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ASSOCIATION OF EPIDIDYMAL ADIPOSE TISSUE HEXOKINASE WITH SUBCELLULAR STRUCTURE

Ø. SPYDEVOLD AND B. BORREBAEK
Institute for Medical Biochemistry, University of Oslo, Oslo (Norway)

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SUMMARY

1. When differential centrifugations were carried out with sucrose homogenates of rat epididymal adipose tissue, it was observed that hexokinase activity was associated with subcellular fractions.

2. Electron microscopy of the precipitates resulting from the differential centrifugations revealed that hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) activity was associated with both mitochondria and microsomes. Mitochondria were specifically identified by treatment with calcium phosphate and gradient centrifugation.

3. Bound hexokinase activity was observed with both inner and outer mitochondrial membranes separated after hypotonic breakage of the mitochondria.

4. The bound hexokinase activity was slowly released in the course of 50 min incubation at 4° when $MgCl_2$, KCl or $(NH_4)_2SO_4$ were added to suspensions of either mitochondria or microsomes. When glucose 6-phosphate was added, hexokinase activity was rapidly released, resulting in a certain partition between soluble and particle-bound enzyme activity which was dependent on the concentration of glucose 6-phosphate.

5. The effect of glucose 6-phosphate was antagonized by inorganic phosphate. This allowed the demonstration of reversible release of bound hexokinase activity. Thus, when enzyme activity was first eluted from the particles by addition of glucose 6-phosphate, a considerable part of the activity was brought back to the particle-bound state by the subsequent addition of inorganic phosphate.

INTRODUCTION

Association of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) with subcellular structure of rat brain was reported in 1945 by UTTER, WOOD AND REINER[1]. In 1953, CRANE AND SOLS[2] demonstrated particulate hexokinase activity in other rat tissues also. Since then, ability of binding hexokinase has been observed with different types of subcellular structure, such as mitochondria (brain tissue[3], rat

liver[4] and ascites cells[5]), microsomes (brain tissue[3]), sarcoplasmic vesicles (skeletal muscle[6]) and plasma membrane (erythrocytes[7]). Currently studying the hexokinase of the rat epididymal fat pad[8,9], we have investigated to what extent the hexokinase activity is bound to the subcellular structure of this tissue. It is shown in the present communication that epididymal adipose tissue hexokinase activity is associated with the mitochondria as well as with the microsomal fraction. Further, the presented effects of ions, glucose 6-phosphate, inorganic phosphate and ATP show that the mechanism of binding of hexokinase to subcellular structures of epididymal adipose tissue resembles that observed in ascites cells[5] and heart muscle[10].

EXPERIMENTAL

Animals and their treatment

Male rats (110–170 g) of local strain, raised and maintained on adequate stock diet were used throughout.

Preparation of homogenates

The rats were stunned by cervical fracture, and then bled. The epididymal fat pads were rapidly excised, blotted on filter paper and weighed. The weighed fat pads were then homogenized with 10 ml homogenizing medium/g in a motor-driven Potter–Elvehjem-type grinder provided with a loose-fitting teflon pestle. The homogenizing medium consisted of 0.24 M sucrose, 20 mM Tris, 5 mM EDTA and 3 mM 2-mercaptoethanol at pH 7.4. 5 or 6 passes were made. In each pass the vessel was pushed firmly up against the pestle until the pestle reached the bottom. The homogenate was then centrifugated at 1000 $\times$ *g* for 5 min to remove cell debris and most of the fat. Further procedures are given in the legends to figures and tables.

Centrifugations

Centrifugations at centrifugal forces between 1000 $\times$ *g* and 20 000 $\times$ *g* were performed with a Model PR-2 International Refrigerated Centrifuge. Centrifugations at 40 000 $\times$ *g* and 100 000 $\times$ *g* were performed with a Model L Spinco Ultracentrifuge, rotor 21 and 40 respectively. Gradient centrifugations were performed with a Model B-35 International Preparative Ultracentrifuge, Rotor Type SB-206.

Centrifugations were performed at 0–2°. Other steps in the preparative procedure were performed in ice bath at 0°.

