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Lactose–Proton Symport by Purified *lac* Carrier Protein†

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ABSTRACT: The *lac* carrier protein of *Escherichia coli* was purified by an improved procedure and its activity assayed by a rapid filter method. Following reconstitution of the carrier by octyl glucoside dilution, proteoliposomes were concentrated by filtration on a microporous filter. Lactose accumulation by adsorbed or entrapped proteoliposomes is driven by an artificially imposed pH gradient (interior alkaline), by a membrane potential (interior negative), or by a combination of both forces. Activity is almost completely abolished by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone or by the competitive inhibitor thiodigalactoside. Addition of lactose to proteoliposomes under appropriate conditions results in alkalinization of the external medium. This effect is not observed with liposomes devoid of *lac* carrier or in the presence of proton conducting agents. The results provide a strong indication that the *lac y* gene product is the only protein in the cytoplasmic membrane of *Escherichia coli* required for lactose–proton symport.

During the last decade, a large body of evidence has accumulated supporting Mitchell's hypothesis (Mitchell, 1961, 1963, 1968) that chemiosmotic phenomena play a central role in active transport [cf. Rosen & Kashket (1978) for a review]. According to the hypothesis, the uptake of many solutes is mediated by specific polypeptide carriers that couple the uphill translocation of substrate across the cytoplasmic membrane to the simultaneous downhill movement of a cation such as H^+ or Na^+ (i.e., symport or antiport). Thus, the concentration gradients of many substrates across the cell membrane are maintained as a consequence of an electrochemical ion gradient generated by the action of various cationmotive pumps (e.g., membrane-bound respiratory chains, proton-translocating ATPases, or Na^+/K^+-ATPases).

The development of facile methods to reconstitute and assay the activity of transport systems is crucial to their isolation and study. Most of the methods described to date are time consuming and poorly suited to the analysis of the large number of fractions typically encountered during the course of a purification. Recently, two transport systems from *Escherichia coli* were solubilized and reconstituted in functional form by octyl β-D-glucopyranoside (octyl glucoside)[1] dilution (Newman & Wilson, 1980; Tsuchiya et al., 1982).[2] Although

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[1] Abbreviations: octyl glucoside, octyl β-D-glucopyranoside; ΔpH, transmembrane pH gradient; ΔΨ, transmembrane electrical potential; $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical proton gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; [^{3}H]NPG, 4-nitro[2-^{3}H]phenyl α-D-galactopyranoside.

[2] Newman, J., & Wilson, T. H. (1981) Abstracts of the Annual Meeting of the American Society for Microbiology, p 158, American Society for Microbiology, Washington, DC.

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proteoliposomes are obtained rapidly by this method, they are sufficiently dilute that a concentration step is required before activity can be assayed. An innovative approach, first utilized to follow the purification of the *lac* carrier (Newman et al., 1981), achieves concentration by trapping the proteoliposomes in a microporous filter. The counterflow activity present in column fractions was assayed directly on filters by using this technique. However, a number of transport systems exhibit low or nondetectable levels of counterflow activity,[3] and assay of such systems after reconstitution requires imposition of an electrochemical ion gradient in order to drive accumulation.

In this report, the rapid filter assay is extended to solute accumulation driven by an artificially imposed pH gradient (ΔpH, interior alkaline) and/or a membrane potential ($\Delta\Psi$, interior negative). Furthermore, lactose-induced proton movements are demonstrated for the first time in proteoliposomes containing purified *lac* carrier. The results demonstrate clearly that lactose transport catalyzed by the *lac* carrier protein, in the absence of other components, is driven by the proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and alkaline). In addition, a modification of the original procedure for the purification of the *lac* carrier protein is described that leads to a 3-fold increase in overall yield and allows the procedure to be scaled up 30-fold.

Experimental Procedures

Materials. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. All other materials were obtained as described (Newman & Wilson, 1980; Newman et al., 1981).

