Insulin Inhibition of Spontaneous Adipose Tissue Lipolysis and Effects upon Fructose and Glucose Metabolism

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SUMMARY

The *in vitro* metabolism of fructose and glucose and the glycerol release by adipose tissue of normal fed, fasted-refed, acutely and chronically diabetic rats were studied and the effects of insulin were investigated.

Glycerol release by adipose tissue of fasted-refed rats exceeded that of normal fed and diabetic rats.

In all four groups of animals that were studied, basal glycerol release was higher with fructose than with an equal concentration of glucose in the medium.

Insulin inhibited glycerol release by adipose tissue in all groups of rats with fructose in the medium. In fed rats a barely significant insulin inhibition occurred also in the presence of glucose, and in fasted-refed rats it was independent of whether a hexose was present in the medium or not.

The following effects of insulin on tissue of fasted-refed rats incubated in the absence of hexose were observed: Inhibition of glycerol release with a dose effect relationship between 10 and 250 μunits insulin per milliliter; partial inhibition of glycogen breakdown during incubation; diminished lactic and pyruvic acid release and a rise of the lactic to pyruvic acid ratio. These same effects were also exerted by the antilipolytic drug 5-methylpyrazole-3-carboxylic acid, which does not stimulate glucose uptake.

At comparable rates of hexose uptake insulin decreased incorporation of hexose-^{14}C into glyceride-glycerol and, conversely, increased fatty acid synthesis. These deviations of hexose-^{14}C metabolism due to insulin could be explained as sequelae of the antilipolytic effect of insulin.

A hypothesis is advanced according to which insulin might block the activity of lipoprotein lipase toward intracellular triglycerides while increasing its activity toward extracellular triglycerides.

INTRODUCTION

Previous work from this laboratory (1, 2) suggests that adipose tissue may be an important site of fructose metabolism despite the absence of a specific fructose pathway (3). Fructose is transported into adipose tissue by a different carrier mechanism from glucose and phosphorylation by hexokinase is possible because virtually no free glucose which would compete for phosphorylation is present intracellularly (2, 4–7).

The metabolic disposal of labeled fructose-^{14}C and glucose-^{14}C by adipose tissue of fed rats with and without insulin stimulation was very similar, and we tentatively concluded that insulin did not affect hexose metabolism in any specific way other than by making more hexose available to the cellular enzyme machinery (2). Similar

studies had led Jeanrenaud and Renold (8) to the same conclusion. The results of Leonards and Landau (9) however suggested that insulin specifically stimulated glycogen synthesis, fatty acid synthesis, and the oxidation of carbon-1 of fructose to $^{14}CO_2$.

We later observed an inhibitory effect of insulin on glycerol release and glyceride glycerol formation which was particularly pronounced and was independent of hexose in tissue of fasted-refed rats. This effect of insulin on lipolysis is analyzed in this paper and viewed to reinterpret new data on the effect of insulin on glucose and fructose metabolism of adipose tissue.

This report extends observations presented elsewhere in preliminary form (10).

METHODS

Some of the methods used have been described elsewhere (2). Glycerol, lactic acid, and pyruvic acid were determined by the enzymic methods of Bergmeyer (11) using the reagents of Biochimica (Boehringer, Mannheim, Germany). The media were deproteinized with sodium hydroxide and zinc sulfate instead of perchloric acid.

Since it is impossible accurately to measure glucose or fructose uptake when their concentrations in the medium are high and the ratio of the volume of the medium to the amount of tissue is unfavorable, an approximation of the hexose uptake was obtained by adding the results of the oxidation of labeled hexose-^{14}C to $^{14}CO_2$ to those of its incorporation into the total lipids, the two of which together account for 70% or more of the actual hexose uptake (2). This value is sometimes referred to as "hexose uptake" in the text and in some figures.

Total lipids were extracted according to Folch *et al.* (12), and the extract was taken to dryness and washed with isooctane:glacial acetic acid (1:1) (13). The fatty acids were obtained by hydrolysis of the total lipids in alcoholic KOH, and incorporation of hexose-^{14}C into the glyceride glycerol fraction was calculated as the difference between the counts in the total lipids and those in the hydrolyzed fatty acids. The free fatty acids in the medium were extracted and titrated according to Gordon (14). The dried fat-free tissue was digested in 30% KOH, and the glycogen was precipitated with cold ethanol and twice reprecipitated before counting. Carrier glycogen was used as a precipitating aid for the tissues with low glycogen content, whereas sodium sulfate was used for tissue of fasted-refed rats. In the latter instance the glycogen was deposited on filter paper, counted, subsequently hydrolyzed in 1 N HCl at 100°C for 2 hr, and determined as glucose using glucose oxidase.

All the radioactive samples were counted on aluminum planchets in a windowless flow counter (Nuclear, Chicago). Uniformly labeled glucose-^{14}C and fructose-^{14}C were purchased from the Radiochemical Centre (Amersham, England), and were chromatographically pure.

Guinea pig anti-insulin serum was produced similarly to Moloney and Coval (15). The details of the procedure may be found in an earlier publication (16).

Crystalline pork insulin with an activity of 23.9 units/mg was kindly supplied by Dr. J. Schlichtkrull (Novo Terapeutisk Laboratorium, Copenhagen). 5-Methylpyrazole-3-carboxylic acid (U-19425), a new hypoglycemic agent (17) synthesized by Upjohn, Kalamazoo, was a gift of Dr. G. C. Gerritsen of the Upjohn Company.

The incubations were carried out in a Warburg apparatus, and the medium used throughout was Krebs-Ringer bicarbonate buffer containing either 200 mg gelatin per 100 ml or albumin[1] in a concentration of 3 or 4 g/100 ml as specified in the legends of the tables and figures. Each experiment was carried out with pooled epididymal adipose tissue of 12 or more rats as described earlier (2, 16).

Four groups of purebred Osborn-Mendel rats were used:

1. Normal fed rats: These rats received

[1] A highly purified albumin preparation, fraction 5, Lot No. 6001 of the Blutspendedienst des Schweizerischen Roten Kreuzes, Bern, which contained 20 μeq free fatty acids per gram and no insulin-like or insulin-inhibitory activity, was used (16).

a regular Purina chow diet ad libitum and were sacrificed by decapitation when they weighed between 130 and 165 g. The weight of the rats used for any one pool did not differ by more than 15 g.

2. Fasted-refed rats: These rats, weighing between 160 and 185 g before fasting, were starved for 72–120 hr and then refed, in most experiments for 24 hr, with a high carbohydrate-low fat diet consisting of oat flakes, carrots, bread, and 10 g of sucrose per 100 ml drinking water.

3. Acutely diabetic rats: Alloxan was injected into the tail vein of rats weighing 115–130 g after a 24-hr fast in a dose of 45 mg/kg. Thereafter the rats had free access to food; 48 hr later, a 10-day course of insulin therapy with 2–4 units of insulin Lente daily was started, during which time the animals gained weight in a normal fashion. The animals were killed in diabetic ketosis 72–96 hr after the last insulin injection, i.e., after 48–72 hr of insulin deficiency. A morning blood sugar of 300 mg/100 ml 48 hr after the last insulin injection and a weight-loss of 10 g or more served as criteria of acute diabetes.

