Transport and Metabolism of Glucose, Insulin Sensitivity and Lipolysis of Epididymal Adipose Tissue of Alloxan-Diabetic Rats after Prolonged Incubation *in vitro**

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Summary. The metabolism of diabetic adipose tissue was studied after prolonged preincubation *in vitro* (16 h) in a medium containing albumin and glucose with and without insulin. The initially decreased glucose uptake and metabolism of diabetic adipose tissue rose to normal levels during incubation. This increase was subject to inhibition by phlorizin, indicating that facilitated glucose diffusion was responsible. The tissue remained insulin-sensitive during prolonged incubation. However, insulin sensitivity did not increase in the same way as basal glucose uptake. — Glycerol release and incorporation of glucose-U-¹⁴C into glyceride-glycerol increased during incubation, and both were inhibited to some extent by insulin. Fatty acid synthesis from glucose-U-¹⁴C increased during incubation but did not reach normal values. — Unlike tissues of fed rats, phosphorylation of glucose rather than transport became the rate-limiting step in glucose metabolism after prolonged incubation *in vitro*. Cycloheximide, but not actinomycin D, inhibited the preincubation effect.

Transport et métabolisme du glucose, sensibilité à l'insuline et lipolyse du tissu adipeux épидидymaire de rats rendus diabétiques par l'alloxane après incubation prolongée in vitro

Résumé. Le métabolisme glucidique du tissu adipeux diabétique après incubation pendant 16 h dans un tampon contenant du glucose et de l'albumine, avec et sans insuline a été examiné. Le métabolisme glucidique d'abord diminué s'élevait à des taux normaux lors de l'incubation. Puisque la phlorizine supprimait cet effet stimulateur de l'incubation la diffusion facilitée du glucose semble être en cause. La sensibilité à l'insuline était maintenue pendant l'incubation, mais elle n'augmentait pas. — La libération de glycérol ainsi que l'incorporation du glucose-U-¹⁴C dans la fraction glycérde-glycérol augmentaient au cours de l'incubation et, alors, étaient inhibées par l'insuline. La synthèse des acides gras à partir du glucose-U-¹⁴C augmentait également mais n'atteignait

pas des valeurs normales. — Tandis que le transport du glucose limite le métabolisme du glucose dans le tissu adipeux normal, la phosphorylation devenait le facteur limitant après l'incubation prolongée. Un agent inhibiteur de la synthèse des protéines, la cycloheximide, inhibait la stimulation du métabolisme du glucose par l'incubation même, tandis que l'actinomycine D était sans effet.

Glucosetransport und -stoffwechsel, Insulinempfindlichkeit und Lipolyse des epididymalen Fettgewebes alloxan-diabetischer Ratten nach längerer Inkubation in vitro

Zusammenfassung. Diabetisches Fettgewebe wurde während 16 Std in einem Puffer mit Albumin und Glucose mit und ohne Insulin inkubiert, und die resultierenden Stoffwechselveränderungen mit Glucose-U-¹⁴C während einer zweiten Inkubation gemessen. Der anfänglich verminderte Glucosestoffwechsel des diabetischen Gewebes normalisierte sich im Verlaufe der Vorinkubation. Da Phlorizin diesen Vorinkubationseffekt auf die Glucoseaufnahme hemmte, darf eine Steigerung der Aktivität des mobilen Carriers dafür verantwortlich gemacht werden. Die Insulinempfindlichkeit des Gewebes blieb während der verlängerten Inkubation erhalten, nahm aber nicht parallel zur basalen Glucoseaufnahme zu. — Die Glycerinabgabe und der Einbau von Glucose-U-¹⁴C in die Glycerid-Glycerol-Fraktion nahm während der Inkubation zu und beide wurden durch Insulin zum Teil gehemmt. Die Fettsäuresynthese aus Glucose-U-¹⁴C wurde gesteigert, normalisierte sich aber nicht vollständig. — Im Gegensatz zu normalem Gewebe wurde an Stelle des Transportes die Phosphorylierung der Glucose zum geschwindigkeitsbestimmenden Faktor des Glucosestoffwechsels. Cycloheximid verminderte den Vorinkubationseffekt auf den Glucosestoffwechsel, während Actinomycin D ohne Wirkung war.

Key-words: Adipose tissue of diabetic rats, prolonged incubation *in vitro*, glucose transport and metabolism, fructose metabolism, insulin sensitivity, lipolysis.