Preparation of Membrane Vesicles. Cells of *E. coli* strain T206, which carry the *lac y* gene in a recombinant plasmid, were grown and induced for the β-galactoside transport system (Teather et al., 1980) in a 1500-L fermentor. Inverted vesicles were prepared by a single passage through a motor-driven French pressure cell as described previously (Newman & Wilson, 1980), except at 5000 lb/in.^{2} instead of 20000 lb/in.^{2}.

Preparation of Acetone/Ether-Washed E. coli Lipid. Crude chloroform/methanol-extracted *E. coli* lipid was acetone precipitated and washed with ether essentially as described (Newman & Wilson, 1980). The acetone-precipitated lipid was collected by centrifugation instead of filtration.

Photoaffinity Labeling of the lac Carrier in T206 Membrane Vesicles. French press vesicles were labeled with 4-nitro[2-^{3}H]phenyl α-D-galactopyranoside ([^{3}H]NPG) (10 Ci/mmol) as described (Kaczorowski et al., 1980). Labeled membrane was prepared at a specific activity of 18 μCi/mg of membrane protein and mixed with unlabeled membrane to yield a final specific acticity of 10–50 nCi/mg of protein.

Purification of lac Carrier Protein. The procedure used is a modification of that described by Newman et al. (1981). All steps were performed at 0–4 °C. T206 French press vesicles (1.0 g of protein) were extracted with 5 M urea as described previously (Newman et al., 1981), except that 0.5 mM phenylmethanesulfonyl fluoride was present during the extraction. The urea-extracted membrane was resuspended by homogenization in a final volume of 140 mL in 50 mM potassium phosphate, pH 7.5. While the solution was stirred, 60 mL of 20% (w/v) sodium cholate was slowly added. The suspension was stirred for 20 min and then centrifuged for 2 h at 35000 rpm in a Beckman Type 35 rotor. The cholate-washed residue was resuspended to a final volume of 200 mL in 10 mM potassium phosphate, pH 5.90, and centrifuged as described above. The urea/cholate-extracted residue was resuspended to a final volume of 81.3 mL in 10 mM potassium phosphate, pH 5.90. While the suspension was stirred, the following additions were made: 96.3 μL of 1.0 M dithiothreitol, 0.72 g of lactose, 7.3 mL of acetone/ether-washed *E. coli* lipid at 50 mg/mL in 2 mM 2 mercaptoethanol, and 8.0 mL of 15% (w/v) octyl glucoside in 10 mM potassium phosphate, pH 5.90. The suspension was stirred for 10 min and then centrifuged for 90 min at 40000 rpm in a Beckman type 60 Ti rotor. The supernatant solution, octyl glucoside extract (volume = 92 mL), contained 65% of the [^{3}H]NPG label present in the original membrane. The pH of the extract was adjusted to pH 6.00 by the dropwise addition of 10 mM K_2HPO_4, 1 mM dithiothreitol, 20 mM lactose, 0.25 mg of washed *E. coli* lipid/mL, and 1.25% octyl glucoside. This extract can be stored for at least several months in liquid nitrogen without significant loss of activity.

A 2.5 × 15 cm column (bed volume 75 mL) of DEAE-Sepharose CL-6B (Pharmacia) was poured and equilibrated with 10 mM potassium phosphate (pH 6.00) at room temperature. After the column was equilibrated, it was cooled to 4 °C, and 100 mL of column buffer (10 mM potassium phosphate, pH 6.00, 1 mM dithiothreitol, 20 mM lactose, 0.25 mg of washed *E. coli* lipid/mL, and 1.25% octyl glucoside) was passed through the column. Thirty milliliters of the octyl glucoside extract was loaded, and the column was then developed with column buffer at a flow rate of 60 mL/h (12 cm/h). *lac* carrier protein eluted slightly after one bed volume and was the only protein that eluted from the column. The yield was 5 mg; thus, the yield from the entire gram of starting material (French press vesicles) was 15 mg. The overall yield of [^{3}H]NPG label relative to the original membrane was 46%.