4. Chronically diabetic rats: Rats weighing 170–190 g were alloxanized in the same manner as the acutely diabetic rats. They were not treated with insulin, however, and were sacrificed 120–144 hr after alloxan administration. The criteria of diabetes were a morning blood sugar above 300 mg/100 ml on the day before sacrifice and a weight loss of 10 g or more. Many rats died before the start of the experiment.

RESULTS

Glycerol Release by Adipose Tissue of Fed, Fasted-Refed, and Diabetic Rats and the Effect of Insulin Thereupon

Glycerol release by tissue of fasted-refed animals was much greater than that of all other groups of rats (Table 1). Tissue of acutely and chronically diabetic rats was more active than tissue of normal animals ($P < 0.005$ and < 0.001, respectively). In all four groups of rats more glycerol was produced with fructose than with glucose in the medium. When the same data were expressed per fat pad instead of per gram tissue, the fat pads of fasted-refed rats still were the most active ones. Glycerol release per fat pad was of approximately the same magnitude in the acutely diabetic and in the normal fed animals and somewhat lower in the chronically diabetic rats.

Inhibition of glycerol release by insulin in the presence of glucose was highly significant in fasted-refed and barely significant in the normal fed rats, whereas an insignificant stimulation was observed in the acutely diabetic group. When fructose was the substrate, insulin inhibited glycerol release in all 3 groups of rats that were studied (Table 1).

Characteristics of the Lipolytic Activity of Adipose Tissue in Fasted-Refed Rats without and with Insulin Stimulation

Relative independence of the presence of glucose. In 5 experiments with pooled epididymal adipose tissue of 72 hr fasted-24 hr refed rats, the basal glycerol release was 6.82 ± 0.49 (mean $\pm$ SEM) and 6.83 ± 0.60 μmoles/g/hr, respectively, in the absence and presence of 200 mg glucose per 100 ml. The glycerol release was inhibited by 1000 μunits insulin per ml to 1.06 ± 0.14 and 1.53 ± 0.17 μmoles/g/hr, respectively, in the absence and presence of glucose ($P < 0.05$). In other experiments, insulin inhibited the glycerol release to between 12 and 22% of the baseline, and inhibition regularly was less marked with glucose in the medium than without it.

Dose response relationship between insulin concentration and inhibition of glycerol release. As Fig. 1 shows, a significant inhibition of glycerol release by adipose tissue of fasted-refed rats was observed at 31 μunits insulin per milliliter. The insulin effect became near maximal at a concentration of 250 μunits/ml. The shape of the curve was independent of whether glucose was present in the medium or not. The output of lactic acid was also suppressed by insulin, but only in the absence of glucose in the medium. Insulin inhibition of lactic acid release varied considerably in magnitude from one to the other tissue

TABLE 1

Glycerol release by pooled epididymal adipose tissue of fed, fasted-refed, acutely and chronically diabetic rats in the presence of glucose or fructose with or without insulin

The rats and the tissue pools were prepared as described under Methods. The incubation medium was Krebs-Ringer bicarbonate buffer containing 3 g, in some experiments 4 g, albumin per 100 ml. Duplicates were run in each experiment. n = number of incubations.

Metabolic state of rats	Additions to medium[a]								Statistical analysis		
	Glucose 200 mg/100 ml				Fructose 200 mg/100 ml						
	No insulin I		Insulin 1000 μU/ml II		No insulin III		Insulin 1000 μU/ml IV		P I ↔ II	P III ↔ IV	P I ↔ III
	Release[a]	n	Release[a]	n	Release[a]	n	Release[a]	n			
Normal fed	1.46 ± 0.20 (0.354)	12	1.02 ± 0.09 (0.230)	10	3.13 ± 0.32 (0.501)	8	0.98 ± 0.27 (0.162)	6	<0.05[b]	<0.01	<0.01
Fasted-refed	6.47 ± 0.43 (0.788)	16	1.76 ± 0.05 (0.207)	16	7.05 ± 1.05 (0.838)	6	1.61 ± 0.04 (0.185)	6	<0.001	<0.001	<0.01[b]
Acutely diabetic	2.99 ± 0.49 (0.377)	10	4.76 ± 1.24 (0.397)	6	4.80 ± 0.74 (0.598)	14	3.06 ± 0.70 (0.264)	8	N.s.	<0.01	<0.01
Chronically diabetic	2.66 ± 0.21 (0.261)	12	2.55 ± 0.24 (0.253)	12	3.28 ± 0.32 (0.318)	12	No data		N.s.	—	<0.01

[a] Glycerol release is stated as micromoles per gram per hour ± standard error of the mean, and (in parentheses) as micromoles per fat pad per hour.
[b] Only the results obtained with the same tissue pools were used for statistical analysis (n = 6).

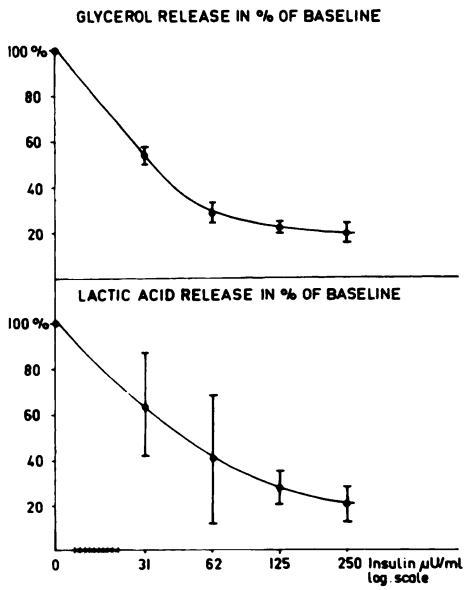

FIG. 1. *Effect of insulin on release of glycerol and lactic acid by adipose tissue of fasted-refed rats in the absence of glucose*

Two pools of epididymal adipose tissue each from 12 rats were used. One group of animals was fasted for 72 hr, the other for 144 hr, and all animals were refed for 24 hr. The Krebs-Ringer bicarbonate buffer contained 200 mg gelatin per 100 ml and no glucose. The results are expressed as the percentage of the value in the absence of insulin. The means of the results of 4 flasks and the SEM are given.

pool, and it became significant at an insulin concentration of 62 µunits/ml.

Release and uptake of free fatty acids. The release of free fatty acids by adipose tissue of fasted-refed rats in the absence of glucose from the medium averaged 5% of that expected theoretically on the basis of a 3:1 ratio of free fatty acids to glycerol (Fig. 2). In other experiments, adipose tissue of fasted-refed rats was incubated in a medium containing 1.58 µeq free fatty acids per milliliter and no glucose. Under these conditions the tissue took up 3.71 ± 0.29 µeq free fatty acids per gram per hour (mean $\pm$ SEM, $n = 8$) in the absence of insulin. Insulin stimulated the free fatty acid uptake to 6.56 ± 0.76 µeq/g/hr ($P < 0.01$). The glycerol release was independent of the concentration of free fatty acids.