Introduction

The decreased glucose metabolism of adipose tissue of fasted rats can be raised by prolonged incubation of the tissue *in vitro* [31, 21, 10].

In a previous study [37, 38] we have shown that the diminished glucose uptake of adipose tissue of alloxan-diabetic rats is stimulated to normal levels within 8 to 16 h during incubation *in vitro* in the absence of insulin.

This report deals with the mechanisms responsible for the partial normalization of the insulin-independent glucose metabolism during incubation *in vitro*. Glucose and fructose transport, hexose-¹⁴C metabolism and adipose tissue lipolysis as influenced by prolonged incubation and by insulin were studied.

Evidence is presented that the increased insulin-independent glucose penetration during prolonged incubation proceeds by way of facilitated diffusion and that phosphorylation rather than transport becomes rate-limiting in overall glucose metabolism.

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Materials and Methods

Glycerol was determined enzymatically using the reagents of Biochimica (Boehringer, Mannheim, Germany). Free fatty acids of the medium were extracted and titrated according to GORDON [27]. Uniformly labelled glucose- ^{14}C and fructose- ^{14}C were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. They were chromatographically pure.

Total lipids were extracted according to FOLCH et al. [13]. The extract was dried, taken up and washed with isooctane: glacial acetic acid (1:1). The fatty acids were obtained by hydrolysis of the total lipids in alcoholic KOH. Incorporation of glucose- ^{14}C into glyceride-glycerol was calculated from the difference between the counts in the total lipids and those in the hydrolyzed fatty acids. The dried fat-free tissue was digested in 30% KOH and the glycogen was precipitated with cold ethanol (95%) and twice reprecipitated before counting. The glycogen content of the tissues was measured by the determination of glucose after hydrolysis of the planchettet glycogen in 1 N HCl. The term "glucose metabolism" often used in the text, denotes the sum of the incorporation of glucose- ^{14}C into CO_2 and total lipids. Incorporation into these two metabolites accounts for 70% or more of the actual hexose uptake [20, 18].

Phlorizin dihydrate was purchased from Fluka AG, Buchs, Switzerland; actinomycin D and cycloheximide from Merck, Sharp and Dohme, West Point, Pa. and from Calbiochem, Los Angeles, Calif., respectively. Crystalline pork insulin with an activity of 23.9 units/mg was a gift of Dr. J. Schlichtkrull, Novo Terapeutisk Laboratorium, Copenhagen.

Experimental procedure

Alloxan diabetes: Alloxan in a dose of 45 mg/kg was injected into the tail vein of pure-bred Osborne-Mendel rats weighing 140–160 g after a 24-h fast. Thereafter the rats had free access to food, and were killed in diabetic ketosis 96 h after the alloxan-injection. Occasionally, 10 ml of Ringer solution containing 0.3 meq of potassium was administered subcutaneously on the third and fourth day after alloxan-injection. Blood sugar values above 500 mg per 100 ml and a weight loss of 10 g or more served as criteria of diabetes. A considerable percentage of rats was lost before the start of the experiment.

Incubation technique: The details of the incubation technique have been described elsewhere [37]. The medium used throughout was a sterile bicarbonate buffer solution (EARLE) [7] containing human albumin¹

¹ Human albumin, fraction V, Lot No. 65/08–10 was purchased from the Blutspendedienst, Swiss Red Cross, Bern. It had neither insulin-like nor insulin-inhibitory activity on the fat pad in concentrations up to 6 g per 100 ml.

in a concentration of 1.5 g per 100 ml. Since pooling of adipose tissue as normally practiced in our laboratory [17] was unfavourable for medium changes and was harmful for the tissue, the entire pads were preincubated and transferred into fresh buffer solution after 8 h. For the final incubation the pads were rinsed, cut into small pieces and pooled. The results of this *final incubation* were compared with those obtained with pools of adipose tissue which had not been preincubated (*initial incubation*).

Adipose tissue takes up considerable amounts of water during prolonged incubations [37, 25, 4]. Therefore, the results of the final incubation refer to the initial weight of the tissue. All incubations were carried out in 25 ml polyethylene flasks (Packard) in a Dubnoff metabolic shaker at 37°C under continuous gassing with a mixture of 95% O_2 – 5% CO_2 .

