Enhancement of Insulin Binding to Rat White Adipocytes at 15 °C by 5,5′-Dithiobis-(2-nitrobenzoic Acid)

INDEPENDENCE OF THE REAGENT’S SULFHYDRYL GROUP REACTIVITY

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Insulin binding to isolated rat white adipocytes at 15 °C, a temperature at which cellular degradation of insulin is negligible, has been found to be described by the Two-step Binding Model: R + I → RI → R'I (Lipkin, E. W., Teller, D. C., and de Haen, C. (1986) J. Biol. Chem. 261, 1702–1711). RI is the initially formed complex between the receptor, R, and insulin, I, and R'I is the complex in an altered state or cellular location. Here the possibility was examined that R'I results from disulfide exchange between the receptor and insulin, an exchange proposed by Clark and Harrison (Clarke, S., and Harrison, L. C. (1983) J. Biol. Chem. 258, 12239–12244) to occur at 37 °C. A number of sulfhydryl reagents representing various chemical reactivities did not affect insulin binding. The exception was 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which enhanced the number of insulin-binding sites up to 2-fold with no effect on the equilibrium constant. The data suggested that this enhancement was due to activation of cystic binding sites pre-existing on the cell surface, possibly by increasing the valency of the receptor from 1 to 2. Insulin binding was also enhanced by structural congeners of DTNB devoid of sulfhydryl reactivity, the simplest one being benzoic acid. It was concluded that the effects were not related to modification of sulfhydryl groups, that modification of sulfhydryl groups on the receptor either did not take place or was without effect on binding, and finally, that disulfide exchange between insulin and the receptor was an unlikely explanation for the formation of R'I. Also, since it is possible to show insulin action at 15 °C, contrary to the proposal by Clark and Harrison (Clark, S., and Harrison, L. C. (1983) J. Biol. Chem. 258, 11434–11437), disulfide exchange does not appear to be necessary for signal transmission by the occupied receptor.

A recent kinetic analysis by Lipkin et al. (1, 2) of insulin binding to isolated rat white adipocytes at 15 °C has provided evidence for the Two-step Binding Model, according to which initial binding of insulin to its receptor is followed by a reversible conversion of the insulin-receptor complex to a second state, i.e. I + R → RI ⇄ R'I. Here I is insulin, R is the insulin receptor, RI is the initial insulin-receptor complex, and R'I is the insulin-receptor complex in a different state or cellular location. The temperature of 15 °C in this study was selected to simplify the kinetic analysis, since at this temperature the fusion of endosomes with lysosomes and thus lysosomal degradation of insulin is blocked (1), yet certain biological responses to insulin are retained (3–5). The chosen temperature thus provides a valid alternative both to studies of insulin action at physiological temperatures, where the process of hormone degradation complicates the kinetic analysis, and to studies with plasma membranes or purified receptor, which may be lacking crucial interaction with cellular proteins.