Glycerol and free fatty acid release and the effect of insulin thereon as a function of the duration of the refeeding period. As shown in Fig. 2 basal glycerol release increased to a maximum between 24 and 36 hr after the beginning of refeeding and thereafter slowly fell off again. Insulin inhibition of glycerol release was most pronounced when basal glycerol release was high. The glycogen content of the tissue determined at the end of incubation was not measurable in fed rats and it was at a maximum level after 48 hr of refeeding fasted rats. The smaller the rats and the longer the fasting period, the higher was the glycogen content and the glycerol release after comparable refeeding periods.

Reversibility of insulin inhibition of glycerol release by the addition of anti-insulin serum in vitro. Glycerol release was determined kinetically with and without insulin in the medium and before and after anti-insulin serum was added to the medium from the side arm of the Warburg vessels (Fig. 3). During the 2 hr of incubation virtually no glycerol was released by the tissue that was under the influence of insulin. At 30 min after the addition of anti-insulin serum, the tissue started again to release glycerol at a rate similar, although not quite equal, to that of the unstimulated tissues.

Consequences of the Antilipolytic Effect of Insulin for the Metabolism of Uniformly Labeled Glucose-^{14}C and Fructose-^{14}C

In the experiment represented in Fig. 4 hexose-^{14}C incorporation into CO_2, total lipids, tissue fatty acids, glyceride glycerol, and glycogen were determined at different rates of hexose uptake by tissue of fed rats. The hexose uptake was forced either by increasing the fructose concentration or by incubating in the presence of both fructose and glucose in the medium, or, alternatively, by the addition of insulin. Hexose incorporation into all these metabolic moieties rose proportionately to the hexose uptake and did not appear to be influenced in any specific way by insulin.

The same experimental design was also used to study the metabolism of tissue of

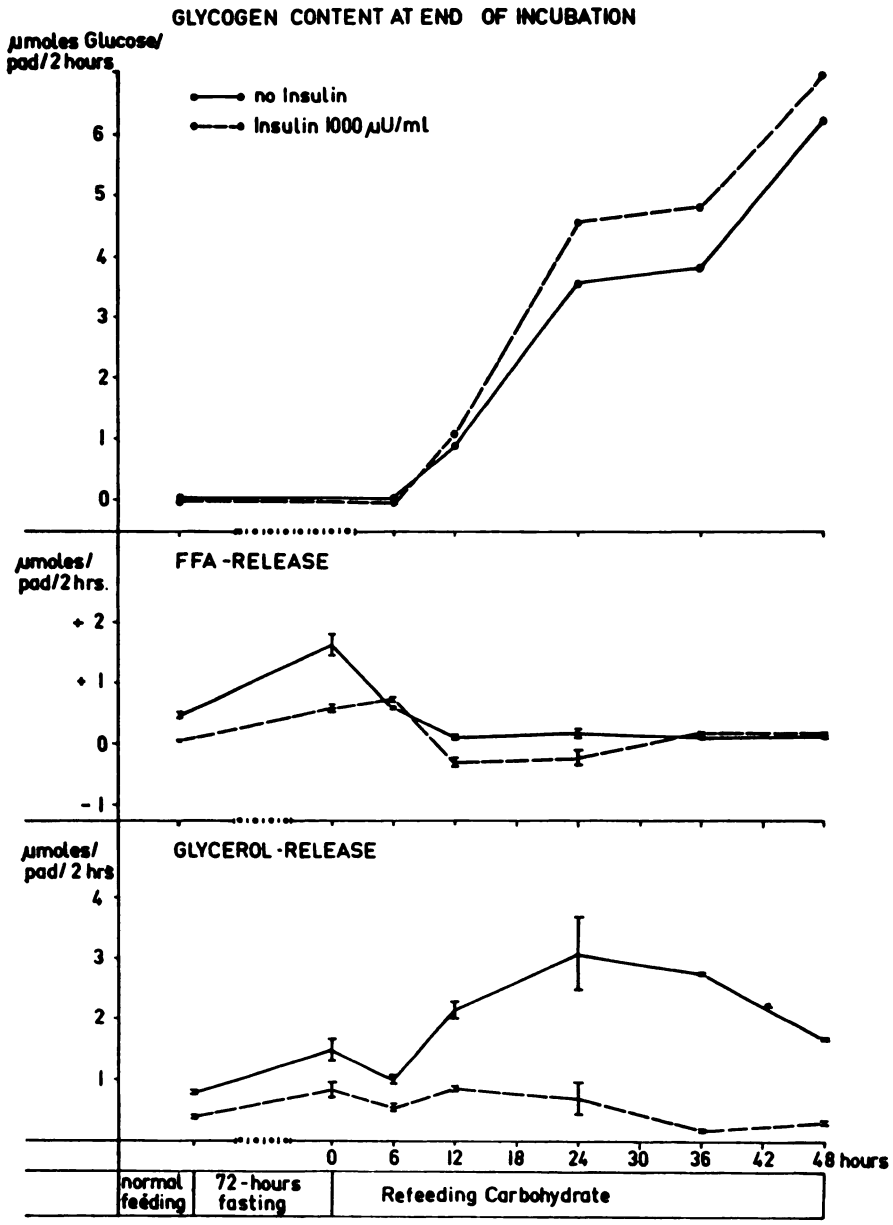

FIG. 2. *Glycerol and free fatty acid release and glycogen content of adipose tissue of fasted-refed rats with and without insulin stimulation as a function of the duration of refeeding*

Two groups of animals with a fasting period of 72 and 120 hr, respectively, were used. The tissue of 4 animals was pooled into 4 flasks, two of which contained insulin. The Krebs-Ringer bicarbonate buffer contained 4 g albumin per 100 ml and no glucose. The incubation lasted 2 hr. The means of the results of two flasks and their range are plotted.

fasted-refed rats (Fig. 5). Incorporation of hexose-^{14}C into $^{14}CO_2$, total lipids, and glycogen rose in proportion to the hexose uptake whether insulin was present or not. With 800 mg fructose per 100 ml in the medium the fructose uptake was similar to that occurring at 200 mg/100 ml together with insulin. In the absence of insulin more