Results

a) Effect of increasing glucose concentrations on fructose- ^{14}C and glucose- ^{14}C metabolism after prolonged incubation. In the range between 10 and 200 mg of glucose per 100 ml the oxidation of glucose to CO_2 and its incorporation into total lipids increased continuously. In a representative experiment in which the concentrations of glucose were 50, 200 and 800 mg per 100 ml respectively, the sums of μmoles of glucose oxidized to CO_2 and incorporated into total lipids per g per h were: 3.50 ± 0.30 (6.46 ± 0.22), 6.29 ± 0.36 (7.74 ± 0.61) and 7.72 ± 0.19 (7.91 ± 0.20) respectively. These values are means of duplicate determinations. The figures in parentheses give the corresponding values in the presence of 1000 μU of insulin per ml. Glucose metabolism was nearly maximal at a glucose concentration of 50 mg per 100 ml in the presence of insulin. At a glucose concentration of 800 mg per 100 ml, glucose metabolism was no longer stimulated by insulin.

Fig. 1 shows the effect of increasing glucose concentrations on fructose- ^{14}C metabolism by adipose tissue of diabetic rats after incubation for 16 h. Glucose in concentrations of 3 and 9 mg per 100 ml neither diminished fructose metabolism nor did it alter its stimulation by 1000 μU of insulin per ml. As the glucose concentration in the medium was further raised, fructose metabolism progressively decreased and insulin accentuated its inhibition. The glucose metabolism increased as the glucose concentration was elevated. At 800 mg of glucose per 100 ml, 6 μmoles of glucose- ^{14}C was metabolized per g per h and an insulin effect was no longer detectable.

b) Inhibition of glucose transport by phlorizin dihydrate during prolonged incubation. The effects of phlorizin on the metabolism of glucose- ^{14}C were investigated in the presence and absence of insulin after different periods of preincubation (Table 1). The

results were statistically evaluated by the analysis of variance [11, 44, 12] (Table 2). The difference between basal and phlorizin-inhibited glucose metabolism (Δ) increased during preincubation. The difference was statistically significant between periods I and II and may have been accidental between periods II and III. Phlorizin inhibited the glucose metabolism of tissue in the presence of insulin more markedly after preincubation than before preincubation.

preincubation with actinomycin D or cycloheximide (Table 3). Actinomycin did not influence the preincubation effect on basal glucose metabolism. In the presence of insulin, actinomycin D slightly stimulated glucose metabolism. Cycloheximide in a concentration of 3.6×10^{-5} M led to a constant and highly significant inhibition of glucose metabolism to 51% of the control values. This effect of cycloheximide was prevented by insulin.

Fig. 1. Effect of various glucose concentrations and of insulin on glucose- $U-^{14}C$ and fructose- $U-^{14}C$ metabolism of adipose tissue of alloxan-diabetic rats after 16 h of preincubation

Epididymal fat pads of 52 alloxan-diabetic rats were preincubated in four consecutive experiments during 16 h with 200 mg per 100 ml. Afterwards they were pooled and incubated for 2 h in the presence and absence of 1000 μU insulin per ml and with hexose concentrations as indicated. The columns give the means of the results of two flasks with their range

Table 1. Inhibition of glucose- $U-^{14}C$ oxidation to CO_2 and of its incorporation into total lipids by phlorizin dihydrate after different periods of preincubation

Epididymal adipose tissue of 24 alloxan-diabetic rats was preincubated for 4, 8 and 12 h respectively. Subsequently the tissue was pooled, and its metabolic activity was tested in a 2-h incubation. No insulin was present in the preincubation medium. The glucose concentration was 200 mg per 100 ml throughout. The results, which give the means of 4 flasks with the standard errors of the mean, are expressed as μ moles of glucose-carbon incorporated into CO_2 and total lipids per g per h

Additions to medium, Final incubation	—	Phlorizin 10 mM	Δ^1	Insulin 1000 μ U/ml	Insulin 1000 μ U/ml, Phlo- rizin 10 mM	$\Delta_{Ins.}^1$	
Preincubation time hours	μ moles of glucose-U- ^{14}C to CO_2 and total lipids per g per h						
I	4	2.51 ± 0.14	0.72 ± 0.08	1.79	4.07 ± 0.12	1.94 ± 0.10	2.13
II	8	3.92 ± 0.18	1.19 ± 0.16	2.73	6.89 ± 0.22	2.59 ± 0.05	4.30
III	12	4.47 ± 0.08	1.56 ± 0.10	2.91	6.55 ± 0.51	2.43 ± 0.43	4.12