One of the questions posed by the analysis is that of the nature of the R'I state. The Two-step Binding Model fitted the binding data better than either models in which the empty receptor exists in two distinct interconvertible states, or a model which allows direct reversible formation of R'I from I and R in addition to its formation from RI. An important requirement of the model is that R'I must return to the RI state before insulin can dissociate from the receptor. A first hypothesis consistent with this requirement envisions R'I as the insulin-receptor complex reversibly internalized by the cell. Estimates in the literature of the extent of internalization at 15 °C range from none (6–8) to as much as 20% of cell-associated insulin after 200 min (9, 10). Preliminary experiments in our laboratory suggest that internalization of insulin at 15 °C is at far too low a level to account for R'I formation. A second hypothesis consistent with the above requirements invokes disulfide interchange between insulin and the receptor, i.e. a process in which a free sulfhydryl group on the insulin receptor would attack one of the disulfide bonds of insulin, creating a disulfide bond between insulin and the receptor and a free sulfhydryl group on insulin. The possibility of disulfide exchange between insulin and the receptor was first raised as an explanation of why uptake of insulin by perfused tissue was inhibited by sulfhydryl group reagents (11). The issue soon became controversial (12–16), in part because the methods employed were inadequate for its resolution. More recently, Clark and Harrison (17–20) have revived the proposal based on experiments with modern techniques. These authors found that a small fraction of cell-associated labeled insulin, upon detergent solubilization, co-migrated during gel filtration with the insulin receptor and was immunoprecipitated by anti-insulin-receptor antibodies (17). Formation of the complex of insulin and receptor was
immunoprecipitated by anti-insulin-receptor antibodies (17). Formulation of the complex of insulin and receptor was blocked by incubation of cells with sulphydryl reagents such as propiolactone or concomitant with insulin exposure, and the complex could reportedly be dissociated by thiols (17). On nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, insulin moved together with some forms of the receptor, and nondissociable insulin, which was subsequently cross-linked to its binding site by disuccinimidyl suberate, moved on reducing gels with the α-subunit of the receptor. It was therefore concluded that insulin formed a disulfide bridge with the binding subunit of certain redox forms of the receptor (19). Moreover, it was suggested that disulfide bridge formation is critical for insulin action, since receptors on erythrocytes and reticulocytes, cells which are unresponsive to insulin, did not form a covalent complex (20). The biological importance of this complex was also suggested by the observation that 5,5'-dithiobis(2-nitrobenzoic acid) inhibited both formation of the complex and insulin action, without affecting the insulin-like activity of anti-insulin-receptor antibodies (18).

The extent of formation of the putative disulfide bond between insulin and the receptor corresponded to most at 10% of the total cell-bound insulin (17). This is substantially less than the fraction of bound insulin in the R'I state at equilibrium at 15 °C, i.e. approximately 60%. However, given the difference in temperature between the two studies, 37 versus 15 °C, the possibility is left open that R'I and the nondissociable insulin-receptor species described previously (17, 18, 21) are the same species. If R'I formation does correspond to formation of a disulfide bond between insulin and the receptor, then insulin binding at 15 °C should be affected by sulphydryl group reagents. This possibility is explored in the present work.

**EXPERIMENTAL PROCEDURES**

**Materials**

[Al41-(3-[125I]iodothyroxine)] insulin was prepared according to Stenzl et al. (22) and had the characteristics described previously (1). Other sources of chemical reagents were as follows: Sigma, 4-chloromercuribenzenesulfonic acid, iodoacetamide, benzo-2-oxa-1,3-diazole-N-oxide = benzofoxuran, 6,6'-dithiodiiodo-2,2'-bipyridine; Aldrich, 4-fluoro-2-nitrobenzoic acid, 5-methyl-2-nitrobenzoic acid; Boehringer Mannheim, and Fluka Biochemicals. The material from these sources is described in a previous work.

**Methods**

**Adipocyte Preparation**—Epididymal fat cells were isolated from Sprague-Dawley rats (200-225 g, Tyler Laboratories, Bellevue, WA) according to Rodbell (23), using materials specified previously (1, 24). Determinations of cell number were made by diluting cell suspensions to approximately 1.5-1.6 × 10^6 cells/ml. Quadruplicate 5-μl samples were dispensed on a hemocytometer, covered with a cover slide and counted under 10-fold magnification. Lipid droplets were clearly distinguishable from adipocytes.