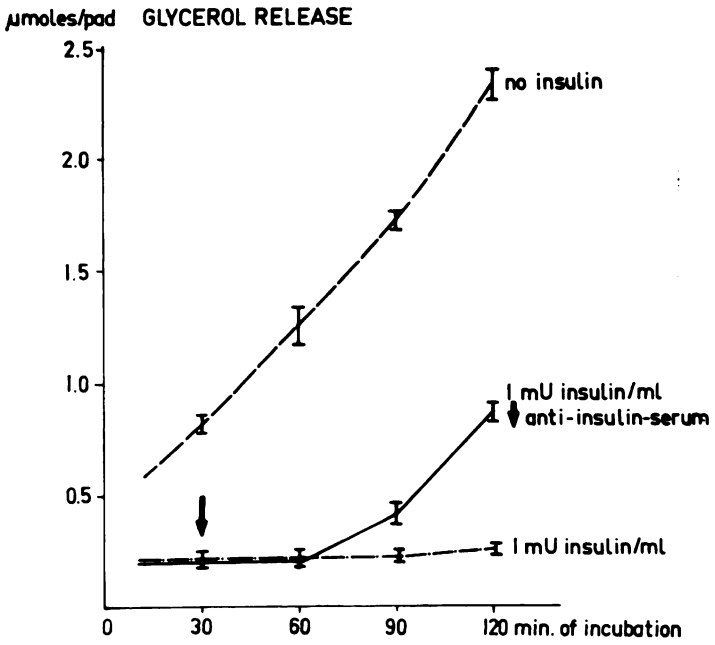

FIG. 3. *Effect of anti-insulin serum on insulin-induced depression of glycerol release by adipose tissue of 120 hr fasted-24 hr refed rats*

A pool of epididymal adipose tissue of 16 rats was used. Each flask contained 2 ml Krebs-Ringer bicarbonate buffer with 3 g albumin per 100 ml and no glucose. Anti-insulin serum, 0.1 ml with an insulin-neutralizing capacity of 50 munits, was added from the side arm of the Warburg vessel 30 min after the start of the incubation, as indicated by the arrow. To the control flasks 0.1 ml normal guinea pig serum was added at the same time. The brackets indicate the results of the two flasks.

carbon-14 was incorporated into glyceride-glycerol and less into the tissue fatty acids. Glycerol and lactic acid release were comparatively lower, and the glycogen content at the end of incubation comparatively higher under insulin stimulation than without it. Newly synthesized glycogen-^{14}C was between 1 and 2% of the total tissue glycogen under all conditions except when the glucose uptake rose to very high levels under insulin stimulation (15%).

Glucose concentrations of 25 and 800 mg/100 ml in the absence of insulin and of 25 mg/100 ml in the presence of three different insulin concentrations were used in another experiment (Fig. 6). Incorporation of carbon-14 into $^{14}CO_2$, total lipids, and tissue glycogen rose in proportion to the sum of the incorporation of glucose-^{14}C into CO_2 plus total lipids. When insulin was absent the tissue incorporated relatively more carbon-14 into the glyceride-glycerol moiety and accordingly less into the fatty acids than the tissue under insulin stimulation. Total tissue glycogen at the termination of the incubation was considerably higher under insulin stimulation than in the nonstimulated fat pads. Glycogen synthesis during incubation amounted to less than 2% of the glycogen content and was, therefore, too small to influence significantly the glycogen balance.

The results of five experiments in which the fructose metabolism by adipose tissue of acutely diabetic rats was studied at 200 mg per 100 ml with and without insulin and at 800 mg per 100 ml are summarized in Fig. 7. The results are presented as the percentage change of the result obtained at the low fructose concentration (200 mg/100 ml) without insulin. The latter was arbitrarily set at 100% and the percentage increase of fructose uptake on the abscissa was correlated with the percentage change

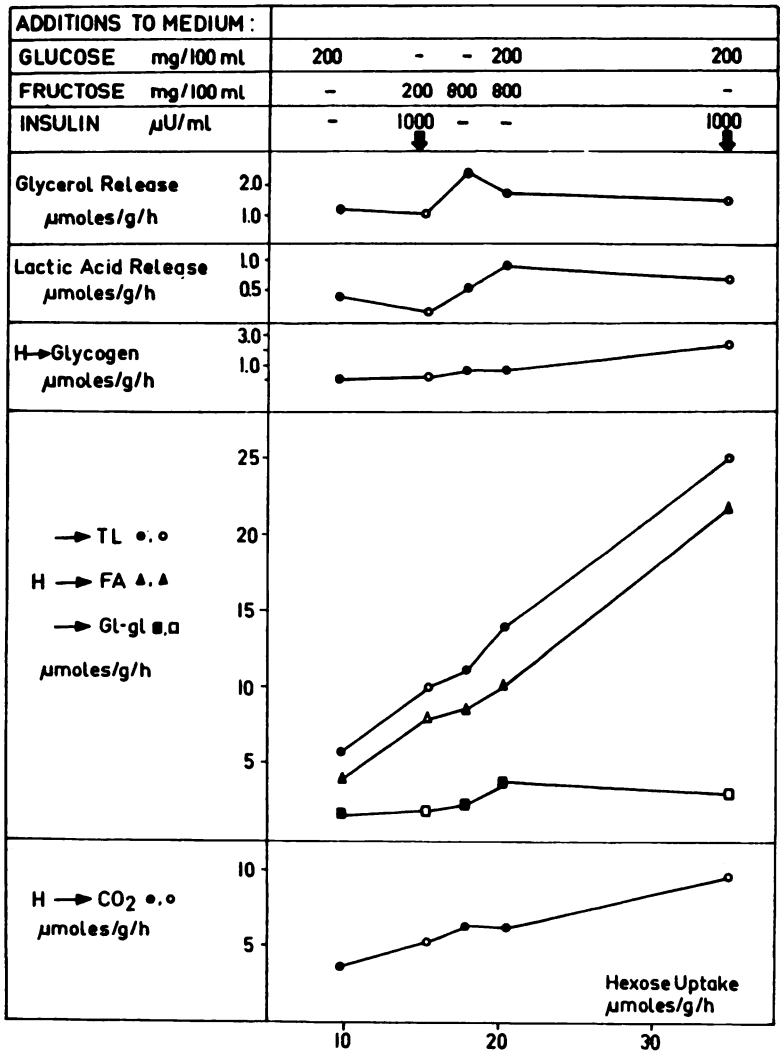

FIG. 4. *Correlation of hexose uptake and hexose metabolism by adipose tissue of fed rats over a wide range of hexose uptake in the presence and absence of insulin*

Pooled epididymal adipose tissue of 12 rats was used. Krebs-Ringer bicarbonate buffer with 3 g albumin per 100 ml was used. The incubation lasted 3 hr. As an estimate of the hexose uptake the results of oxidation of labeled hexose-^{14}C to CO_2 and those of incorporation into total lipids were added together and plotted on the abscissa. The means of the results of 2 flasks are given. The open symbols denote the presence of insulin. *H* = hexose; *TL* = total lipids; *FA* = total tissue fatty acids; *Gl-gl* = glyceride glycerol.

of various metabolic indices on the ordinate. Oxidation of carbon-14 to $^{14}CO_2$ increased to the same extent whether the fructose uptake was forced by increasing its concentration or, alternatively, by adding insulin, whereas the response of the tissue was different with regard to all other metabolic indices registered in Fig. 7. Thus insulin significantly decreased the glycerol and lactic acid release and the incorporation of carbon-14 into glyceride-glycerol, whereas raising the fructose concentration to 800 mg/100 ml resulted in an increase in all these metabolic indices above the level