Reconstitution of the lac Carrier. Bath-sonicated liposomes (0.7 mL), prepared as described (Newman & Wilson, 1980) but without lactose, were mixed with 50 μL of 15% octyl glucoside (in 50 mM potassium phosphate, pH 7.5) and 2.33 mL of a column fraction containing pure *lac* carrier (approximately 0.2 mg of protein/mL). The mixture was blended on a vortex mixer and incubated on ice for 10 min. The suspension was drawn into a pipet and squirted into a beaker containing 106 mL of 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol at room temperature. Immediately following dilution, the resultant suspension was stirred with a magnetic stir bar.

For experiments involving the measurement of lactose-induced proton movements, the column fraction was diluted before reconstitution with column buffer to give a protein concentration of 60 μg/mL. Following detergent dilution, the proteoliposomes were collected by centrifugation for 2 h at 40000 rpm in a Beckman type 60 Ti rotor and resuspended with 50 mM potassium phosphate, pH 7.5, containing 1 mM dithiothreitol to a final protein concentration of 0.3 mg of protein/mL. The resuspended proteoliposomes were rapidly frozen, thawed at 30 °C, and sonicated in a conical plastic tube for 10 s by using a bath-type sonicator (80 W, 80 Hz, generator model G80-80-1, tank model T80-80-1-RS from laboratory Supplies Co., Inc., Hicksville, NY).

Rapid Assay of Lactose Accumulation. (A) $\Delta\Psi$ Driven. A 0.5-mL aliquot of proteoliposomes was adjusted to 2 μM (final concentration) in valinomycin and filtered on a 25-mm diameter GSTF filter (Millipore, 0.2-μm pore size), using a chimney with a 9-mm internal diameter (Newman et al., 1981). The vacuum pump was turned off and the vacuum beneath the filter allowed to dissipate. A 0.15-mL aliquot of 50 mM *sodium* phosphate, pH 7.5, containing 1.8 μCi of

[3] M. L. Garcia and H. R. Kaback, unpublished experiments.

[1-^{14}C]lactose (59 mCi/mmol) at a final concentration of 0.2 mM was placed on top of the filter. After given periods of time, the lactose solution was filtered, and the narrow-diameter chimney was removed and replaced with a normal chimney (16-mm internal diameter). The filter was then washed with 5 mL of ice-cold 50 mM sodium phosphate, pH 7.5, and counted by liquid scintillation.

(*B*) *ΔpH Driven.* Proteoliposomes containing valinomycin were filtered as described above, and 0.15 mL of 50 mM potassium phosphate, *pH 5.5*, containing 1.8 μCi of [1-^{14}C]-lactose (59 mCi/mmol) at a final concentration of 0.2 mM was placed on top of the filter. After the incubation period, the filter was washed with 5 mL of ice-cold 50 mM potassium phosphate, pH 5.5, and counted.

(*C*) *$\Delta\bar{\mu}_{H^+}$ Driven.* Proteoliposomes containing valinomycin were filtered as described above, and 0.15 mL of 50 mM *sodium* phosphate, *pH 5.5*, containing 1.8 μCi of [1-^{14}C]lactose (59 mCi/mmol) at a final concentration of 0.2 mM was placed on top of the filter. After the incubation period, the filter was washed with 5 mL of ice-cold 50 mM sodium phosphate, pH 5.5, and counted.

A blank to correct for the presence of [^{3}H]NPG-labeled carrier, obtained by filtering proteoliposomes in the absence of labeled lactose, was subtracted from all values. More than 80% of the [^{3}H]NPG label was retained by the filters, indicating that most of the proteoliposomes were retained.[4]

Lactose-Induced Proton Influx. The pH measurements were performed in a closed electrode vessel which was jacketed and maintained at 30 °C during all experiments. The vessel was continuously flushed with a stream of water-saturated nitrogen. By means of a lateral inlet, 2.5 mL of 150 mM KCl, 10 mM $MgSO_4$, and 60 μL of proteoliposomes containing 20 μg of *lac* carrier were added to the vessel. This solution was adjusted to 5 μM in valinomycin. Proton movements were initiated by the addition of an aliquot of a freshly prepared stock solution of 0.5 M lactose to a final concentration of 10 mM. The lactose stock solution was first carefully adjusted to the pH of the proteoliposome suspension with a KOH solution. The suspension was stirred throughout the course of the experiment with a magnetic stir bar. A Radiometer pH meter (pHm84) connected to a Radiometer pH electrode (GK 2401 B) and a Radiometer chart recorder (REC 61 Servograph) were used to monitor the pH continuously. Calibration of the measured pH changes was performed at the conclusion of the experiment by addition of 10 μL of a 1 mM HCl solution.