**Insulin Binding Kinetics**—The protocol used for studies of insulin binding kinetics was an adaptation of that described by Lipkin et al. (1, 2). All binding studies were performed in a modified Krebs-Ringer-phosphate-HEPES buffer, pH 7.4, containing the following components: 10 mM NaHCO₃, 30 mM HEPES, 128 mM NaCl, 1.4 mM CaCl₂, 1.4 mM MgSO₄, 5.2 mM KCl. In addition, the buffer contained 10 mM d-glucose, 0.7 mg/ml (500 μM) bacitracin, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 4% (w/v) bovine serum albumin. In general (Figs. 1, 3, 4, 6) time courses of insulin binding were studied by adding fat cell suspensions (8 × 10^6 to 4 × 10^7 cells/ml final concentration) to binding buffer, pre-equilibrated at 15 °C in 50-mL polystyrene tubes (Falcon) with labeled insulin (1 × 10⁻⁶ to 1 × 10⁻⁸ M) and reagents as indicated. Water-soluble reagents were freshly prepared at 100 mM to avoid degradation, which is a possibility with DTNB (25), and titrated to pH 7.4. Cell suspensions were agitated by gentle back-and-forth movement in a Dubnoff water bath at 60 cycles/min and 15 °C. Prior to removal of aliquots for binding measurements, cell suspensions were rendered homogeneous by rapid swirling. Time courses were followed by removing aliquots (150 μl of the cell suspension and adding these to microcentrifuge tubes (450 μl) preloaded with dithionyl phosphate (100-150 μl). After centrifugation in a Microfuge (Beckman) at 9800 g for 10 s, cell pellets overlying the oil layer were excised by cutting through the oil layer with a 10-gm stainless steel probe, and the pellets were counted for radioactivity.

Dissociation of previously bound labeled insulin was induced by adding unlabeled insulin (1 × 10⁻⁸ M final concentration) to cell suspensions; the dilution caused by addition of unlabeled insulin was 1%. The time course of dissociation of labeled insulin was followed by removing aliquots of the cell suspension by the methods described above.

**Equilibrium Binding**—All equilibrium experiments (Tables I, III, and IV) were performed in Eppendorf tubes (1.5 ml) incubated at 15 °C in a shaking water bath (2). Unless otherwise noted incubations were for 4 h. Shorter times (2 h) were used as indicated with reagents that caused significant cell loss within 4 h. For Tables I and II, which ranged from 2 × 10⁻¹² to 1 × 10⁻⁶ M, insulin binding at each insulin concentration was measured in quadruplicate in the absence or presence of 2.5 mM DTNB. In Tables III and IV insulin concentrations were 1 × 10⁻⁶ M in 4-h incubations and 1 × 10⁻³ M in 2-h incubations.

Benzofoxuran was dispersed from stock solutions in dimethyl sulfoxide at 100-fold the final concentration. To ensure its effective dispersion, the reagent was added to the incubation mixture containing buffer and labeled insulin and vigorously mixed prior to addition of the cell suspension. Control experiments with 1% dimethyl sulfoxide were run in parallel; although significant (p < 0.01) inhibition of binding was observed with this dimethyl sulfoxide concentration, cell viability was not compromised, but DTNB in the presence of the solvent still significantly (p < 0.01) enhanced insulin binding.

**Data Analysis**—The kinetic analysis presented in Tables I and III was performed using the program SAAM (26) to fit the observed data to the Two-step Binding Model described in the introduction. Data were statistically weighted as (observed value)⁻¹. The calculated values returned by this program are indistinguishable from values calculated using the programs previously described (2).

**RESULTS**

DTNB was initially chosen for exploration of the possibility that the two-step process of insulin binding at 15 °C identified previously (1, 2) described disulfide interchange between insulin and the receptor. The initial experiment was designed to explore whether DTNB would affect the kinetics of insulin binding, and if so, whether the effect would be on the rates of association or dissociation. Labeled insulin (1 × 10⁻⁶ M) was allowed to associate with adipocytes for 150 min in the presence or absence of 2.5 mM DTNB. At 150 min net dissociation of labeled insulin was observed by the addition of unlabeled insulin at a final concentration of 1 × 10⁻⁴ M while maintaining the concentration of DTNB at 2.5 mM, and in one case addition of DTNB (2.5 mM) and unlabeled insulin for 150 min, a concentration which had not been exposed to DTNB during the association phase. The results are presented in Fig. 1. There was a marked enhancement of insulin binding when DTNB and insulin were simultaneously presented to adipocytes (O versus □, ▽). This could reflect an increased number of binding sites or tighter binding of insulin. The initial rate of association of insulin did not appear to be significantly altered by the presence of DTNB. In contrast DTNB reduced the rate of dissociation of labeled insulin in the presence of a large concentration excess of unlabeled insulin (O versus □). When DTNB was added only at the initiation of dissociation to-
TABLE I