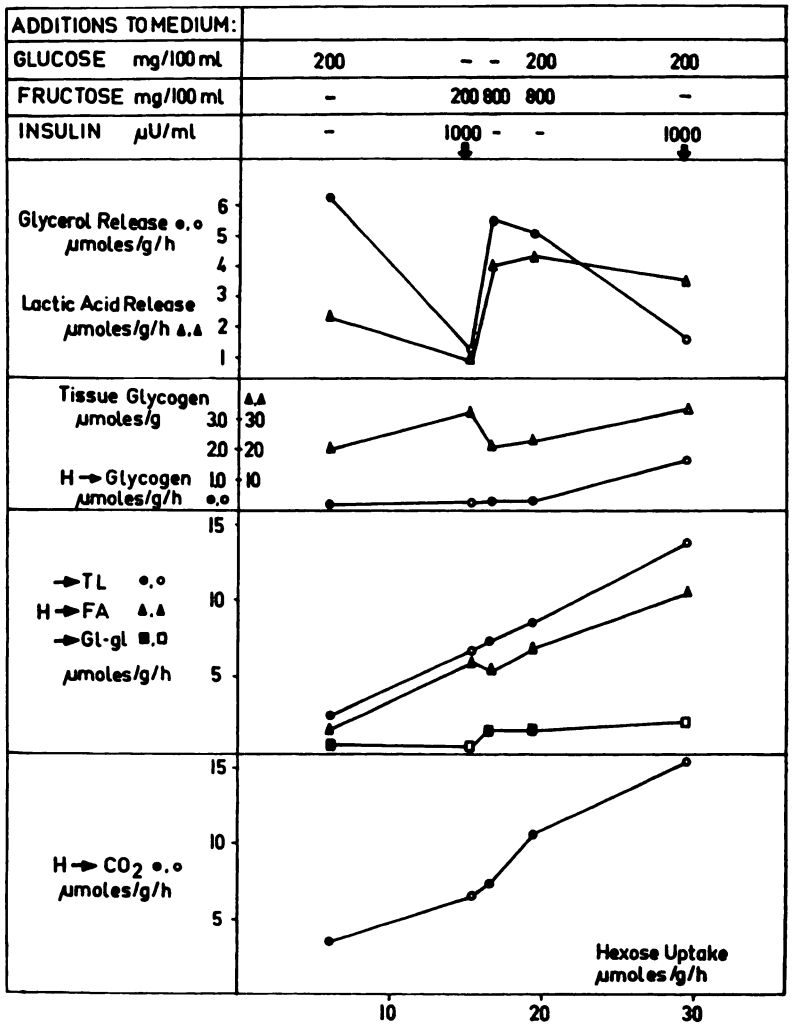

FIG. 5. *Correlation of hexose uptake and hexose metabolism by adipose tissue of 120 hr fasted-24 hr refed rats over a wide range of glucose uptake in the presence and absence of insulin*

The conditions were identical with those described in the legend for Fig. 4 with the exception that 12 fasted-refed rats were used. The open symbols denote the presence of insulin. Abbreviations same as in Fig. 4.

at the low fructose concentration. Fatty acid synthesis was more markedly stimulated by insulin than by the high fructose concentration.

Mechanism of Insulin Action on Lipolysis

Table 2 shows results on the effect of insulin on the release of lactic and pyruvic acid by adipose tissue of fasted-refed rats. In the absence of a carbohydrate substrate in the medium insulin caused a relatively greater decrease of the pyruvic than of the lactic acid release, whereby the ratio of lactic to pyruvic acid rose significantly. At a comparable glucose uptake this ratio was significantly greater in the presence of insulin than in its absence. When the glucose concentration was raised to 200 mg/100 ml, insulin no longer affected the ratio of lactic to pyruvic acid. With fructose as substrate

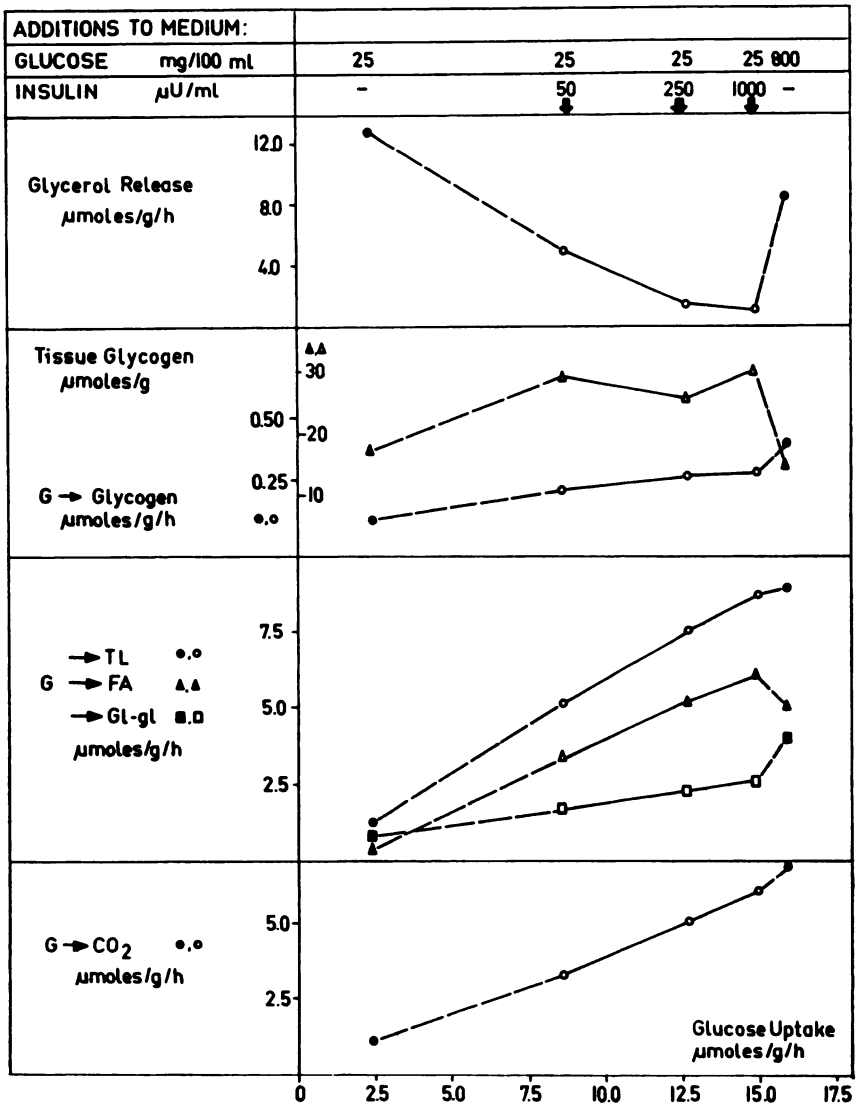

FIG. 6. *Correlation of glucose uptake and glucose metabolism by adipose tissue of 120 hr fasted-24 hr refed rats over a wide range of glucose uptake in the presence and absence of insulin*

Epididymal adipose tissue of 8 rats was pooled into 15 flasks. The means of the results of 3 flasks are given. The conditions were identical to those described in the legends for Figs. 4 and 5. The open symbols denote the presence of insulin. G = glucose; other abbreviations same as in Fig. 4

insulin suppressed the release of lactic and pyruvic acid to the same extent, leaving their ratio unchanged.