¹ difference between values of basal and of phlorizin inhibited glucose "uptake".

c) Effect of actinomycin D and of cycloheximide on the stimulation of glucose metabolism during prolonged incubation. Oxidation of glucose- $U-^{14}C$ to CO_2 and its incorporation into total lipids were measured after a

d) Metabolic fate of glucose- $U-^{14}C$, glycogen metabolism and lipolysis before and after prolonged incubation. Incorporation of glucose- $U-^{14}C$ into CO_2 , total lipids and glycogen, and the response of the tissue to insulin

were studied before and after a preincubation of 16 h (Table 4). Under basal conditions initial glucose metabolism of the diabetic adipose tissue was 1.74 μ moles per g per h (Table 4, line 1) compared with a mean of 3.83 μ moles per g per h in tissue of normal rats under similar conditions. 1000 μ U of insulin per ml stimulated the glucose metabolism of the diabetic tissue 4-fold. After preincubation without insulin the basal glucose metabolism rose to normal levels but the tissue did not become more sensitive to insulin. Insulin (250 μ U per ml) in the preincubation medium did not seem to influence the basal glucose uptake during the final incubation, but it increased the insulin sensitivity of the

Table 2. Analysis of variance and variance ratio test of the results presented in Table 1. Variance ratio = F

Source of variation	Degrees of freedom	Mean Square — Insulin	+ Insulin
P Preincubation periods	2	4.102	7.042
A Agent (control, phlorizin)	1	36.852	74.132
Interaction P · A	2	0.717 ¹	2.927 ³
P · A, Periods I versus II and III	1	1.401 ²	5.817 ⁴
P · A, Periods II versus III	1	0.032	0.037
Error	18	0.067	0.343

¹ F_1 0.717/0.067 $pF_1 < 0.001$.

² F_2 1.401/0.067 $pF_2 < 0.001$.

³ F_1 (insulin) 2.927/0.343 pF_1 (ins.) < 0.01 .

⁴ F_2 (insulin) 5.817/0.343 pF_2 (ins.) < 0.001 .

resulted in an increase of glucose incorporation into fatty acids to 9% of total glucose metabolism (line 3). Insulin in the final incubation stimulated fatty acid synthesis of preincubated tissue 3-fold, i.e. to 17% of

Table 3. Effect of actinomycin D and of cycloheximide during prolonged incubation on glucose metabolism of adipose tissue of alloxan-diabetic rats during final incubation

The preincubation lasted 16 h, the final incubation 2 h. The glucose concentration was 200 mg per 100 ml throughout. The medium for the final incubation did not contain cycloheximide or actinomycin D, whereas the insulin concentration was the same as that indicated for the preincubation medium. The results of the control incubation ($\pm$ insulin) were set as 100%. Students t -test was used for statistical analysis

Additions to preincubation medium	Sum of glucose-U- ¹⁴ C to CO ₂ + total lipids in % of control $\pm$ SEM (n)	p versus control
No addition (control)	100	—
Cycloheximide 3.6 $\times 10^{-5}$ M	51.05 $\pm$ 3.56 (9)	< 0.0005
Actinomycin D 5 $\times 10^{-5}$ M	102.80 $\pm$ 5.85 (7)	> 0.35
Insulin 1000 μ U/ml (control)	100	—
Cycloheximide 3.6 $\times 10^{-5}$ M + Insulin 1000 μ U/ml	102.83 $\pm$ 4.95 (9)	> 0.30
Actinomycin D 5 $\times 10^{-5}$ M + Insulin 1000 μ U/ml	118.33 $\pm$ 6.19 (7)	< 0.025

Table 4. Comparison of glucose-U-¹⁴C metabolism before and after prolonged incubation of epididymal adipose tissue of alloxan-diabetic rats, with and without insulin

Initial incubation: Fat pads of 6 alloxan-diabetic rats were pooled immediately after their removal, and incubated for three hours in the presence of 200 mg of uniformly labelled glucose-U-¹⁴C per 100 ml with and without 1000 μ U of insulin per ml. — *Final incubation:* Fat pads of 12 alloxan-diabetic rats were preincubated for 16 h in the absence of any labelled substrate with 200 mg of glucose per 100 ml, without and with 250 μ U of insulin per ml. At the end of the preincubation these tissues were washed and pooled, and incubated for three hours in the same medium as used for the initial incubation. — The results give the means of triplicate determinations $\pm$ SEM