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Kd</th>
<th>Bmax</th>
<th>10^6 s*</th>
</tr>
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<tr>
<td></td>
<td>DTNB</td>
<td>DTNB</td>
<td>DTNB</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td>sites/cell</td>
<td>100%</td>
</tr>
<tr>
<td>1</td>
<td>4.4</td>
<td>4.7</td>
<td>220</td>
</tr>
<tr>
<td>2</td>
<td>5.9</td>
<td>6.0</td>
<td>160 (100%)</td>
</tr>
</tbody>
</table>

- Trapping constant fitted as described before (1) and normalized to 10^6 cells/incubation.

The effect of DTNB on equilibrium binding of insulin. Equilibrium binding of insulin to adipocytes (9 x 10^6 cells/ml) was measured after 4 h at 15 °C in the presence or absence of 2.5 mM DTNB. Percent saturation of binding sites in the presence (■) and absence (○) of DTNB was calculated from fitting the data to a single-site model as described (2).

The effect of preincubation of adipocytes or insulin with DTNB on subsequent insulin binding. Adipocytes (1.1 x 10^6 cells/ml) were incubated at 15 °C in binding buffer for 70 min in the presence (△) or absence (○, □, △) of 2.5 mM DTNB. At zero time labeled insulin (3 nM final concentration) was added to the cell suspension along with binding buffer (○) or binding buffer plus DTNB at a concentration yielding 2.5 mM in the final incubation (△, □, △). In one case (◣) the insulin was preincubated for 70 min with DTNB (2.5 mM) before addition to the cell suspension. Aliquots of the cell suspension were harvested at the indicated times as described under "Experimental Procedures."

The increase in binding sites due to DTNB would represent receptors newly recruited to the cell surface, one would expect that the action of DTNB requires substantial time. This possibility was tested by preincubating adipocytes with or without DTNB (2.5 mM) for 70 min, at which point labeled insulin was added and the kinetics of association were followed. It can be seen (Fig. 3) that there was no increase in the rate of association or in the equilibrium level reached when adipocytes were exposed to DTNB prior to insulin (△), compared to a control in which DTNB was presented to cells simultaneously with insulin (○). Both of these cases, as well as a control in which the insulin was preincubated with DTNB for 70 min before exposure to adipocytes (◣), gave superimposable rates and levels of association of insulin, which were, however, markedly larger than the DTNB-free control (○).

These data suggest that if DTNB can act in the absence of insulin to increase the number of receptors, that process must occur on a time scale substantially shorter than 3 min, the first time point measured in this experiment.

The reversibility of the DTNB-induced increase in receptor number was tested. Adipocytes preincubated with DTNB for 70 min, then washed for less than 5 min to remove the DTNB, showed insulin binding characteristics identical to those of...
values obtained in the absence of DTNB (Table 5). The rates of association of insulin were followed for direct equilibrium binding analyses, performed in parallel with the kinetic experiments (data not shown). The agreement constants calculated from the kinetic constants according to Equation 5 of Ref. 2 yielded 3.6 and 6.5 nM, respectively, in good agreement with values of 4.7 and 6.4 nM, determined in direct equilibrium binding analyses, performed in parallel with the kinetic experiments (data not shown). The agreement between kinetics and equilibrium experiments suggests that the Two-step Binding Model, which assumes complete reversibility, is an adequate description of insulin binding even in the presence of DTNB. As already noted the equilibrium constants in the presence of DTNB were very similar to values obtained in the absence of DTNB (Table I). They were slightly lower than the average of 8.4 ± 1.5 nM found previously (1) but still within 2 S.D. of that estimate. For purposes of comparison, the kinetic constants describing insulin binding in the absence of DTNB are also presented in Table II. The kinetic constants in the presence of DTNB differed slightly from those previously determined in the absence of DTNB (2). In view of the estimated error associated with those constants and the slight shifts of $K_d$ to lower values observed in recent experiments, the changes seen may be considered negligible.

