitromethane to inhibit insulin binding to fat cell plasma membranes (Cuatrecasas, 1971b). The role of receptor tyrosines in insulin binding was reexamined in the present studies by using tetranitromethane and *N*-acetylimidazole, reagents with specificity for reaction with tyrosine (Sokolovsky et al., 1966; Riordan et al., 1965). As shown in Table II, tetranitromethane treatment (10 min, 10 mM) causes insulin binding to fall to 59% of control values, and insulin affords some protection against this action. *N*-Acetylimidazole has no significant effect on insulin binding. Thus, the inhibition of insulin binding due to tetranitromethane may implicate tyrosine residue participation in insulin binding (Cuatrecasas, 1971b), but these data are difficult to interpret (see Discussion).

Discussion

The data presented here show that diethyl pyrocarbonate inhibits insulin binding in a time- and dose-dependent manner (Figures 1 and 2). Scatchard analysis (Figure 4) reveals that this inhibition is due to a loss in receptor number rather than a loss in receptor affinity for hormone. The presence of insulin during diethyl pyrocarbonate treatment affords nearly complete protection against subsequent inhibition of tracer binding (Figures 1 and 2; Table I). The simplest interpretation of these data is that diethyl pyrocarbonate inhibits insulin binding by reacting with a receptor residue directly involved in hormone–receptor interaction, that is, at the insulin binding site of the receptor. It cannot be completely ruled out, however, that diethyl pyrocarbonate reaction occurs at a site distal to the insulin binding site and this reaction inhibits binding by freezing a receptor conformation unfavorable for hormone–receptor interaction.

Since diethyl pyrocarbonate reacts preferentially with the imidazole moiety of histidine residues under the conditions of this study (Miles, 1977), it is highly likely that this amino acid moiety of the insulin receptor is being modified in the present study. This reagent has been used to implicate an essential role for histidine in a large number of biological systems (Miles, 1977; Padan et al., 1979; Fujioka et al., 1980; Cromartie, 1981; McKinely et al., 1981; Tso & Zalkan, 1981). However, diethyl pyrocarbonate can also react with lysyl and tyrosyl groups under certain conditions (Miles, 1977), and care must be taken to rule out these possibilities. Reagents that preferentially react with lysyl residues such as acetic anhydride (Cuatrecasas, 1971b) and succinimidyl butyrate (Pilch & Czech, 1979) have little if any effect on insulin binding. Moreover, the reaction of diethyl pyrocarbonate with amino groups is irreversible whereas hydroxylamine can to a considerable extent reverse the effect of diethyl pyrocarbonate on insulin binding (Table I). The pH dependency of the ability of diethyl pyrocarbonate to inhibit insulin binding further supports the notion of histidine involvement (Figure 3). Binding inhibition is substantially greater above pH 7.0 where the imidazole of histidine would be unprotonated and therefore react to a greater extent (Miles, 1977). Thus, it is unlikely that diethyl pyrocarbonate is reacting to a substantial degree with an insulin receptor lysine residue.

Tyrosine residues of the insulin receptor have been implicated as necessary for hormone binding based on the ability of the tyrosine-reactive reagents tetranitromethane and 1*H*-tetrazolediazonium to inhibit insulin binding to adipocyte plasma membranes (Cuatrecasas, 1971b). No attempt was made to prevent receptor modification by incubating with insulin, and it cannot therefore be determined from these previous studies if the binding inhibition was due to tyrosine modification at the insulin binding site or if inhibition was due to a reaction that occurred distal to the binding site and thus altered the receptor structure such that hormone binding was diminished. Moreover, the tetrazole compound will also modify histidine residues (Sokolovsky & Vallee, 1966), further complicating interpretation of these studies (Cuatrecasas, 1971b). So that these ambiguities could be resolved, liver plasma membranes were reacted with tetranitromethane in the presence and absence of insulin in protocols essentially identical with those employing diethyl pyrocarbonate. Tetranitromethane inhibited insulin binding and the presence of insulin during tetranitromethane exposure offered a small degree of protection against this effect (Table II). These results tend to confirm those of Cuatrecasas but must be interpreted with caution because *N*-acetylimidazole, another tyrosine-preferring reagent (Riordan et al., 1965), is without effect on insulin binding (Table II). Furthermore, tetranitrommethane can cross-link proteins (Martinson & McCarthy, 1975), and it has been previously shown that cross-linking of membrane proteins with the bifunctional amino group reagent disuccinimidyl suberate inhibits insulin binding