Finally, the results of an experiment in which the effects of insulin were compared with those of another antilipolytic drug, 5-methylpyrazole-3-carboxylic acid (U-19425), are given in Table 3. The effects of both substances were qualitatively similar. Glycerol release was markedly inhibited by insulin as well as by U-19425, both of which brought about a rise of the ratio of lactic to pyruvic acid. The breakdown of tissue glycogen during incubation was partially blocked, and the corrected net gas exchange was higher in the presence

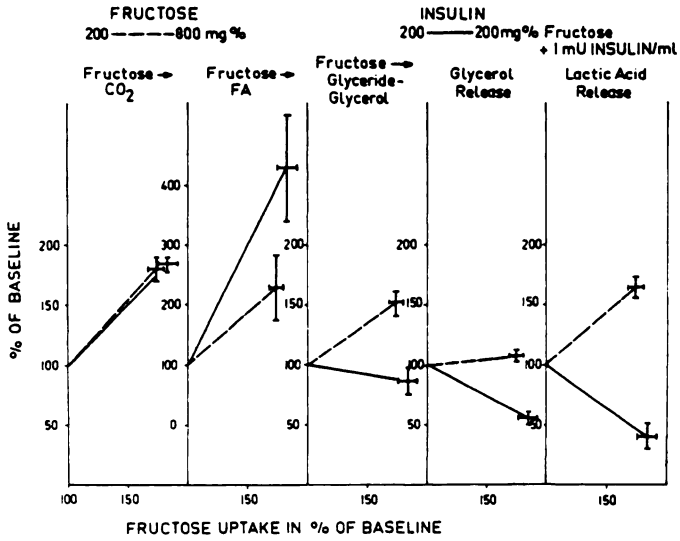

FIG. 7. *Comparison of the effects of insulin and increasing fructose concentration on adipose tissue metabolism of acutely diabetic rats at similar rates of fructose uptake*

The abscissa denotes the percentage increase of the fructose uptake over and above that occurring at 200 mg fructose per 100 ml, and the ordinate the concomitant increase of the respective metabolic indices. Fructose uptake was computed from the incorporation of fructose-^{14}C into $^{14}CO_2$ and total lipids. The results obtained at a fructose concentration of 200 mg/100 ml were set at 100%. Five pools of tissue from 12–16 rats were used for the data on the insulin effects, and the same plus five additional pools for those on increased fructose concentration. The means and the SEM are given.

of these antilipolytic substances than in their absence. Assuming that glycogen was the major precursor of lactic acid, pyruvic acid and α-glycerophosphate, their results were totaled and compared with the decrease of glycogen during incubation. Considerably more glycogen was broken down during incubation than could be accounted for by the sum of the above-mentioned metabolites.

DISCUSSION

The release of free fatty acids from adipose tissue is governed by the rate of lipolysis and by the rate of reesterification of the free fatty acids (18–21). The latter depends on the availability of carbohydrate, i.e., under most conditions on the uptake of glucose furnishing α-glycerophosphate. Glycerol release on the other hand reflects the rate at which monoglycerides are hydrolyzed, and since this rate is supposed to be determined by the enzymic hydrolysis of triglycerides, which proceeds at a slower rate than that of the monoglycerides (22, 23), the glycerol release may also be taken as an approximation of lipolysis at the level of the triglycerides. It appears likely that reesterification of the diglycerides is quantitatively unimportant and that it does not interfere to any great extent in the assessment of lipolytic activity as judged by the glycerol release (24).

Glycerol release per gram of tissue was higher in acutely diabetic than in normal fed rats, but about equal when the considerable shrinkage of the tissue was taken into account and the results were expressed per fat pad. This does not, however, preclude free fatty acid release by the diabetic adipose tissue since it has been demonstrated that glucose uptake is considerably decreased at this stage of diabetes and that reesterification of free fatty acids may become limiting (5). In this connection it has proved useful to correlate glycerol release with glucose uptake. The molar ratios of glycerol release to glucose uptake were found to average 0.52 with a SEM of ± 0.03 in normal fed, 1.38 ± 0.18 in acutely dia-

TABLE 2

Effect of insulin on the release of lactic and pyruvic acid by adipose tissue of fasted-refed rats in the absence and presence of glucose or fructose

Krebs-Ringer bicarbonate buffer containing 200 mg gelatin per 100 ml served as incubation medium. Each figure represents the mean of the results obtained in several experiments. The SEM is also given. *n* is equal to the number of incubations, and *n* divided by 2 is equal to the number of experiments with different tissue pools. The variations of lactic and pyruvic acid release and of the ratio of the two from pool to pool were considerable. However, the changes due to insulin were quite consistent, and they were used for statistical analysis.

Additions to medium	*n*	Lactic acid release (μmoles/pad/hr)	Pyruvic acid release (μmoles/pad/hr)	Ratio lactic:pyruvic acid	Per cent change of ratio in presence of insulin	*P*
No glucose	12	0.364 ± 0.039	0.061 ± 0.008	6.50 ± 0.39	—	—
No glucose + insulin 1000 μU/ml	12	0.228 ± 0.031	0.017 ± 0.003	15.49 ± 2.09	+127.1 ± 26.7	<0.0005
Glucose 800 mg/100 ml	6	0.705 ± 0.105	0.145 ± 0.055	6.57 ± 1.79	—	—
Glucose 25 mg/100 ml + insulin 1000 μU/ml	6	0.407 ± 0.068	0.056 ± 0.025	11.50 ± 3.87	+65.6 ± 13.6	<0.005
Glucose 200 mg/100 ml	2	0.226	0.028	8.07	—	—
Glucose 200 mg/100 ml + insulin 1000 μU/ml	2	0.310	0.036	8.61	+7.0	—
Fructose 800 mg/100 ml	6	0.603 ± 0.055	0.065 ± 0.0064	9.57 ± 0.63	—	—
Fructose 200 mg/100 ml	6	0.398 ± 0.037	0.050 ± 0.010	8.80 ± 1.09	—	—
Fructose 200 mg/100 ml + insulin 1000 μU/ml	8	0.245 ± 0.025	0.026 ± 0.003	9.53 ± 0.42	−1.8 ± 1.8	N.s.

betic, and 2.81 ± 0.45 in chronically diabetic rats (5).

Bally *et al.* (13) have demonstrated that lipolysis in adipose tissue under glucagon or ACTH stimulation and in the absence of glucose is a self-limiting process which may be partially reactivated by the addition of glucose to the medium and more completely and rapidly by the addition of glucose plus insulin. It appears that under these circumstances glucose and insulin did furnish a substrate or coenzyme necessary for the lipolytic enzymes to be fully active. Under such conditions the glycerol release would of course no longer reflect the potential activity of the lipolytic enzymes.