Examination of the concentration-effect relationship for DTNB action on insulin binding (Fig. 6) revealed that a

Fig. 4. The effect of preincubation with DTNB on subsequent insulin binding in the presence or absence of DTNB. Adipocytes (4 x 10^6 cells/ml) were incubated at 15°C in binding buffer in the absence (panel A) or absence (panel B) of 2.5 mM DTNB for 70 min. Cells were then extensively washed in DTNB-free binding buffer and assayed for binding of insulin (1 x 10^-9 M) in the absence (□, □) or presence (○, □) of 2.5 mM DTNB. Dissociation or bound labeled insulin was induced as in Fig. 1. Data are mean ± S.D. of duplicate determinations within a typical experiment.

FIG. 5. The effect of DTNB on insulin binding at varying insulin concentrations. Adipocytes (8 x 10^6 cells/ml) were incubated at 15°C in a shaking water bath with 2.5 mM DTNB and labeled insulin at final concentrations of 1.3 x 10^-10 M (○), 3.4 x 10^-10 M (□), 1.1 x 10^-9 M (△), 3.1 x 10^-9 M (●), and 3.1 x 10^-8 M (△). At the indicated times aliquots were processed as described under "Experimental Procedures." Data are the mean ± S.E. of quadruplicate determinations from a representative experiment. Solid line indicates the fit to the data using the kinetic constants given in Table II.

<table>
<thead>
<tr>
<th>Kinetic constants</th>
<th>Experiment 1 (Fig. 5)</th>
<th>Experiment 2</th>
<th>No DTNB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-4} k_{on}$ (mol^-1 s^-1)</td>
<td>2.5</td>
<td>2.8</td>
<td>1.6 x 1.4</td>
</tr>
<tr>
<td>$10^{4} k_{on}$ (s^-1)</td>
<td>3.2</td>
<td>5.6</td>
<td>3.4 x 1.6</td>
</tr>
<tr>
<td>$10^{4} k_{off}$ (s^-1)</td>
<td>1.0</td>
<td>1.2</td>
<td>3.2 x 1.5</td>
</tr>
<tr>
<td>$10^{6} k_{off}$ (s^-1)</td>
<td>4.1</td>
<td>4.4</td>
<td>2.0 x 1.5</td>
</tr>
</tbody>
</table>

*Data from Ref. 2, Table II.
roughly 50% increases in number of binding sites, as required by a 100% increase at a maximally effective DTNB concentration. Experiments with NaCl at three times the corresponding concentration of DTNB to match the ionic strength increases due to DTNB showed lower binding than salt-free controls (data not shown).

The alterations in the dissociation of labeled insulin after addition of a large concentration (1 × 10^{-6} M) of unlabeled insulin caused by DTNB (Fig. 1), which was also observed in experiments using various concentrations of labeled insulin ranging from 1.6 × 10^{-10} to 10^{-8} M (data not shown), remained the sole result discrepant with the interpretation offered. The phenomenon was only observed in the presence of very high insulin concentrations, where insulin is known to dimerize significantly (27, 28). We suggest that DTNB interacts with insulin or its dimer at these high concentrations resulting in a substantial reduction of the concentration of free insulin, capable of competing with labeled insulin for binding to the receptor. This phenomenon does not appear to occur in the insulin concentration range used to characterize binding by the other experimental designs.

In order to determine whether the enhancement of insulin binding by DTNB was a common property of all sulfhydryl reagents or just a subset thereof, a variety of such reagents were tested for their ability to affect equilibrium binding of insulin (Table III). Incubation conditions were such that the average cell lysis at the end of the incubation was not significantly different from that of the control incubation. The reagents tested had no consistent effect on insulin binding. The enhancement seen with 6,6'-dithiodinicotinic acid, which did not reach statistical significance, could be due to sulfhydryl reactivity similar to DTNB. Alternatively, the enhancement may reflect other similarities between these compounds, i.e., that both are aromatic acids (see Table IV). In either event, the effect of 6,6'-dithiodinicotinic acid is substantially less than that of DTNB.