(Pilch & Czech, 1979) whereas monofunctional amino group reagents do not (Pilch & Czech, 1979; Cuatrecasas, 1971b). In fact, extensive cross-linking of liver plasma membrane proteins does occur after tetranitromethane treatment (10 mM, 10 min) as determined by electrophoretic analysis (data not shown). Thus, inhibition of insulin binding by this tyrosine-reactive reagent may occur as a result of an intra- or inter-protein cross-link rather than by receptor binding site modification. Taken together, the data from the present study and previous studies strongly support the notion of histidine is the primary target of diethyl pyrocarbonate modification rather than lysine or tyrosine. It cannot be ruled out, however, that modification of these latter residues also occurs to some extent. A completely unambiguous demonstration that diethyl pyrocarbonate modifies primarily or only insulin receptor histidine will require pure insulin receptor in a fully functional form with respect to insulin binding as is discussed in detail below.

Much is known about the structure of insulin and the way in which modifications of hormone structure effect hormone binding and action (Gliemann & Gammeltoft, 1974; Pullen et al., 1976; DeMeyts et al., 1978). These data implicate a receptor binding region of the hormone consisting of many hydrophobic groups (A chain Tyr-19 and B chain Val-12, Tyr-16, Phe-24, Phe-25, and Tyr-26) as well as more polar residues (A chain Gly-1, Gln-5, and Asn 21). A mechanism of insulin binding was proposed from these studies in which hydrophobic interactions between receptor and hormone were stabilized by hydrogen bonding between these two entities (Pullen et al., 1976). The thermodynamics of insulin–receptor interaction are consistent with this hypothesis (Waelbroeck et al., 1979). The present chemical data can also be accommodated by this model. Receptor histidine residues demonstrated here to be critical for hormone binding may be involved in the hydrogen bonding postulated as stabilizing hydrophobic interactions between hormone and receptor (Pullen et al., 1976). Most other polar amino acids can be ruled out as unlikely to be present at or near the insulin binding site of the receptor. These include residues with carboxyl, amino, and sulfhydryl functionality (Cuatrecasas, 1971b) as well as the guanidino group of arginine (P. Pilch, unpublished data).

The data presented here suggest that an insulin receptor histidine residue is critical for hormone binding. Similar conclusions have been reached concerning the essential role of histidine in numerous biological systems. Padan et al. (1979) treated *Escherichia coli* membrane vesicles with diethyl pyrocarbonate and observed inhibition of lactose translocation. The pH profile of diethyl pyrocarbonate reaction and the reversibility of this process by hydroxylamine were interpreted as implicating histidine(s) in lactose/proton symport by *E. coli* membrane vesicles. Recently, it was demonstrated that the infectivity of Scrapie agent could be dramatically reduced by diethyl pyrocarbonate treatment (McKinley et al., 1981). Treatment of Scrapie agent with hydroxylamine restored infectivity and suggested histidine modification was responsible for loss of activity due to diethyl pyrocarbonate. In these two studies, as well as in the present case, modification of histidine was not directly demonstrated. Nevertheless, several lines of indirect evidence support the notion of insulin receptor histidine modification. These are (1) the demonstration of histidine modification by diethyl pyrocarbonate for numerous soluble enzymes (Miles, 1977; Fujioka et al., 1980; Cromartie, 1981; Tso & Zalkin, 1981), (2) the increased inactivation of insulin binding when histidine would be expected to undergo deprotonation (Figure 3), and (3) the partial reversal of diethyl pyrocarbonate action by hydroxylamine (Table I).

