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# Sedimentation characteristics of vesicles associated with insulin-sensitive intracellular glucose transporter from rat adipocytes

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**The sedimentation characteristics of vesicles associated with the insulin-sensitive intracellular glucose transporter from rat adipocytes were studied. The method used was sucrose density gradient centrifugation, which was carried out under non-equilibrium and equilibrium (isopycnic) conditions. The glucose transport activity was determined by the reconstitution method. As reported previously, the sedimentation velocity of the intracellular glucose-transport activity was considerably slower than that of the counterpart in the plasma membrane. It was found, however, that the specific gravity of the slow-sedimenting glucose-transport activity was almost identical to that of the activity in the plasma membrane ($d = 1.118–1.122$). It is concluded that the intracellular glucose transport activity is associated not with low-density microsomal vesicles, but with unidentified slow-sedimenting vesicles that have a specific gravity similar to that of the plasma membrane.**

As is well known, insulin stimulates transport of glucose across the plasma membrane of muscle and fat cells [1]. Although the mechanism of this hormonal action is still obscure, these hormone-sensitive cells are equipped with two groups of insulin-sensitive glucose-transport activities. One of them is associated with the plasma membrane while the other is bound to some unidentified, intracellular membranes, possibly a specific group of vesicles [2–5]. When the cells are exposed to insulin, the glucose transport activity in the plasma membrane is increased while that associated with the intracellular membranes is reduced [2–7]. Based on this and other observations, it has been suggested that insulin may induce translocation of glucose transporters from an intracellular site to the plasma membrane [2–7]. A possible mechanism of this translocation is exocytotic fusion of the transporter-carrying vesicles with the plasma membrane [8]. However, a positive proof of the translocation is yet to be obtained, and the above theory of insulin action is still controversial (e.g., Ref. 9).

Upon differential centrifugation of the cell homogenate, the intracellular glucose-transport activity (or the intracellular vesicles associated with the activity) is concentrated into a slow-sedimenting fraction that is also enriched with a portion of the cellular NADH dehydrogenase activity (a marker of the endoplasmic reticulum) [2] and of UDPgalactose : *N*-acetylglucosamine galactosyltransferase (galactosyltransferase, a marker of the Golgi apparatus) [3,4,10]. Therefore, it could be

Abbreviations: galactosyltransferase, UDPgalactose : *N*-acetylglucosamine galactosyltransferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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postulated that the intracellular transport activity is associated with either the Golgi apparatus or low-density microsomal vesicles. However, the exact nature of the membranes that carry the intracellular glucose transporter is still unknown. In our present study, we examined the sedimentability of the transport activity by using sucrose density gradient centrifugation under both non-equilibrium and equilibrium (i.e. isopycnic) conditions. A part of our present study has been reported in an abstract form [11].

The sources of materials used in our present study were listed in our previous publications [5,7]. Isolated adipocytes were prepared by the collagenase method [12] from epididymal and perirenal adipose tissue of Sprague-Dawley rats (180–240 g). The cells from six rats were combined and homogenized in 40 ml of Buffer A (0.25 M sucrose in 10 mM Tris-HCl supplemented with 1 mM EDTA/Na, pH 7.5) at 13–15°C in a Dounce tissue grinder (Type B, 8 strokes). The homogenate was first fractionated (at 2°C) into several fractions by differential centrifugation as shown in Fig. 1. Subsequently, Fraction P-2 (the plasma-membrane enriched fraction) and Fraction P-4 (a slow-sedimenting microsomal fraction) were subjected to linear sucrose density gradient centrifugation under various conditions as specified later. The sucrose solutions used for the preparation of density gradients were buffered with 10 mM Tris-HCl and supplemented with 1 mM EDTA/Na, pH 7.5; the concentration of sucrose is reported in weight %. The centrifugation was carried out at 2°C in a Beckman's SW 41 rotor (swinging bucket type; $r_{av}$ = 110.2 mm); the length of the sucrose density gradient was 84 mm. After the centrifugation, the sucrose solution was collected from the bottom and divided into 17 fractions (0.65 ml each). Each fraction was then divided into two portions; one of them was used for the assay of glucose transport activity, while the other was employed for determination of (a) the activity of 5′-nucleotidase or that of galactosyltransferase and (b) the concentrations of protein and sucrose. The glucose transport activity was determined by the reconstitution method [13], 5′-nucleotidase was assayed by the method of Avruch and Wallach [14], galactosyltransferase activity was measured as described by Fleischer [15], the concentration of sucrose was determined by refractometry, and that of protein was estimated by the method of Bradford [16]. The standard used for the protein assay was crystalline bovine serum albumin. In each set of experiments, the preparation of adipocytes and fractionation of subcellulular components were completed in one working day, except when sucrose density gradient centrifugation was carried out overnight (for 18 h). When the fractionation was completed in one day, all the sample preparations were frozen at −70°C and analyzed the next day. When the sucrose density gradient centrifugation was carried out overnight, all the samples were analyzed (without freezing) on the second day. All the enzyme activities measured in this study were not significantly affected by freezing, but some of them were reduced up to 15% when Fractions P-2 and F-4 (in Fig. 1) were kept refrigerated at 4°C for 18 h in the presence of 20% w/w sucrose (data not shown). All the results presented in this report were confirmed by repeating identical experiments