Given the lack of effect similar to those of DTNB of so many sulfhydryl reagents, it was of interest to determine whether the effect of DTNB required its ability to react with sulfhydryl groups or whether other features of its structure were responsible for enhancement of insulin binding. To test this a number of compounds similar to parts of the structure of DTNB were examined for their potentiation of insulin binding. Binding of labeled insulin (1 × 10^{-9} M) was assayed in the absence or presence of excess unlabeled insulin (10^{-8} M) and in the presence of 0, 2.5, or 25 mM compound as indicated (Table IV). Insulin binding was enhanced by 2.5 mM DTNB and 2-nitrobenzoic acid. Other modified nitrobenzolic acid compounds at 2.5 mM were without consistent effect on insulin binding. At a higher concentration (25 mM), 5-methyl-2-nitrobenzoic acid and benzoic acid, as well as DTNB and 2-nitrobenzoic acid, increased insulin binding. The increases in insulin binding at 25 mM reagent were not caused by an increase in osmolarity of the incubation medium, since sucrose at 25 mM had no effect on insulin binding (results not shown). These observations, coupled with the insensitivity of insulin binding to a wide range of sulfhydryl reagents, strongly suggest that the enhancement of insulin binding caused by DTNB is due to its benzoic acid moiety and enhanced by certain substituents on that moiety, rather than to its sulfhydryl group reactivity.

**DISCUSSION**

The goal of this study was to clarify whether disulfide interchange between insulin and its receptor and thus reversibly covalent binding was part of the mechanism of insulin binding to adipocytes at 15 °C. The earlier kinetic analysis (2) and disulfide exchange share the characteristics of constraining formation and loss of the secondary insulin-receptor complex, R'I, to a pathway through an intermediate primary and noncovalent insulin-receptor complex, RI. Saviolakis et al. (21) and Clark and Harrison (17, 19) have reported formation of insulin covalently bound to the receptor in studies at 37 °C. Evidence for disulfide interchange between insulin and receptor was presented (17, 19). Moreover, it was suggested that disulfide exchange was part of the mechanism whereby insulin triggered biological responses (18). Chemical modification of insulin has demonstrated that the disulfide bridges in positions A6-A11 (29) and A7-B7 (30-32) were not required for biological activity, although only a limited spectrum of biological activities were examined. A role of the disulfide bridge in position A20-B19 in biological activity remains a possibility. It is important for present purposes that none of the chemical modification studies have ruled out participation of disulfide exchange in binding per se.

In the course of their studies, Clark and Harrison (17, 18) studied the effect of DTNB on insulin binding at 37 °C. They found no effect on apparent equilibrium binding of insulin to adipocytes when DTNB and insulin were presented simultaneously to cells at 37 °C. In contrast the present data show that DTNB strongly enhanced insulin binding at 15 °C. The possibility exists that a temperature-dependent conformational change in the receptor results in altered sensitivity to DTNB. If so, the change must be tacit with respect to receptor function, since at 15 °C adipocytes are still insulin-responsive (3-5). A more likely possibility is that the difference between the present results and those of Clark and Harrison (17, 18) is due to intracellular degradation of insulin at 37 °C masking DTNB-induced changes in binding to the receptor at the cell surface. At 15 °C insulin internalization and degradation are demonstrably negligible (1) and thus effects on cell surface binding are more readily observed.

If total insulin concentrations did not exceed 10^{-9} M, the effect of DTNB on insulin binding at 15 °C was an increase in the number of binding sites, without significant changes in either binding equilibrium or kinetics. At maximally effective DTNB concentration, binding sites increased at best by about 100%. The effect of DTNB was not ascribable to the elevation of the ionic strength, since NaCl at equivalent ionic strength did not mimic DTNB. Indeed above the base-line ionic strength of the incubation medium (213 mM) insulin binding to receptor is expected to be affected very little by ionic
strength increases, and even inhibition has been observed (33–35). The rapidity of onset and reversal of action of DTNB argues strongly against a mechanism involving translocation of receptors between cytoplasmic compartments and the plasma membrane. Our data therefore favor a mechanism whereby DTNB activates cryptic binding sites pre-existing on the receptor. Such sulfhydryl groups could be on the receptor or on insulin after disulfide interchange. Since the disulfide interchange mechanism introduces an irreversible step, DTNB would have been expected to alter net binding rates significantly and to impair the ability to describe the system satisfactorily by the Two-step Binding Model. Thus, the agreement of the kinetic with the equilibrium analysis of binding in the presence of DTNB, and the lack of significant differences in rate constants in the presence of DTNB argue strongly against the chemical modification mechanism hypothesized.