Hexokinase assay

A modification of the method of DiPietro and Weinhouse[11] was used. The amount of glucose 6-phosphate formed from glucose by the enzyme was determined by following the appearance of NADPH in the presence of purified glucose-6-phosphate dehydrogenase and $NADP^+$. Changes in absorbance were measured spectrophotometrically at 340 mμ with a RK Complete Hilger-Gilford reaction-kinetics spectrophotometer. Each cuvette contained 80 mM histidine–HCl, 80 mM Tris, 9 mM EDTA, 9 mM $MgCl_2$, 7 mM ATP, 2 mM $NADP^+$, 65 m units/ml of purified glucose 6-phosphate dehydrogenase and 100–200 μl/ml of tissue preparation, in aqueous solution at pH 8.0. The reaction was started by addition of glucose to a concentration of 50 mM. The final volume of the cuvette was 2.0 ml, and the reaction was carried out at 30°. The absorb-

ances of 4 samples were recorded sequentially in automatic rotation at 8-sec intervals for the first 10 min after zero-order kinetics were observed. The extinction coefficient of $6.22 \cdot 10^6$ cm^2/mole for NADPH was used to convert changes in absorbance to amount of NADPH formed.

Separation of mitochondrial membrane fractions

The mitochondrial membranes were separated according to PARSONS, WILLIAMS AND CHANCE[12]. The mitochondrial pellet (the precipitate obtained after centrifugation step 3 in Table I) from 40 ml of adipose tissue homogenate was carefully washed

TABLE I

HEXOKINASE ACTIVITIES OF THE FRACTIONS SEPARATED BY DIFFERENTIAL CENTRIFUGATIONS OF THE EPIDIDYMAL ADIPOSE TISSUE HOMOGENATE

The homogenate was first centrifuged for 5 min at 1000 × *g*. The infranatant was then further centrifugated stepwise as indicated in the table. Hexokinase activities and protein values were determined in the supernatants after each step of centrifugation. Thus, the data given for mitochondrial, intermediate and microsomal fractions represent the amounts of hexokinase activity and protein which were removed (spun down) from the suspension at the indicated centrifugation intervals. Hexokinase activity is expressed as mμmoles NADPH formed per min per ml of supernatant (or infranatant), protein as mg per ml of supernatant, and specific hexokinase activity as mμmoles NADPH formed per min per mg protein. The data (presented with S.E. values in column 4 and 6) represent the means of 8 observations.

Fraction	*Centrifugation interval*	*Hexokinase activity*	*Hexokinase activity as per cent of total*	*Protein*	*Specific hexokinase activity*
Total*		121	100	1.53	79 ± 5
Mitochondrial	5 min at 1 000 × *g*— 10 min at 10 000 × *g*	34	28 ± 3	0.21	162 ± 9
Intermediate	10 min at 10 000 × *g*— 10 min at 20 000 × *g*	6	5 ± 1.2	0.22	23 ± 4
Microsomal	10 min at 20 000 × *g*— 45 min at 100 000 × *g*	7	6 ± 0.9	0.19	32 ± 4
Soluble**		74	61 ± 5	0.91	81 ± 7

* Values measured in the infranatant after centrifugation of the homogenate for 5 min at 1000 × *g*.

** Values measured in the supernatant after 45 min at 100 000 × *g*.

3 times, resuspended in 20 ml homogenizing medium and recentrifuged at 10 000 × *g* for 10 min. The resulting precipitate was resuspended in 2 ml of 20 mM phosphate buffer (pH 7.2) containing 0.02% bovine serum albumin, diluted to 250 ml with the same buffer, allowed to stand for 20 min at 4° and then centrifuged at 40 000 × *g* for 20 min. This resuspension in the hypotonic buffer with the following centrifugation was then repeated and the final pellet was resuspended in 3 ml 20 mM phosphate buffer (pH 7.2). 2 ml of the suspension was layered on the top of a discontinuous sucrose gradient consisting of 2 ml 1.5 M sucrose ($P = 1.192$), 2 ml 1.1 M sucrose ($P = 1.142$) and 2 ml 0.74 M sucrose ($P = 1.094$). The gradient was centrifugated at 115 000 × *g* for 60 min. A pellet at the bottom of the tube and 3 turbidity bands at the interfaces

appeared. The centrifugation tube was then frozen at −20°, and different fractions were collected by cutting the frozen tube in 8 pieces (see Fig. 3).

Cytochrome oxidase

Cytochrome oxidase activity was determined according to SMITH[13].

Electron microscopy

The pellets were fixed in osmium tetroxide according to CAULFIELD[14]. The specimens were dehydrated in acetone and embedded in Araldite according to WEBSTER *et al.*[15]. Sections were cut with glass knives on a LKB Ultrotome and stained with lead acetate. Electron micrographs were obtained with a Siemens Elmiskop I.