The differences of basal glycerol release conditioned by the hexose which were observed in the experiments reported in Table 1 might also be interpreted in the light of the different availability of a lipolytic substrate or coenzyme. The spontaneous lipolysis of the tissues was in each instance greater with fructose than with glucose as a substrate, whereas the glycerol release under insulin stimulation was not significantly different. The knowledge about the nature of the lipolytic processes in adipose tissue, however, is too scarce at the present time to permit any further fruitful discussion.

As shown in Fig. 3 glycerol release by adipose tissue of fasted-refed rats containing large amounts of readily available

TABLE 3

Comparison of the effects of insulin and of another antilipolytic agent, 5-methylpyrazole-3-carboxylic acid, on the metabolism of adipose tissue of fasted-refed rats in the absence of glucose

Epididymal adipose tissue of 14 rats was pooled into 14 flasks containing 2.5 ml Krebs-Ringer bicarbonate buffer with 1.5 g albumin per 100 ml and no glucose. Initial tissue glycogen was determined in two flasks before incubation. The results of the net gas exchange were corrected for acid production during incubation (16). Each figure represents the mean of the results of a quadruplicate incubation with the standard deviation. In the case of the initial glycogen the range is given. The incubation lasted 2 hr.

	Additions to medium					
	I	II	III	*P* values		
Parameter	None	Insulin, 1000 μU/ml	5-Methylpyrazole-3-carboxylic acid, 10^{-5} M	I ↔ II	I ↔ III	II ↔ III
1 Initial glycogen (μmoles/g)	—	175.4 ± 3.9	—	—	—	—
2 Final glycogen (μmoles/g)	91.0 ± 6.9	105.3 ± 2.6	112.5 ± 7.4	<0.01	<0.01	<0.15
3 Glycerol release (μmoles/g)	33.25 ± 3.20	4.67 ± 0.31	3.79 ± 0.08	<0.001	<0.001	<0.005
4 Lactic acid release (μmoles/g)	8.26 ± 0.67	6.44 ± 0.17	6.59 ± 0.39	<0.005	<0.005	<0.30
5 Pyruvic acid release (μmoles/g)	1.01 ± 0.16	0.22 ± 0.07	0.36 ± 0.05	<0.001	<0.001	<0.01
6 Ratio lactic:pyruvic acid	8.42 ± 2.04	24.20 ± 1.65	18.80 ± 3.82	<0.001	<0.005	<0.05
7 Sum of 3, 4, and 5	42.52 ± 3.21	11.33 ± 0.46	10.74 ± 0.29	<0.001	<0.001	<0.10
8 2 × (1 − 2) − 7[a] μmoles 3-C-units	126.3	128.9	115.1	—	—	—
9 Corrected net gas exchange (μl/g)	4.6 ± 35.6	93.0 ± 24.0	77.4 ± 52.1	<0.01	<0.05	<0.35

[a] This term denotes the 3-carbon fragments arising from the breakdown of glycogen (initial minus final glycogen times two) which are not accounted for by the formation of α-glycerophosphate, lactic acid, and pyruvic acid (sum of values in lines 3, 4, and 5).

endogenous substrate, i.e., glycogen, proceeded in a linear fashion during several hours of *in vitro* incubation in the absence of glucose. We therefore do not feel that the above-mentioned mechanism of the self-limitation of glycerol release through a shortage of lipolytic substrate or coenzyme plays any role as a factor controlling lipolysis by adipose tissue of fasted-refed rats, which will be discussed forthwith.

The elevated glycerol release of tissue of fasted-refed rats also reported by Jungas and Ball (25) is somewhat surprising since these animals absorb large amounts of carbohydrate, exhibit an elevated blood sugar, and no longer appear to be in need of stored energy. In fact, under the *in vitro* conditions used in our studies and in agreement with other investigators (21, 26) tissue of fasted-refed rats may take up considerable quantities of free fatty acids from the medium despite the absence of glucose. Therefore the high rate of spontaneous lipolysis of this tissue *in vitro* might not reflect its true physiological significance in the whole organism. It is conceivable that the enzyme responsible for the elevated lipolysis *in vitro* might be mainly concerned with the hydrolysis of serum lipids in the intact organism. Thus it has been demonstrated that the activity of adipose tissue lipoprotein lipase is low during fasting and in the diabetic state and high in fed and fasted-refed rats (27–31), and it

is believed that this enzyme is responsible for the hydrolysis of serum lipids, enhancing their uptake by the tissue in the form of fatty acids (32, 33). Lipoprotein lipase activity toward exogenous lipids and its release from the tissue is increased by incubation of adipose tissue with glucose and insulin, whereas in our experiments endogenous lipolysis was inhibited by insulin. If these results are to be brought together, one is led to speculate that insulin might cause a reorientation of this enzyme at the cell surface in such a way that lipoprotein lipase would now attack triglycerides at the outer surface of the cell membrane. Since the adipose tissue of fasted-refed rats is under insulin stimulation in the intact organism at all times and in contrast to our *in vitro* conditions, the physiological function of this enzyme might indeed be the splitting of the serum triglycerides rather than of the endogenous triglycerides. The location of this enzyme on or near the cell membrane is supported by our finding that insulin inhibition of glycerol release is readily reversed by anti-insulin serum.

The adipose tissue lipase, which is stimulated by epinephrine and the other lipolytic hormones and the activity of which is high in fasting and diabetic animals [for references see reviews by Hollenberg (34) and Vaughan and Steinberg (23)] does not appear to play an important role in these antilipolytic effects of insulin, since inhibition of lipolysis was least pronounced in tissue of diabetic rats. According to Jungas and Ball (35) and Bally *et al.* (13), insulin partially inhibits the basal glycerol release of fed rats both in the presence and absence of glucose, but may have an opposite effect when the same tissue is stimulated by epinephrine or other lipolytic hormones. A glucose-independent antilipolytic effect of insulin in normal fed rats, an effect much less marked than the one observed in fasted-refed rats (10, 25), has been described by several investigators (35–38).

A significant inhibition of glycerol release by adipose tissue of fasted-refed rats was observed at an insulin concentration as low as 10 μunits/ml. and inhibition became near maximal at 250 μunits/ml. Thus the dose-response relationship closely resembles that which we and others have obtained with glucose uptake, net gas exchange and other metabolic indices of insulin action in comparable experiments with fed rats (16). Insulin-induced inhibition of glycerol release was readily reversible when anti-insulin serum was added to the medium, again in analogy to the prompt reversibility by anti-insulin serum of the insulin effects on net gas exchange as an index of glucose transport (16).

Tissue of fasted-refed rats seems to drain on its glycogen to reesterify the free fatty acids, and the loss of glycogen during incubation amply accounted for the lactic and pyruvic acid released plus the α-glycerophosphate required to replace all the glycerol released into the medium. Insulin partially inhibited the glycogen breakdown (10, 25). A rather large amount of glycogen which was degraded during incubation could not be accounted for by these metabolites. The positive net gas exchange of the tissue indicated that net formation of fatty acids occurred presumably from glycogen since no other readily available source of carbohydrate was present (25). It appears likely that the substrate for respiration was mostly glycogen, and that fatty acids were used only to a minor extent.