The evidence above alone does not rule out the possibility that DTNB exerts its action by modification of sulfhydryl groups on the receptor not involved in disulfide interchange.

### TABLE III

<table>
<thead>
<tr>
<th>Reagent†</th>
<th>Ref.</th>
<th>Type of sulfhydryl modification</th>
<th>% Change in insulin binding with reagent (2.5 mM)</th>
<th>% Change in insulin binding with DTNB (2.5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Chloromercuribenzenesulfonic acid&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(44)</td>
<td>Mercaptide formation</td>
<td>3 ± 18 (n = 4)</td>
<td>60 ± 13</td>
</tr>
<tr>
<td>Iodoacetamide&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>Alkylation</td>
<td>−5 ± 4 (n = 4)</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>4-Fluoro-2-nitrobenzoic acid&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(45, 46)</td>
<td>Arylation</td>
<td>4 ± 4 (n = 4)</td>
<td>55 ± 16</td>
</tr>
<tr>
<td>Benzofuroxane&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>Oxidation</td>
<td>−11 ± 7 (n = 3)</td>
<td>57 ± 12</td>
</tr>
<tr>
<td>6,6'-Dithiodinicotinic acid&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(47)</td>
<td>Sulphydryl-disulfide exchange</td>
<td>7 ± 4 (n = 3)</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>2-Nitro-5-thiocyanobenzoic acid</td>
<td></td>
<td>Cyanlation</td>
<td>−8 ± 16 (n = 3)</td>
<td>63 ± 3</td>
</tr>
</tbody>
</table>

* Sulfhydryl group reactivity of these compounds is documented in Torchinsky (43) or in the references cited.
† Reagents documented to have limited cell permeability.
‡ Reagents tested at 2.5 mM final concentration for effect on binding of 1 × 10⁻⁸ M labeled insulin for 2 h at 15 °C. The shorter incubation time, compensated for in part by the higher insulin concentration, was chosen to minimize effects of the reagent on cell viability.
§ In a single experiment, 4-fluoro-2-nitrobenzoic acid tested at 25 mM significantly (p < 0.05) enhanced binding of 10⁻⁹ M insulin at 4 h at 15 °C, consistent with the results presented in Table II.
☆ In order to get optimal dispersion, benzofuroxan was added from a 250 mM stock solution in dimethyl sulfoxide to the incubation buffer containing labeled insulin and was vigorously mixed prior to addition of the cell suspension. Binding was measured relative to a control containing 1% dimethyl sulfoxide.

### TABLE IV

**Effects of DTNB and structural congeners on insulin binding to adipocytes**

| Reagent | % Total insulin Trapped insulin Bound insulin P |
|---|---|---|---|
| | Total in pellet | in pellet | in pellet | |
| Control | 7.44 ± 0.09 | 0.79 ± 0.01 | 6.65 ± 0.05 | |
| DTNB 2.5 mM | 10.05 ± 0.20 | 0.98 ± 0.01 | 9.07 ± 0.10 | <0.005 |
| DTNB 25 mM | 12.15 ± 0.17 | 1.65 ± 0.03 | 10.50 ± 0.09 | <0.001 |
| 5-Methyl-2-nitrobenzoic acid 2.5 mM | 7.79 ± 0.09 | 0.69 ± 0.01 | 7.10 ± 0.05 | N.S.* |
| 5-Methyl-2-nitrobenzoic acid 25 mM | 9.85 ± 0.23 | 0.66 ± 0.01 | 9.19 ± 0.12 | <0.005 |
| 2-Nitrobenzoic acid 2.5 mM | 8.47 ± 0.09 | 0.66 ± 0.01 | 7.81 ± 0.05 | <0.010 |
| 2-Nitrobenzoic acid 25 mM | 15.66 ± 0.38 | 0.66 ± 0.01 | 13.00 ± 0.19 | <0.001 |
| Benzoic acid 2.5 mM | 7.81 ± 0.05 | 0.70 ± 0.01 | 7.11 ± 0.03 | N.S. |
| Benzoic acid 25 mM | 8.38 ± 0.05 | 0.65 ± 0.02 | 7.74 ± 0.03 | <0.005 |