Incubation procedure

Particle suspensions were incubated (with linear, horizontal agitations at 80 cycles/min and 4 cm amplitude) in glass tubes without gassing at 4°.

Chemical analysis

Protein was determined by the method of LOWRY *et al.*[16].

Materials

The disodium salt of ATP (neutralized with KOH before use), the monosodium salt of $NADP^+$ and antimycin A were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Glucose-6-phosphate dehydrogenase was obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Glucagon-free insulin, recrystallized 10 times, was a gift from Novo Terapeutisk Laboratorium, Copenhagen, Denmark. Araldite was obtained from Ciba Ltd., Basle, Switzerland.

RESULTS

Preparation and identification of the subcellular fractions

To our knowledge, no detailed characterization of the fractions obtained by differential centrifugations of the epididymal adipose tissue homogenate prepared as described in EXPERIMENTAL has been reported. It was therefore necessary to perform electron microscopic examinations of the precipitates. The whole homogenate was not suitable for enzyme assays due to tissue debris and high fat content. Consequently, we used the infranatant resulting from centrifugation of the homogenate at 1000 × *g* for 5 min as the starting material. 3 precipitates were obtained from the infranatant by stepwise centrifugations, at 10 000 × *g* for 10 min (precipitating the mitochondrial fraction shown in Fig. 1A) followed by 20 000 × *g* for 10 min (precipitating the intermediate fraction shown in Fig. 1B) and finally at 100 000 × *g* for 45 min (precipitating the microsomal fraction shown in Fig. 1C).

Hexokinase activities of the subcellular fractions

As is shown in Table I, hexokinase activity was present in all the investigated fractions. The data are based on measurements of hexokinase activities in the supernatants from the stepwise centrifugations rather than on measurements of enzyme

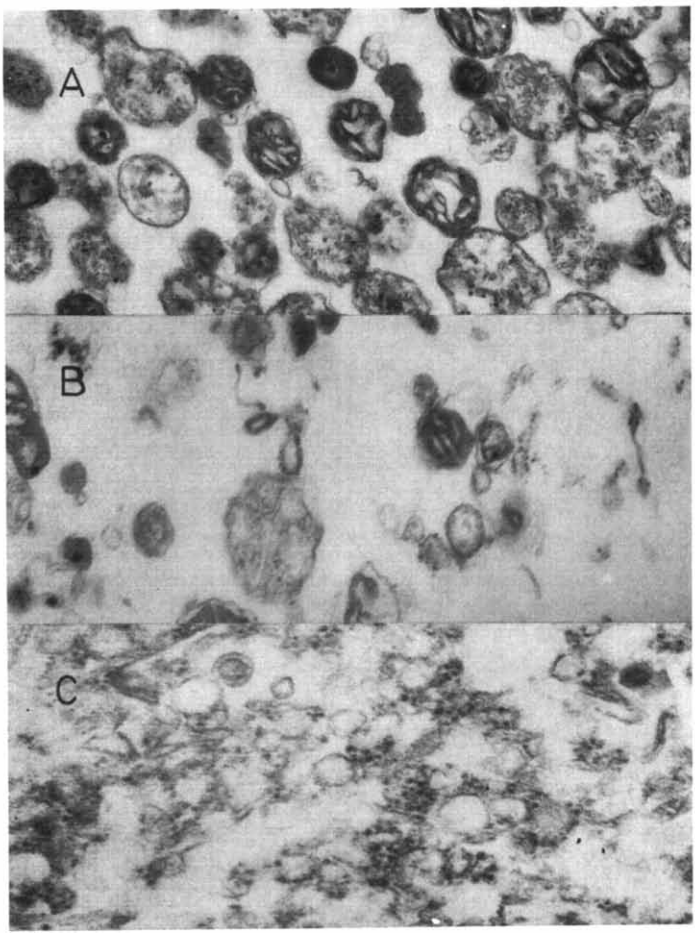


Fig. 1. Electron micrographs (see EXPERIMENTAL) of the precipitates resulting from the differential centrifugations described in the text and in Table I. A, the mitochondrial fraction (× 13 000). B, the intermediate fraction (× 32 000). C, the microsomal fraction (× 50 000). (The electron micrographs were performed at the Institute of Anatomy, University of Oslo.)

activities in the isolated resuspended particle fractions. The binding to the particles was labile so that partial loss (release) of hexokinase activity occurred during the isolation procedures. Considerable variations in total adipose tissue hexokinase activity (from rat to rat) were observed, while the relative subcellular distribution and specific activities (presented together with the S.E. values) were fairly constant.