Several groups have now reported the purification of the insulin receptor (Jacobs et al., 1977; Harrison & Itin, 1980; Siegel et al., 1981). In theory, it should be feasible to use radioactive diethyl pyrocarbonate (Melchior & Fahrney, 1970) and pure receptor to demonstrate the stoichiometry of histidine modification and possibly to identify the polypeptide comprising, in part, the insulin binding domain [for example, see Hegyi et al. (1974)]. However, the purification schemes cited above yield apparently pure receptor with only 10–20% of the expected insulin binding capacity. It would not be possible to determine with certainty either reaction stoichiometry or the receptor polypeptide being modified with these preparations. Preliminary results with partially pure soluble receptor (purified ca. 100-fold) indicate a greatly increased sensitivity to diethyl pyrocarbonate [>90% inhibition of binding with 1 mM diethyl pyrocarbonate for 5 min at 23 °C (P. Pilch, unpublished data)] as compared to plasma membranes (Figure 1). These results indicate that it should eventually be feasible to describe aspects of the biochemical basis of insulin–receptor interaction by using diethyl pyrocarbonate and functionally pure receptor in a manner similar to that well described for soluble enzymes. Efforts are currently in progress to obtain pure insulin receptor in a fully functional form.

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5′-Deoxypyridoxal Interaction with Dexamethasone Receptor: A New Probe for Structure and Function of Steroid Receptors†

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ABSTRACT: 5′-Deoxypyridoxal, a vitamin B-6 analogue, increased the rate of dissociation of [^{3}H]dexamethasone from HeLa S_3 cytoplasmic glucocorticoid receptor complexes in vitro. This effect was achieved at millimolar concentrations of 5′-deoxypyridoxal, suggesting a low-affinity interaction of 5′-deoxypyridoxal with receptor. Loss of [^{3}H]dexamethasone-receptor binding in the presence of 5′-deoxypyridoxal was pH dependent, and a plot of K_{diss} vs. pH fit a simple sigmoidal titration curve with an inflection point at pH 7.8, suggesting that deprotonation of a single functional group on 5′-deoxypyridoxal increases K_{diss}. Loss of [^{3}H]dexamethasone binding in the presence or absence of unlabeled steroid also increased with pH, but no inflection point occurred over the range of pH tested. A titration of 5′-deoxypyridoxal indicated a pK of 7.94 for the pyridinium proton, suggesting deprotonation of the pyridinium nitrogen may account for the pH dependence of K_{diss} of dexamethasone from receptor. 5′-Deoxypyridoxal also caused a decrease in nuclear [^{3}H]-dexamethasone-receptor binding when incubated with whole HeLa S_3 cells at 37 °C. Furthermore, 5′-deoxypyridoxal was effective in reducing nuclear binding of dexamethasone when added either simultaneously with [^{3}H]dexamethasone or after achievement of equilibrium of steroid with receptor. The reduction in nuclear [^{3}H]dexamethasone binding is highly specific for 5′-deoxypyridoxal. Several analogues of this compound, including 5′-deoxypyridoxamine, were ineffective. In addition, this effect was reversible following removal of extracellular 5′-deoxypyridoxal. Under these conditions, 5′-deoxypyridoxal was competitive with dexamethasone for binding to nuclear receptor, with $K_I = 8.1 \times 10^{-6}$ M. Scatchard plot analysis of dexamethasone-receptor binding in the presence or absence of 5′-deoxypyridoxal was consistent with an apparent reduced affinity of [^{3}H]dexamethasone for receptor, which again suggests competitive interaction or allosteric interaction mediated dissociation. Glucocorticoids are known to stimulate alkaline phosphatase activity within HeLa S_3 cells. In whole cell incubations, 5′-deoxypyridoxal was effective in reducing the dexamethasone-induced increase in alkaline phosphatase activity by 60% under conditions in which cell viability and cell growth were not affected.

Pyridoxal 5′-phosphate, the coenzymatically active form of vitamin B-6, is known to alter the molecular properties of several steroid hormone receptors, including the rat uterine estrogen receptor (Muldoon & Cidlowski, 1980), rat thymus (Cidlowski & Thanassi, 1979), HeLa S_3 glucocorticoid receptor (O'Brien & Cidlowski, 1981), and avian progesterone receptor (Nishigori & Toft, 1979). It appears that pyridoxal phosphate reacts with an amino group of a specific lysine residue(s) in a covalent Schiff-base linkage. 5′-Deoxypyridoxal, a vitamin B-6 analogue, also appears to affect the properties of steroid hormone receptors (O'Brien et al., 1980). In preliminary studies it was reported that millimolar concentrations of 5′-deoxypyridoxal caused a loss of [^{3}H]dexa-

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