Protein Determination. Protein was assayed by a modification of the method of Schaffner & Weissmann (1973), by using bovine serum albumin as the reference standard (Newman et al., 1981).

Results

$\Delta\bar{\mu}_{H^+}$-Driven Lactose Accumulation. Once trapped within the pores of a microporous filter, proteoliposomes containing the *lac* carrier can be exposed to buffers of any desired composition, and a transmembrane pH gradient can be established simply by placing a solution at a pH different from that of the proteoliposomes on the filter. Thus, proteoliposomes in potassium phosphate at pH 7.5 were concentrated on filters and then incubated with potassium phosphate at pH 5.5 containing [1-^{14}C]lactose (Figure 1). Rapid accumulation

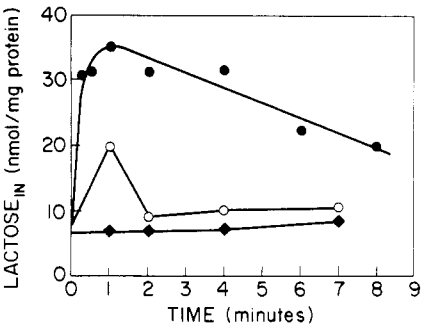

FIGURE 1: ΔpH-driven lactose transport in proteoliposomes reconstituted with purified *lac* carrier. A column fraction containing pure *lac* carrier (ca. 0.2 mg of protein/mL) was reconstituted as described under Experimental Procedures. A 0.5-mL aliquot of proteoliposomes containing 2 μM valinomycin was filtered on a 25-mm GSTF (Millipore) filter by using a 9-mm internal diameter chimney. The vacuum was allowed to dissipate. A 0.15-mL aliquot of 50 mM potassium phosphate, pH 5.5, containing 1.8 μCi of [1-^{14}C]lactose at a final concentration of 0.2 mM was placed on top of the filter, and the proteoliposomes were assayed at a given time as described under Experimental Procedures. A blank to correct for the presence of [^{3}H]NPG-labeled carrier, obtained by filtering proteoliposomes that were not incubated with [1-^{14}C]lactose, was subtracted from all points. (●) proteoliposomes assayed as described above; (○) proteoliposomes assayed in the presence of 20 μM CCCP; (♦) proteoliposomes assayed in the presence of 10 mM thiodigalactoside.

of lactose occurs in a very short period of time at a rate that is difficult to measure accurately, but is at least 120 nmol min^{-1} (mg of protein)$^{-1}$. The level of lactose accumulated remains constant for about 1 min and then slowly decreases, due presumably to dissipation of the pH gradient and subsequent equilibration of lactose between the internal and external compartments. Lactose accumulation is abolished by the addition of thiodigalactoside and drastically diminished by the protonophore CCCP. Although a small amount of lactose accumulation is observed during short incubation times in the presence of CCCP, this may indicate that the carrier responds to ΔpH more rapidly than the latter can be dissipated by the protonophore. Values obtained in the presence of thiodigalactoside are due to nonspecific binding of lactose to the proteoliposomes and the filter and were not subtracted in these studies so that the "signal to noise ratio" is readily apparent. This background is constant and does not vary significantly with the time of incubation.

A transmembrane electrical potential was estalished by entrapping proteoliposomes containing potassium phosphate, pH 7.5, on filters and then incubating them with sodium phosphate, pH 7.5, in the presence of the potassium ionophore valinomycin. Efflux of K^+ from the proteoliposomes results in the generation of a $\Delta\Psi$ (interior negative). As shown in Figure 2, imposition of $\Delta\Psi$ in this fashion leads to marked, transient accumulation of lactose with a time course similar to that observed in isolated membrane vesicles (Schuldiner & Kaback, 1975). Moreover, accumulation is inhibited by CCCP and does not occur when the proteoliposomes are incubated with equimolar potassium phosphate. As in the case of ΔpH-driven transport, the initial rate of lactose accumulation observed here is too rapid to estimate accurately. The maximum level of lactose accumulated also remains relatively constant for a period of about 1 min and then rapidly declines with time. Interestingly, the rate of decline is about 8-fold greater than that observed during ΔpH-driven transport (cf. Figure 1).