Identification of mitochondrial-bound hexokinase

To establish whether the hexokinase in the adipose tissue mitochondrial fraction was truly bound to the mitochondria and not to other types of particles sedimented in the same fraction, a method used by ROSE AND WARMS[5] for a similar purpose was employed. This method is founded upon the observations by GREENAWALT, ROSSI AND LEHNINGER[17], that mitochondria which had been pre-incubated under conditions of calcium phosphate uptake sedimented to a region of higher density in a linear

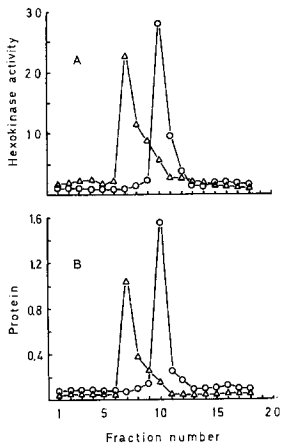


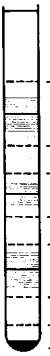
Fig. 2. Distribution of hexokinase activity and mitochondria on a linear (0.25 M to 3.0 M) sucrose gradient. Mitochondria from epididymal fat pads of 3 rats were suspended in 20 ml of a mixture consisting of 10 mM Tris, 10 mM $MgCl_2$, 6.7 mM succinate, 2 mM ATP, 4 mM $CaCl_2$, 4 mM NaH_2PO_4 and 0.25 M mannitol at pH 7.2. The suspension was divided in 2 and incubated with (○) and without (△) antimycin A (1 μl/ml) for 20 min. After centrifugation at 10 000 × g for 10 min, the mitochondrial pellet was gently washed with sucrose and then resuspended in 1 ml 0.25 M sucrose and placed at the top of a sucrose gradient according to GREENAWALT, ROSSI AND LEHNINGER[17]. After centrifugation at 110 000 × g for 180 min the gradients were fractionated by collecting equal counts of drops from holes in the bottom of the tubes. 0.6 ml was collected in each tube. Protein and hexokinase activity were determined in each fraction. Hexokinase activity is presented as mμmoles NADPH formed per min and protein as mg, both per whole fraction (0.6 ml).

sucrose gradient than when the process of calcium phosphate uptake had been blocked by antimycin A. It is clearly shown in Fig. 2B that the protein peak (representing particulate material) migrated to a higher density region in the gradient after calcium phosphate uptake. This effect is specific for the mitochondria, and since a corresponding change was observed with the hexokinase activity peak (Fig. 2A), it is evident that the hexokinase was associated with the mitochondria.

Hexokinase activities of inner and outer mitochondrial membranes

Inner and outer mitochondrial membranes were separated as described in EXPERIMENTAL. As is shown in Fig. 3, considerable amounts of hexokinase activity were observed in Fractions 1, 2, 4, 6 and 8. The distribution of turbidity on the gradient (Fig. 3) is in correspondence with that found by PARSONS, WILLIAMS AND CHANCE[12] working with rat liver mitochondria. According to their results, Fraction 4 consisted of outer membrane material, Fraction 6 contained inner membrane with outer membranes still attached, the pellet in Fraction 8 consisted of inner membranes contaminated with a small amount of outer membrane material, and Fraction 2 contained some outer membrane material and lipid droplets. The specific cytochrome oxidase activities in the different fractions presented in Fig. 3 are in accordance with a similar distribution in the preparation from adipose tissue.

Probably, the enzyme activity in Fraction 1 represents hexokinase activity which was solubilized during the experiment. Enzyme activity in Fractions 2 and 4 (Fig. 3) indicated hexokinase associated with outer mitochondrial membranes. The hexokinase in Fraction 8 can hardly be explained by contamination with outer



Fraction number	Volume (ml)	Protein	Hexokinase activity	Specific hexokinase activity	Specific cytochrome *c* oxidase activity
1	1.8	17.3	19.4	30	—
2	1.5	18.2	13	19	—
3	0.6	1.7	1.0	—	—
4	1.0	7.8	10.6	36	0.3
5	0.6	3.8	3	—	—
6	1.3	42.2	46	29	0.32
7	0.6	3.0	1	—	—
8	0.6	6.0	6.0	28	5.08
		100	100		

Fig. 3. Mitochondrial membrane fractions separated on a discontinuous sucrose gradient (see EXPERIMENTAL). The 3 distinct turbid bands that appeared at the interfaces are indicated by hatched areas. The filled area at the bottom represents the pellet. The gradient was frozen and cut into 8 pieces at the places marked by dotted lines. Protein and hexokinase activity are presented as per cent of total. The total amount of protein was 1.85 mg and total hexokinase activity was 48.6 mμmoles of NADPH formed per min. Specific hexokinase activity is presented as mμmoles NADPH formed per min per mg protein. Specific cytochrome *c* oxidase is presented as mmoles reduced cytochrome *c* oxidized per min per 10 mg protein.