		Insulin Incorporation of glucose-U- ¹⁴ C in μ moles/g/h into							
		1000 μ U/ml	CO ₂	Total lipids	Fatty acids	Glyceride-glycerol	Glycogen	Total (CO ₂ + TL + Glycogen)	
Initial	no	—	0.90 $\pm$ 0.06	0.66 $\pm$ 0.04	0.06 $\pm$ 0.01	0.60 $\pm$ 0.04	0.18 $\pm$ 0.02	1.74 $\pm$ 0.10	1
	preincubation	+	3.39 $\pm$ 0.13	2.17 $\pm$ 0.15	1.24 $\pm$ 0.03	0.93 $\pm$ 0.12	2.05 $\pm$ 0.09	7.61 $\pm$ 0.28	2
Final	preincubation	—	2.82 $\pm$ 0.03	3.13 $\pm$ 0.06	0.55 $\pm$ 0.01	2.58 $\pm$ 0.06	0.29 $\pm$ 0.01	6.23 $\pm$ 0.09	3
	without insulin	+	4.08 $\pm$ 0.05	4.15 $\pm$ 0.15	1.64 $\pm$ 0.13	2.51 $\pm$ 0.12	1.45 $\pm$ 0.09	9.68 $\pm$ 0.24	4
	preincubation	—	3.65 $\pm$ 0.21	3.08 $\pm$ 0.27	0.95 $\pm$ 0.19	2.13 $\pm$ 0.11	0.10 $\pm$ 0.02	6.83 $\pm$ 0.49	5
	with insulin	+	6.38 $\pm$ 0.18	7.01 $\pm$ 0.38	4.59 $\pm$ 0.23	2.42 $\pm$ 0.29	0.32 $\pm$ 0.06	13.71 $\pm$ 0.43	6

tissue. Fatty acid synthesis from glucose-U-¹⁴C in diabetic adipose tissue initially amounted to 3.4% of the sum of glucose incorporation into CO₂, total lipids and glycogen. It was stimulated 20-fold by insulin (Table 4, line 1 and 2). Preincubation of the tissue

the total glucose metabolism. When the preincubation medium contained insulin, fatty acid synthesis rose to 14%, and was further enhanced to 33% when insulin was also present during the final incubation.

Incorporation of glucose-U-¹⁴C into glyceride-gly-

cerol during the initial incubation was slightly increased by insulin. Preincubation led to a more marked increase, and insulin in the final incubation medium was without effect.

The glycogen content of adipose tissue of alloxan-diabetic rats was too small to be measured accurately. After 16 h of preincubation without insulin the glycogen content was approximately 2 μ moles of glucose per g of tissue. Insulin in the initial and final incubation had practically no effect on glycogen content (Fig. 2). When the tissue was in contact with insulin throughout the entire period of incubation (initial and final) the glycogen content was increased.

Insulin stimulated glucose incorporation into glycogen during the initial incubation approximately 10-fold (Table 4). Sixteen hours of preincubation resulted in a slight increase of basal incorporation into glycogen, which was stimulated 5-fold by insulin, while the glycogen content of the tissue was unchanged (Fig. 2). Preincubation of adipose tissue with insulin resulted in a decrease of basal glucose incorporation into glycogen, and the effect of insulin in the final incubation was small.

Fig. 2. Comparison of the glycogen content, glycerol and free fatty acid release and of the free fatty acid content of adipose tissue of alloxan-diabetic rats before (initial) and after (final) prolonged incubation. Results from the same experiment as in Table 4. The results give the mean and SEM of three flasks. For incubation conditions see legend to Table 4

Glycerol release per g of adipose tissue of diabetic rats (Fig. 2, initial) was increased above that of normal rats. Insulin enhanced the glycerol release in diabetic tissue but not in normal tissue. Preincubation resulted in an increase of basal glycerol release, which was markedly inhibited by insulin. This effect of insulin was more pronounced in tissue that had been preincubated with insulin.

Glucose uptake was correlated with fatty acid synthesis over a wide range of glucose uptake under various conditions that influence the glucose uptake (insulin, hexose concentration, preincubation with and without insulin). The results of a representative experiment are shown in Fig. 3. The arbitrarily drawn curve in Figure 3 indicates that the tissues which were in contact with insulin incorporated relatively more glucose- $U-^{14}C$ into fatty acids than those incubated without insulin at corresponding levels of glucose uptake.