When proteoliposomes loaded with potassium phosphate, pH 7.5, are incubated with sodium phosphate, pH 5.5, in the presence of valinomycin, both a ΔpH (interior alkaline) and

[4] Since the proteoliposomes are not retained by cellulose acetate filters (Millipore) of the same pore size, it is likely that adsorptive phenomena rather than entrapment are involved.

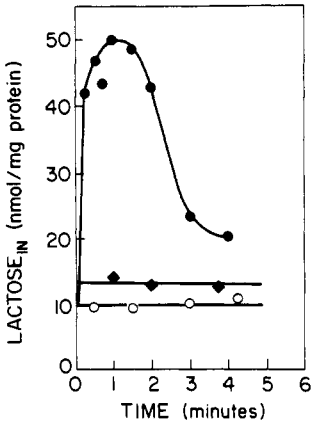

FIGURE 2: $\Delta\Psi$-driven lactose transport in proteoliposomes reconstituted with purified *lac* carrier. A column fraction containing pure *lac* carrier (ca. 0.2 mg of protein/mL) was reconstituted as described under Experimental Procedures. Proteoliposomes containing 2 μM valinomycin were assayed as described in Figure 1 except that the solution placed on the filter was 50 mM sodium phosphate, pH 7.5, containing 1.8 μCi of [1-^{14}C]lactose. (●) Proteoliposomes assayed as described above; (♦) proteoliposomes assayed in the presence of 20 μM CCCP; (○) potassium-loaded proteoliposomes assayed in equimolar potassium phosphate buffer at the same pH.

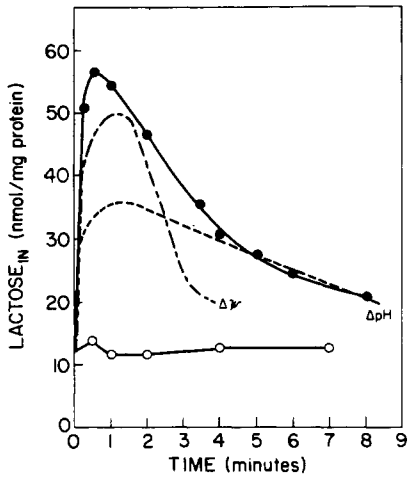

FIGURE 3: $\Delta\bar{\mu}_{H^+}$-driven lactose transport in proteoliposomes reconstituted with purified *lac* carrier. A column fraction containing pure *lac* carrier (ca. 0.2 mg of protein/mL) was reconstituted as described under Experimental Procedures. Proteoliposomes were assayed as described in Figure 1 except that the solution placed on the filter was 50 mM sodium phosphate, pH 5.5, containing 1.8 μCi of [1-^{14}C]lactose at a final concentration of 0.2 mM. (●) Proteoliposomes assayed as described above; (○) proteoliposomes assayed in the presence of 20 μM CCCP. The dashed lines correspond to the accumulation observed when transport is driven by either ΔpH or $\Delta\Psi$ alone (cf. Figures 1 and 2, respectively).

a $\Delta\Psi$ (interior negative) are generated. Under these conditions, it is apparent that significantly more lactose is accumulated than in the presence of ΔpH or $\Delta\Psi$ alone (Figure 3). Furthermore, the time course of $\Delta\bar{\mu}_{H^+}$-driven lactose uptake roughly approximates the sum of the time courses observed in the presence of $\Delta\Psi$ and ΔpH independently.