TABLE II

RELEASE OF HEXOKINASE ACTIVITY FROM THE ISOLATED PARTICULATE FRACTIONS

The particulate fractions were obtained by centrifugations as described in the text and in Table I. The precipitates were suspended in the homogenizing medium (0.24 M sucrose, 20 mM Tris, 5 mM EDTA and 3 mM 2-mercaptoethanol at pH 7.4). The suspensions were distributed among different tubes (3 ml in each) which were incubated at 4° for the indicated time periods. Particle-bound hexokinase activity was determined in each sample by estimating the difference in activity before and after centrifugation at 10 000 × *g* for 10 min (mitochondria) and at 100 000 × *g* for 60 min (microsomes).

Addition	*Per cent particle-bound hexokinase activity*			
	Mitochondrial			*Microsomal*
	After 5 min incubation	*After 30 min incubation*	*After 50 min incubation*	*After 60 min incubation*
No addition	80	74	71	88
2 mM Na_2HPO_4	82	76	73	90
0.01 mM glucose 6-phosphate	53	49	44	63
0.04 mM glucose 6-phosphate	32	28	30	22
0.2 mM glucose 6-phosphate	6	3	5	
0.2 mM ATP	78	65	68	63
2 mM ATP	17		16	
0.13 M KCl		25	14	0
0.07 M $MgCl_2$		39	20	0
0.03 M $(NH_4)_2SO_4$	68	31	13	

membrane material, since the specific hexokinase activity of this fraction was comparable to that of the outer membrane fraction (Fraction 4 in Fig. 3). It seems therefore that both outer and inner membranes of the epididymal adipose tissue mitochondria bind hexokinase.

Effect of ions, glucose 6-phosphate and ATP on the binding of hexokinase activity to subcellular particles

As shown by ROSE AND WARMS[5], ascites cell mitochondrial-bound hexokinase was released from the particles by certain cations and small-molecular compounds. Some of the effectors described by these workers were tested in the present investigation in order to see whether the hexokinase-binding property of adipose tissue is of the same type as that of ascites cell mitochondria. As is evident from Table II, hexokinase activity was solubilized when the particle suspensions were incubated at 4° in the presence of magnesium chloride, potassium chloride, ammonium sulfate, ATP and glucose 6-phosphate. With the inorganic salts, 50–70% of the bound hexokinase was released after 30 min while 75–85% was released after 50 min incubation. With glucose 6-phosphate, the enzyme activity was rapidly released, reaching a certain partition between soluble and particle-bound enzyme activity (dependent on the glucose 6-phosphate concentration) already after 5 min of incubation. This partition was almost constant up to 50 min of incubation. These results are in accordance with the observations by ROSE AND WARMS[5], who have suggested the existence of an equilibrium between mitochondrial-bound and soluble hexokinase in ascites cells, the equilibrium constant being influenced by glucose 6-phosphate. Particle-bound hexokinase activity was rapidly released also in the presence of ATP (Table II), although at a much higher concentration than with glucose 6-phosphate. It should be noted that the data in Table II represent the amount of the hexokinase activity which was associated with the particles. No difference in total hexokinase activity was observed, indicating that the decrease in particle-bound activity really represented a release of hexokinase molecules and not deactivation of firmly bound enzyme.

Rebinding of previously released hexokinase activity.

Evidence for reversibility between mitochondrial-bound and soluble hexokinase activity was confirmed as the effect of glucose 6-phosphate was antagonized by inorganic phosphate. In the experiments described in Fig. 4, mitochondrial-bound hexokinase activity was first eluted by the addition of glucose 6-phosphate. The steady partition between bound and soluble enzyme activity was reached very rapidly (open circles) as the elution was almost completed at zero incubation-time. This means that the elution process took place during the centrifugation after addition of glucose 6-phosphate. When inorganic phosphate was added to the glucose 6-phosphate-containing suspension after 10 min of incubation, a considerable amount of the previously eluted hexokinase activity was brought back to the mitochondrial-bound state (filled circles in Fig. 4). This rebinding of the enzyme activity appears immediately at the figure (Fig. 4), indicating that the process was completed during the centrifugation. As shown in Table III, similar antagonistic effects of glucose 6-phosphate and inorganic phosphate were observed with the microsomes also.