Discussion

Many of the effects of insulin on adipose tissue metabolism may result from accelerated glucose entry into the cells by way of facilitated glucose diffusion (for review see RENOLD et al. [43]). However, insulin affects glycerol and free fatty acid release [20], protein

Fig. 3. Glucose- $U-^{14}C$ incorporation into fatty acids as a function of glucose "uptake" by adipose tissue of alloxan-diabetic rats after 16 h of preincubation

Epididymal adipose tissue of 20 alloxan-diabetic rats was incubated in the presence and absence of 250 μ U of insulin per ml, and with 200 mg of glucose per 100 ml. At the end of the preincubation these tissues were washed, pooled and incubated for 2 hours with uniformly labelled glucose- $U-^{14}C$ in concentrations of 50, 200 and 800 mg per 100 ml in the presence and absence of 1000 μ U of insulin per ml. Each dot represent the results of one flask

metabolism [30, 3, 26] and the resting electrical membrane potential in the absence of glucose [1], so that not all effects of insulin can be explained by its action on glucose transport.

Diabetic adipose tissue has a markedly diminished glucose metabolism, which can be restored to normal by insulin therapy *in vivo* (for review see WINEGRAD et al. [46]). Metabolic abnormalities similar to those found in diabetic adipose tissue also occur when cellular carbohydrate starvation is due to fasting. These are normalized by refeeding. Whether insulin reverses the diabetic abnormalities only by relieving cellular carbohydrate starvation, or whether other effects of the hormone are essential cannot be decided, since it is rarely possible to obtain reproducible rates of glucose uptake under physiological conditions by means other than by insulin. Stimulation of glucose metabolism of rat adipose tissue *in vitro* without insulin has been achieved by hyperosmolarity of the medium [34] and by increasing the hexose concentrations [32].

Severe derangements of adipose tissue metabolism of alloxan-diabetic rats were observed when no insulin was given for at least 48 h [45, 16]. For the restoration of these "diabetic" abnormalities of adipose tissue metabolism 12 or more hours of insulin treatment *in vivo* was required [46, 28]. In a preceding paper we have described a method for the prolonged incubation of adipose tissue *in vitro* [37]. The diminished glucose uptake of the tissue of alloxan-diabetic rats was normalized within 8 to 16 h of incubation in the absence of insulin. The same experimental model was used to study whether or not the normalization of glucose uptake during prolonged incubation *in vitro* without insulin may also lead to a reversal of other diabetic alterations of adipose tissue metabolism.

An important difference between normal adipose tissue and diabetic adipose tissue after "normalization" by prolonged incubation became apparent when the fructose metabolism was investigated. Fructose is transported into the adipose cell by a separate transport system for which glucose does not compete. Apparently, fructose is phosphorylated by the same adipose tissue hexokinase as glucose. The affinity of this phosphorylating enzyme is much greater for glucose than for fructose [18, 29, 6, 24]. The lack of competition at the hexokinase level between these two hexoses in tissue of normal fed rats at physiological glucose concentrations is due to the virtually complete absence of free (non-phosphorylated) glucose within the cell. When maximal rates of glucose uptake are induced by high glucose concentrations (810 mg of glucose per 100 ml) and insulin stimulation (1000 μ U per ml), the fructose metabolism of adipose tissue is depressed [18]. In tissues of alloxan-diabetic rats the suppression of fructose phosphorylation becomes significant at much lower rates of glucose uptake [16]. After 16 h of incubation, phosphorylation became already limiting at glucose concentrations between 30 and 100 mg per 100 ml (Fig. 1), i.e. when the glucose uptake corresponded

to that of nonstimulated normal tissue. It seems likely that the activity of the phosphorylating system known to be depressed in diabetic tissue [36, 2] did not increase in proportion to the glucose uptake during the incubation period so that phosphorylation rather than transport became rate limiting for glucose metabolism. Therefore, an accurate estimation of glucose transport rates can not be derived from results of glucose-U- 14 C metabolism. Nevertheless, it is quite clear that insulin stimulated glucose transport of preincubated tissue to a relatively small extent, even at low glucose concentrations (3 and 9 mg per 100 ml) which did not inhibit fructose metabolism (Fig. 1).