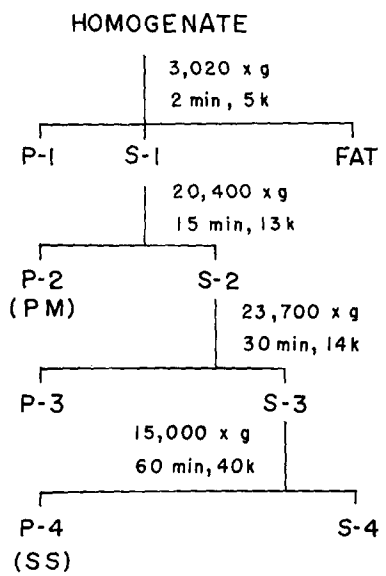


Fig. 1. Preliminary fractionation. Homogenate of adipocytes was fractionated into several fractions by differential centrifugation. The rotors used were Beckman's JA 20 (for the first three centrifugations) and 75 Ti (for the last centrifugation). The time for centrifugation includes the period of acceleration, but not that of deceleration. The *g*-values indicate the maximum levels attained at the steady state. $k$ = 1000 rpm; PM = fraction enriched with the plasma membrane; and SS = fraction enriched with slow-sedimenting microsomes.

several times on different occasions with different batches of adipocytes.

In the first set of experiments shown in Fig. 2, Fractions P-2 and P-4 (see Fig. 1) were subjected to linear sucrose density gradient centrifugation for 40 min at 35000 rpm ($160000 \times g_{av}$). Under these conditions, the two groups of glucose transport activities were concentrated into two different locations. Thus, as shown in Fig. 2A, the glucose transporter in Fraction P-2 was fractionated, along with the 5′-nucleotidase (a plasma membrane marker), into Fractions 3-7. The sucrose concentration at the estimated summit of their peaks was 28% ($d = 1.118$) *. In contrast, as reported in Fig. 2B, the glucose transporter in Fraction P-4 was concentrated, together with galactosyltransferase, into Fractions 14-16. The sucrose concentration at the estimated summit of their peaks was 16% ($d = 1.064$).

In the next set of experiments reported in Fig. 3, Fractions P-2 and P-4 were centrifuged for 18 h at 35000 rpm. Under these conditions, the two glucose transport activities were concentrated into almost identical fractions. The sucrose concentration at the estimated summit of the peak in Fig. 3A was 28% ($d = 1.118$), and that in Fig. 3B was 29% ($d = 1.122$). The locations of these peaks in a sucrose density gradient were not significantly altered when the centrifugation time was increased to 24 h. This indicated that both types of the transporter-carrying vesicles had been brought down to their isopycnic points in 18 h.

The data in Figs. 2B and 3B also indicate that the position of the peak of glucose transporter was identical to that of galactosyltransferase in both cases. However, when the centrifugation (at 40000 rpm) was terminated at either 1.5 or 3.0 h, the peak of glucose transporter was found slightly, but significantly, ahead of that of galactosyltransferase (data not shown). This observation was consistent with the data obtained in our previous experiment with rat cardiac myocytes [5].

Earlier, Oka and Czech [17] concluded that the

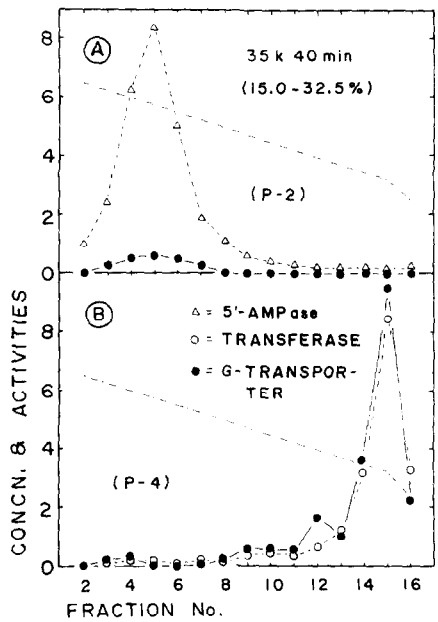


Fig. 2. Sucrose density gradient centrifugation (non-equilibrium centrifugation). Fraction P-2 and P-4 (see Fig. 1) were centrifuged for 40 min in a Beckman's SW 41 rotor as specified in the figure. The unit of glucose transport activity (G-transport (●)) is 0.5 ×nmol glucose taken up in 20 s per min per ml of the fraction; that of 5′-nucleotidase activity (5′-AMPase (△)) is 0.25×nmol/min per ml; that of galactosyltransferase activity (transferase (○)) is 20×nmol/min per ml; and that of sucrose (·–·–·) is 0.2×% (w/w). The transport and enzyme activities were normalized to 24.6 mg protein (average amount from six rats) in Fraction S-1 (in Fig. 1).