* N.S., not statistically significant.
with insulin. However, it is unlikely that such chemical modification would have been reversible on the time scale on which reversal of DTNB action on binding site numbers was observed. A second approach was used to examine whether the ability of DTNB to increase the number of insulin-binding sites was related to its reactivity as a sulfhydryl reagent. A number of other sulfhydryl group reagents were tested for their ability to alter insulin binding. The reagents were chosen to represent a range of different chemistries of sulfhydryl modification. It was also necessary that the reagents cause only minimal lysis of adipocytes over periods of time required for insulin binding studies. In particular, N-ethylmaleimide at 2.5 mM, a concentration lower than that used by Clark and Harrison (17), proved unsuitable since in our hands it caused virtually complete cell lysis within 60 min. The lytic effects of N-ethylmaleimide also precluded any experiments with this reagent which could have detected a change in receptor sulfhydryl status upon insulin binding, as has been noted by Maturo et al. (38). Of the eight sulfhydryl reagents tested which were not toxic, only DTNB affected equilibrium insulin binding to adipocytes (Tables I and III). This unique sensitivity to DTNB suggests that if DTNB was to act by sulfhydryl modification, the putative sulfhydryl groups would have to be in an unusually restrictive microenvironment. It has been noted that upon binding to the insulin receptor, insulin becomes less accessible to anti-insulin antibodies (39, 40). Given a binding site for insulin which is deeply buried in the receptor, it would be reasonable that the proposed insulin-reactive sulfhydryl group on the receptor would also be inaccessible to a surface probe. Other membrane proteins have been described to have selective sensitivity to sulfhydryl reagents. A thiol group of the ion channel of the nicotinic acetylcholine receptor was observed to be more sensitive to N-benzylmaleimide and N-butylmaleimide than to N-ethylmaleimide (41), and the band 3 protein of the erythrocyte membrane has a sulfhydryl group which could be labeled by DTNB but not by N-ethylmaleimide or p-chloromercuribenzenesulfonate (42).

The involvement of a sulfhydryl group with special selectivity for DTNB in the action of this compound is not supported by the observation that structural cogeners of DTNB lacking in sulfhydryl reactivity were able to enhance insulin binding. The effect of these reagents suggests that DTNB's effects were also independent of sulfhydryl modification. If indeed the DTNB effects reported here were not mediated by sulfhydryl reactivity, then the fact that a large number of sulfhydryl reagents were devoid of effects on insulin binding may be taken as strong evidence against participation of disulfide interchange in insulin binding at 15 °C in general. Further, if in fact insulin binding induces a change in the sulfhydryl status of the receptor, as suggested by Maturo et al. (38), such a change must be tacit with respect to the kinetics of insulin binding. More specifically, the data argue against identification of the kinetically identified R'I species with a disulfide interchange-mediated covalent complex between insulin and its receptor. Although our study at 15 °C cannot rule out disulfide exchange and covalent complex formation at 37 °C, it appears that, contrary to the proposal of Clark and Harrison (18), formation of such complexes must not be a prerequisite for biological action, since fat cells still respond to insulin at 15 °C (3-5) when these complexes appear to be absent. The fact that a compound as simple as 2-nitrobenzoic acid at 2.5 mM was able to enhance insulin binding is of some interest. Should this phenomenon occur at 37 °C, and should the increased binding favor biological action, the site involved could become a target for drug development.

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REFERENCES

DTNB Enhancement of Insulin Binding