Intact membranes are prerequisites for the effect of insulin on sugar transport. Prolonged incubation *in vitro* might well damage the plasma membrane so that glucose might freely diffuse into the cell. Therefore, we felt obliged to characterize hexose transport. The data reported in Tables 1 and 2 indicate that phlorizin, a selective inhibitor of carrier-mediated glucose transport [35, 5, 15] significantly inhibited the preincubation effect on glucose metabolism. It is concluded that prolonged incubation *in vitro* stimulated facilitated diffusion. The same conclusion was reached by FESSLER, RUBINSTEIN and BECK [10] using space measurement of 3-O-methylglucose in adipose tissue of fed and fasted rats. The stimulation of glucose transport by insulin after prolonged incubation was also inhibited by phlorizin. The intimate mechanism of the stimulation of glucose transport by prolonged incubation is as yet unknown. It is conceivable that the swelling of the extracellular space during incubations *in vitro* [37, 25, 4] may lead to structural and functional changes of the membranes. The glucose carrier in free adipose tissue cells is saturated at relatively low glucose concentrations. This may be due to an increased accessibility of transport sites for glucose. Glucose transport in muscle is stimulated by anoxia, muscular work and by some inhibitors of glycolysis [41, 40, 39]. These effects are also inhibited by phlorizin, which indicates that stimulation of facilitated diffusion can result from changes of energy metabolism.

One of the glucose-independent effects of insulin is the inhibition of lipolysis. In the presence of glucose, insulin reduces reesterification of fatty acids despite the simultaneous stimulation of glucose uptake [20]. In contrast, the stimulation of glucose metabolism by prolonged incubation was accompanied by a 4-fold rise of glycerol release (4), (Fig. 3), and a corresponding increase of glucose-U- 14 C incorporation into glyceride-glycerol (Table 4, line 3). The amount of free fatty acids in the medium and tissue was approximately the same before and after prolonged incubation.

Net glycogen synthesis seemed to occur during prolonged incubations in the absence of insulin (Fig. 2). Insulin stimulated the incorporation of glucose-U- 14 C into glycogen about 10-fold during the initial incubation, and 5-fold after preincubation without insulin. When insulin was present throughout the entire incu-

bation period, incorporation of glucose into glycogen was decreased while glycogen content was higher than under any other conditions of incubation. This is best explained by an inhibitory action of insulin on phosphorylase. These results are compatible with those obtained in fasted-refed rats, which indicate that phosphorylase activity and the availability of glucose-6-phosphate regulate the glycogen content of adipose tissue [14, 19]. A stimulation of glycogen-synthetase activity by insulin as reported by JUNGAS [33] was not demonstrable in these experiments. The glycogen content of "diabetic" adipose tissue after prolonged incubation was small compared with the marked accumulation of glycogen in adipose tissue of fasted-refed rats *in vivo*.

Impaired glucose utilization finally leads to depressed lipogenesis in adipose tissue of alloxan-diabetic rats. Insulin administered *in vivo* normalizes the glucose incorporation into fatty acids within 12 or more hours [46, 28]. The incorporation of glucose-carbon into fatty acids was raised by prolonged incubation *in vitro*, but it remained below normal, even when insulin was present during preincubation and final incubation. The highest value of fatty acid synthesis from glucose- $U\text{-}^{14}C$ was 33% of the total glucose metabolism (Table 4, line 6). A correlation between fatty acid synthesis and glucose metabolism over a large range is shown in Figure 3. At comparable rates of glucose metabolism, fatty acid synthesis from glucose- $U\text{-}^{14}C$ was somewhat higher with insulin than without insulin. Unfortunately, this effect of insulin on lipogenesis could not be evaluated in a statistically meaningful way. It would be of interest to measure the activity of the enzyme system which is responsible for the desaturation of stearic to oleic acid. Its activity is reduced in adipose tissue of diabetic animals, reflecting the decreased rate of total lipid biosynthesis [22].

The normalization of the metabolic defects of adipose tissue metabolism by insulin *in vivo* involves *de novo* synthesis of enzymes, in particular hexokinase [2] and stearate-desaturating enzyme [22, 23]. Actinomycin D did not inhibit the effect of prolonged incubation *in vitro* on glucose metabolism (Table 3) indicating that RNA-synthesis was not involved [42]. On the other hand, the preincubation effect was consistently decreased by cycloheximide (Table 3), which would be compatible with the hypothesis that protein synthesis may be of some importance in the preincubation effect [8, 9]. It is most puzzling that insulin abolished the inhibitory effect of cycloheximide, particularly in view of the recent report of WOOL and CAVICCHI, [47] who demonstrated an effect of insulin on protein synthesis in muscle ribosomes.

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