slow-sedimenting glucose transporter in adipocytes is somehow sequestered from the extracellular buffer. Thus, using tritiated cytochalasin B as the labeling agent, these investigators observed that photolabeling of glucose transporter in the plasma membrane was inhibited by 50 mM ethylidene glucose at 15 °C, but that of the transporter in the 'low-density' microsomal fraction was not. They interpreted these data as indicating that under the given conditions ethylidene glucose interacted only with those glucose transporters that were exposed to the cell surface and protected them from photolabeling. It appears, therefore, that a portion of glucose transporters in adipocytes is bound to intracellular membranes, which are either vesicles in nature or readily convertible into vesicles by homogenization. When the cell homogenate is fractionated by differential centri-

* The position of the summit of a peak was estimated by extrapolating the lines on both sides of the peak. Note that the points in the figure represent the average activities in the respective fractions, and that the highest point in a peak does not necessarily show the location of the summit.

fugation, these vesicles are concentrated into a slow-sedimenting (or high-speed pellet) fraction (Refs. 2–7 and Figs. 1 and 2). This fraction is often referred to as a low-density microsomal fraction (e.g., Refs. 6, 9, 10 and 17), but our present data indicate that the specific gravity of these vesicles is similar, if not identical, to that of the plasma membrane (Fig. 3). Since it is generally accepted that the sedimentation velocity of small particles is a function of their size and specific gravity [18], the above data may indicate that the size of the vesicles associated with the intracellular glucose transporter is considerably smaller than that of the vesicles derived from the plasma membrane. In fact, Simpson et al. [10] observed by electron microscopy that the 'low-density' microsomal fraction was enriched with very fine vesicles. It is conceivable, however, that the sedimentation velocity of biological vesicles may also be affected by other factors, such as the shape or electric charges of the vesicles. The finding that the specific gravity of the slow-sedimenting vesicles with glucose transport activity is similar to that of the plasma membrane (Fig. 3) is consistent with the

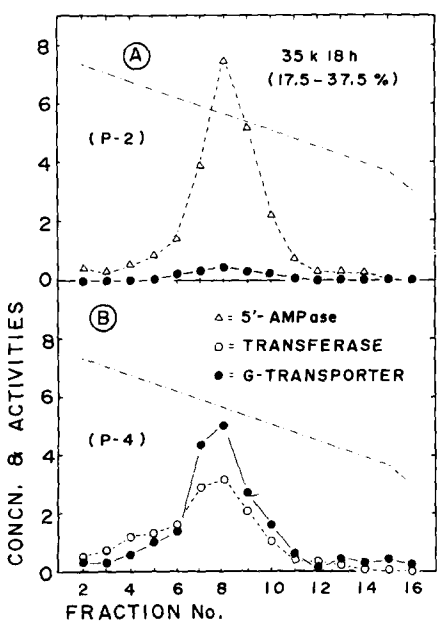


Fig. 3. Sucrose density gradient centrifugation (equilibrium or isopycnic centrifugation). Fractions P-2 and P-4 (see Fig. 1) were centrifuged for 18 h at 2° C as specified in the figure. See Fig. 2 for the units of the enzyme activities.

view that the glucose transporter is recycled between an intracellular site and the plasma membrane by endo- and exocytotic reactions. It should be noted in this connection that the specific gravities of cellular membranes are not necessarily identical; for example, the specific gravity of mitochondria in rat adipocytes (approximately, $d = 1.17$, which is equal to that of 38% sucrose (data not shown)) is considerably larger than that of the plasma membrane, despite the fact that they are both basically made of lipid bilayers. Finally, our present data do not prove, nor disprove, the hypothesis that the intracellular glucose transporter is associated with the Golgi apparatus. Although we found that the glucose transporter and galactosyltransferase in Fraction P-4 can be partially separated under selected conditions (data not shown), it is still obscure whether a majority of the transport activity is associated with the Golgi apparatus or some other type of vesicles that have similar sedimentation characteristics to those of the Golgi apparatus.

In conclusion, it was unfortunate that a fraction similar to our present Fraction P-4 was referred to as a low-density microsomal fraction by Karnieli et al. [6]. Their terminology, which has been widely used in the field (e.g., Refs. 9, 10 and 17), is not entirely incorrect, but is misleading. We suggest, therefore, that the fraction enriched with intracellular glucose transporter should be designated as either a high-speed pellet fraction [5] or slow-sedimenting microsomal fraction.

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