Lactose-Induced Proton Movements. Lactose-induced proton symport catalyzed by purified *lac* carrier was demonstrated by monitoring the rise in pH induced by the addition of lactose to proteoliposomes. Proteoliposomes were diluted 40-fold into an unbuffered medium, resulting in a suspension with a final pH of 7.2. Valinomycin was added to prevent the generation of a $\Delta\Psi$ (interior positive) during lactose–proton symport. When lactose is added to the proteoliposome suspension, transient alkalinization of the medium is observed,

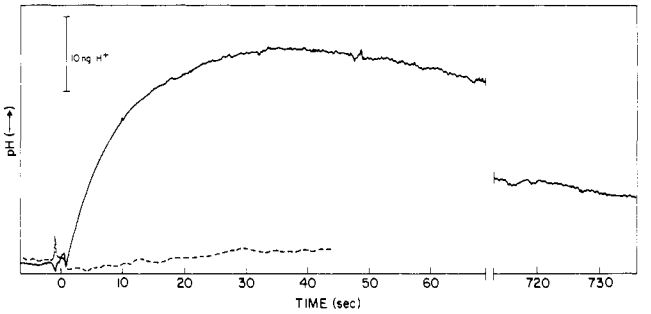

FIGURE 4: Lactose-induced proton movements in proteoliposomes with purified *lac* carrier. A column fraction containing pure *lac* carrier was diluted to 0.08 mg of protein/mL and reconstituted, and the proteoliposomes were collected as described under Experimental Procedures. Proteoliposomes containing 20 μg of *lac* carrier were added to a closed electrode vessel containing 2.5 mL of 150 mM KCl, 10 mM $MgSO_4$, and 5 μM valinomycin. The final pH was approximately 7.2. The electrode vessel was continuously flushed with water-saturated nitrogen and maintained at 30 °C. At time zero, an aliquot of a 0.5 M lactose solution adjusted to the same pH as the proteoliposome suspension was added to a final concentration of 10 mM. The pH of the solution was monitored continuously as described under Experimental Procedures. In parallel experiments (indicated by the dashed line), liposomes formed in the absence of *lac* carrier were assayed. Similar results were obtained with proteoliposomes incubated in the presence of the proton conducting reagents nigericin (300 nM) and CCCP (20 μM). The pH changes were calibrated at the conclusion of the experiment by the addition of 10 μL of a 1 mM HCl solution.

and the pH tracing reaches maximum displacement in about 0.5 min and returns to the base line after approximately 15 min (Figure 4). The initial velocity of the pH change is directly proportional to the amount of *lac* carrier incorporated into the liposomes (data not shown), and lactose-induced proton influx is not observed with liposomes formed in the absence of *lac* carrier (Figure 4). Finally, nigericin or CCCP abolishes lactose-induced alkalinization when added prior to the disaccharide (data not shown). Clearly, the results demonstrate that influx of protons (or efflux of hydroxide) accompanies the carrier-mediated influx of lactose.

Discussion

These experiments demonstrate that either a ΔpH or a $\Delta\Psi$ can drive lactose transport, a conclusion that is in complete agreement with numerous studies conducted with right-side-out membrane vesicles (Schuldiner & Kaback, 1975; Ramos & Kaback, 1977; Kaczorowski et al., 1979; Robertson et al., 1980). Moreover, since purified *lac* carrier protein was utilized in these studies, the results demonstrate clearly that no other protein is needed in order to couple lactose transport to $\Delta\bar{\mu}_{H^+}$. Thus, the observations presented here are in agreement with the conclusions of Newman et al. (1981) and stand in contradistinction to the suggestion by Hong (1977) and Plate & Suit (1981) that certain mutants with pleiotropic defects in solute–proton symport result from the alteration of a polypeptide common to all symport systems (such as a proton-translocating subunit). A more likely possibility is that such mutants are defective in a component of a common regulatory system.

The procedure described originally for the purification of the *lac* carrier (Newman et al., 1981) was substantially improved with a few minor modifications. First, the cholate-insoluble membrane residue was collected at higher centrifugal force, resulting in an increased yield of *lac* carrier. Second, solubilization of the *lac* carrier with octyl glucoside was carried out at a greater protein concentration. This permits application of larger amounts of carrier to the DEAE-Sepharose column.

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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