It should be noted that the indicated times in Tables II and III and Fig. 4 must be taken with some precaution. With an incubation temperature as low as 4°, the

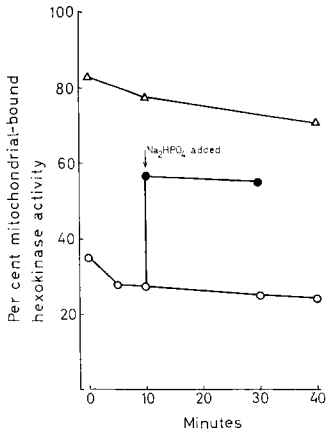


Fig. 4. The rebinding of previously eluted mitochondrial-bound hexokinase activity by the addition of inorganic phosphate. The mitochondrial suspension was prepared as described in Table II. The suspension was divided among several glass tubes (1 ml in each) which were incubated for different time periods at 4°, either with no further addition (△), or with glucose 6-phosphate added to a concentration of 0.04 mM before incubation (○). To some of the tubes containing glucose 6-phosphate, Na_2HPO_4 was added to a concentration of 10 mM after 10 min of incubation (●). Mitochondrial-bound hexokinase activity was determined by estimating the difference in activity before and after centrifugation at 10 000 × *g* for 10 min. The data represent the means of 3 observations.

length of the centrifugation procedure following the incubation period probably represented a significant addition to the times indicated.

DISCUSSION

It could be questioned whether the reversible release/rebinding of hexokinase to subcellular structure observed *in vitro* is relevant in the intact cell *in vivo*. The data reported here do not give any answer to this problem. However, as proposed by SIEKEVITZ[18], association of enzymes with subcellular structure may play an important role in the regulation of metabolic pathways. Recently, MARGRETH, MUSCATELLO AND ANDERSON-CEDERGREN[19] have emphasized this view considering the regulation of glycolysis in muscle tissue. The possibility that intracellular translocation of hexokinase is important in the regulation of carbohydrate metabolism of adipose tissue is further strengthened by the finding of a difference in the amount of mitochondrial-bound enzyme between fasted and carbohydrate-fed rats[9], and by the observations of increased mitochondrial-bound hexokinase activity in whole epididymal fat pads incubated in the presence of glucose and insulin[9].

The finding of bound hexokinase both in inner and outer mitochondrial membranes (Fig. 3) is not quite in accordance with the results of ROSE AND WARMS[5]. These workers have presented strong arguments for the view that in ascites cells, bound hexokinase is confined to the external mitochondrial surface. However, the possibility of random transformation of hexokinase activity from outer to inner membrane fractions during the separation procedure employed in the present study is not ruled out.

We have made several attempts to determine which sub-fraction of the microsomal pellet binds the hexokinase. Unfortunately, the detergents (deoxycholate, dodecyl sulfate and isooctanol) employed in the separation procedures appeared to be inhibitors of the hexokinase. However, as is evident from the presented data, the mechanisms of the binding of hexokinase to mitochondria on the one hand, and to microsomes on the other are of similar nature, as they are acted upon in the same way by the effectors. It is then tempting to suggest that the enzyme is bound to similar kind of material in the two different subcellular fractions. Possibly, we are dealing with a more or less general hexokinase-binding property of cellular lipoprotein membranes.

The effects of ionic strength, ATP, glucose 6-phosphate and inorganic phosphate on the association of hexokinase activity with mitochondria in the epididymal adipose tissue reported here correspond well to the results of ROSE AND WARMS[5] with ascites cells, and of HERNANDEZ AND CRANE[10] with heart tissue. It is therefore likely that the conclusions reached by ROSE AND WARMS[5] for the binding of hexokinase to the mitochondria of ascites cells also are valid for the similar phenomenon in adipose tissue, that there exists a reversible transformation between free and mitochondrial-bound hexokinase in the intact cell which is controlled by glucose 6-phosphate and inorganic phosphate (and probably other metabolites) by mechanisms related to their effects on the catalytic activity of the hexokinase. Also the effect of ions probably represents a significant control mechanism, at least the concentration of KCl employed in the experiments described in Table II is within the physiological range.

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