Insulin influenced the ratio of lactic to pyruvic acid only when no or little carbohydrate was taken up from the medium. It is conceivable that at high rates of lipolysis and reesterification the reduction of dihydroxyacetone-phosphate to α-glycerophosphate may determine the availability of NADH in the cell to some extent. At high glucose and fructose concentrations in the medium insulin did not induce a significant increase of this ratio, possibly because the above-mentioned process became quantitatively less important in determining the redox potential at these high rates of carbon flow.

Are there any reflections of these antilipolytic effects of insulin on glucose and fructose metabolism? In earlier studies (2, 5) and in agreement with Jeanrenaud and Renold (8), we failed to note any difference between the metabolic disposal of

glucose and fructose whether their uptake was forced by increasing the hexose concentrations in the medium or by adding insulin. This view was contested by Fain (7), however, who demonstrated that comparatively less fructose was incorporated into glyceride-glycerol under insulin stimulation, an effect confirmed in our experiments with fasted-refed rats. Furthermore, Leonards and Landau (9) showed that insulin selectively stimulated glycogen synthesis, oxidation of fructose-1-^{14}C to $^{14}CO_2$, and fatty acid synthesis from fructose. The interpretation of their data is, however, open to criticism since a correlation of the metabolic indices with total fructose uptake was not attempted. Such a correlation, however, is indispensable, since the percentage of fructose and glucose incorporated into each of these metabolic moieties clearly depends on the magnitude of the hexose uptake (5, 39).

The arguments of Leonards and Landau (9) have again been taken up in this study. Figure 4 shows an experiment with adipose tissue of normal fed rats in which the variations of hexose-^{14}C metabolism from 10 to 37 μmoles/g/hr were brought about by changing the hexose concentrations in the medium or by adding insulin. The incorporation of hexose-^{14}C into CO_2, total lipids, fatty acids, and glycogen was proportional to the hexose uptake whether insulin was present or not.

For comparison the same experiment was carried out with tissue of fasted-refed rats (Fig. 5). Whereas incorporation of hexose-^{14}C into glycogen, CO_2, and total lipids was not specifically affected by insulin, incorporation into glyceride glycerol was relatively smaller with insulin than at the same rate of hexose metabolism in the absence of insulin.

These specific insulin effects were also observed with glucose as a substrate. Figure 6 shows a strict proportionality between the hexose uptake and hexose-^{14}C incorporation into CO_2, total lipids, and glycogen, whereas less ^{14}C was incorporated into glyceride glycerol and comparatively more into the fatty acids under the influence of insulin than in its absence.

Insulin decreases the availability of carbohydrate for tissue metabolism since it partially blocks glycogenolysis (10, 25). It also reduces dilution of hexose-^{14}C by unlabeled carbon from glycogen. This complex situation may be compared with another one, in which fructose-U-^{14}C served as a substrate with and without unlabeled glucose in the medium (2). Despite the fact that glucose carbon diluted fructose-^{14}C, fatty acid synthesis from labeled fructose was actually increased by glucose. This phenomenon is best explained by the fact that the absolute and relative increase of fatty acid synthesis from carbohydrate due to a rise of the hexose uptake exceeds that of α-glycerophosphate synthesis manyfold and more than compensates for the dilution of ^{14}C by unlabeled carbon. If inhibition of glycogenolysis were the primary effect of insulin, fructose-U-^{14}C would, above all, be used to replace glycogen for the reesterification of free fatty acids. Since this is clearly not the case, the obvious explanation for the specific insulin effects on the metabolic distribution of hexose-^{14}C in adipose tissue of fasted-refed rats is that of a primary inhibition of lipolysis diminishing the requirement for α-glycerophosphate.

In this respect it must be borne in mind that insulin stimulated the fatty acid synthesis from hexose over and above the level expected in the absence of insulin at comparable rates of hexose uptake only in those tissues in which it inhibited lipolysis, i.e., in all four groups of rats with fructose in the medium, but only in fed and in fasted-refed rats with glucose as a substrate.

The concept of a primary inhibition of lipolysis is also supported by findings on adipose tissue of acutely diabetic rats. Here, the effects of insulin on fructose-U-^{14}C metabolism were qualitatively the same although glycogen was not present in detectable quantities (Fig. 7).

The stimulation of fatty acid synthesis above that expected solely on the basis of an increased hexose uptake may also be interpreted as a sequence of the primary antilipolytic insulin effect for two more reasons. Under the influence of insulin, less dihydroxyacetone-phosphate is reduced to

α-glycerophosphate resulting in a rise of the intracellular ratio of NADH to NAD^+ which is reflected by an increase of the ratio of lactic to pyruvic acid in the medium. Furthermore, insulin probably decreases the concentration of intracellular free fatty acids. Both conditions favor fatty acid synthesis. In this respect it is of interest that 5-methylpyrazole-3-carboxylic acid, which inhibits lipolysis of adipose tissue of fasted-refed rats to approximately the same degree as insulin without increasing hexose transport, shares with insulin all its other effects. Thus, it inhibited glycogen breakdown and led to a rise of the lactic to pyruvic acid ratio. Lately we have also observed that this drug inhibited glyceride glycerol synthesis from fructose-^{14}C and stimulated fatty acid synthesis in the same way as insulin (unpublished observation).

It is now generally agreed that insulin exerts several effects on adipose tissue which are not secondary to increased glucose transport. Beigelman and Hollander (40, 41) have established a dose-response relationship between the increase of adipose tissue resting membrane potential and insulin concentrations of 1–1000 μunits/ml, an effect that is not influenced by glucose and that is readily reversible by the *in vitro* addition of anti-insulin serum. Stimulation of leucine uptake and metabolism in adipose tissue was reported by Goodman (42), and increased incorporation of labeled histidine-^{14}C into adipose tissue protein was reported by Krahl (43), both insulin effects being independent of glucose.

All these insulin effects deserve a great deal of attention since their pursuit may yield the secret of the mechanism of insulin action. They do not, however, seem to play a role nearly as important for the energy metabolism of the whole organism as the insulin-induced acceleration of glucose transport. Were it not for the recent report of Zierler and Rabinowitz (44), who demonstrated that insulin injected intra-arterially in man decreased the release of free fatty acids from adipose tissue of the forearm at small doses of insulin that did not detectably increase glucose uptake, we would not even have thought it opportune to discuss a possible physiologic role of the direct antilipolytic action of insulin that is not mediated through increased reesterification of free fatty acids. These authors tentatively postulated that less insulin might be necessary to alter the fixed charge of the membrane, making it impermeable for charged molecules such as free fatty acids, than to increase its permeability to uncharged molecules such as glucose. Our results may not fit into this hypothesis, since even non insulin-stimulated tissue of fasted-refed animals releasing large quantities of glycerol did not give off, but rather took up, free fatty acids from the medium. Nevertheless, they demonstrate that human adipose tissue *in vivo* may respond to insulin in a way similar to adipose tissue of fasted-refed rats both with increased reesterification of free fatty acids and with a direct inhibition of the lipolytic processes, an effect well worthy of further investigation.

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