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## Effect of Insulin Pretreatment on Glucose and Lipid Metabolism of Liver Slices from Normal Rats.* (31362)

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The mechanisms controlling the dependence of hepatic lipogenesis on active carbohydrate metabolism continue to be the subject of investigation, which has inevitably involved consideration of the role of insulin in this relationship(1-4). These studies, however, have not dealt with the relative participation of glucose and insulin in the metabolism of hepatic tissue or with the effects of insulin and glucose on relative incorporation of glucose into its various metabolic pathways.

It has been suggested that elevated levels of plasma triglyceride observed in patients with the clinical syndrome of carbohydrate-induced hypertriglyceridemia(5) may result from increased hepatic production and secretion of triglyceride-rich lipoproteins(6). It is uncertain what role, if any, the abnormal glucose tolerance and elevated levels of plasma insulin found in these patients(7,8) play in altering hepatic lipid metabolism and plasma triglyceride levels. It is possible that increased hepatic triglyceride production may result from the effects of hyperinsulinemia and increased glucose concentration acting together on an otherwise normal liver.

To examine the relationship between glucose concentration and insulin action on the metabolism of glucose by the liver, the effect of insulin pretreatment and glucose concentration on the incorporation of glucose into $CO_2$ and various lipid fractions by liver slices from normal rats has been studied.

*Methods.* Male Sprague-Dawley rats weighing between 200-300 g were fed *ad libitum* until the time of sacrifice. One group was pretreated by subcutaneous injection of 1-2 units of ultralente insulin per kilogram of body weight, 20 hours prior to sacrifice. Liver slices were prepared using a Stadie Riggs hand microtome. All tissue was incubated in Krebs-Ringer bicarbonate buffer, pH 7.4. Medium glucose concentration was either 4.0 mg/ml or 0.4 mg/ml, and all incubations were carried out at both concentrations. For ease of expression, these concentrations will be designated as "high" and "low." Tracer amounts of glucose-$6^{14}C$ were added to the buffer so that the initial specific activity of the media was the same regardless of glucose concentration.

Three to four hundred mg of tissue from each rat were placed in 3 ml of media containing glucose at both "high" and "low" concentrations, and the amount of glucose-$6^{14}C$ recovered as $CO_2$ or lipid was determined for each animal. All studies were done in triplicate at each glucose level. Incubations were carried out for 2 hours in a metabolic shaker at 37°. Medium glucose was measured enzymatically (glucose oxidase)

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before and at the end of the 2-hour incubation of liver slices from control and treated animals.

$CO_2$ formed during the incubation period was collected according to the method of Snyder and Godfrey(9). Its radioactive content was determined in 0.4% P.P.O. and 0.01% P.O.P.O.P. with a Packard liquid scintillation spectrometer.† Glucose-6$^{14}$C incorporation into tissue lipid was determined at the end of the 2-hour incubation period. The liver slice was completely disrupted by sonification in the incubation medium, and total lipid was extracted in chloroform:methanol according to the method of Bligh and Dyer (10). Aliquots of the total lipid extract were analyzed for radioactive content as above. Extracted lipids were separated by thin layer chromatography (silica gel G), with 15% ethyl ether in petroleum ether (b.p. 60-70°) as solvent. Triglyceride was eluted and extracted from the plate in a large volume of ethyl ether using the apparatus described by Goldrick and Hirsch(11). The triglyceride was analyzed for its radioactive content as above. An aliquot of the total lipid fraction was dried under nitrogen and fatty acid methyl esters were formed by interesterification according to the procedure of Stoffel *et al*(12). The methyl esters were isolated by extraction with petroleum ether (b.p. 30-60°) and their radioactivity determined. The counts remaining in the water phase were assumed to be derived from glycerol. Glucose-6$^{14}$C recovered in the fatty acid and glycerol moiety of total lipid thus represents label contributed by fatty acids and glycerol of mono-, di-, and triglycerides, phospholipid, and to a small degree cholesterol. Although differences exist in fatty acid composition of triglyceride and phospholipid(13), Chernick and Scow have shown that the ratio of $^{14}$C glucose incorporated into fatty acid/glycerol is the same in the total lipid, triglyceride, and phospholipid fractions of rat liver(14). Thus, estimation of the relative amount of glucose incorporated into fatty acid and glycerol moiety of lipid can be made in this manner.

† A variable amount of counts was recovered in hyamine in control flasks, *i.e.*, those without tissue, indicating isotopic impurity. All counts were therefore corrected for this error.

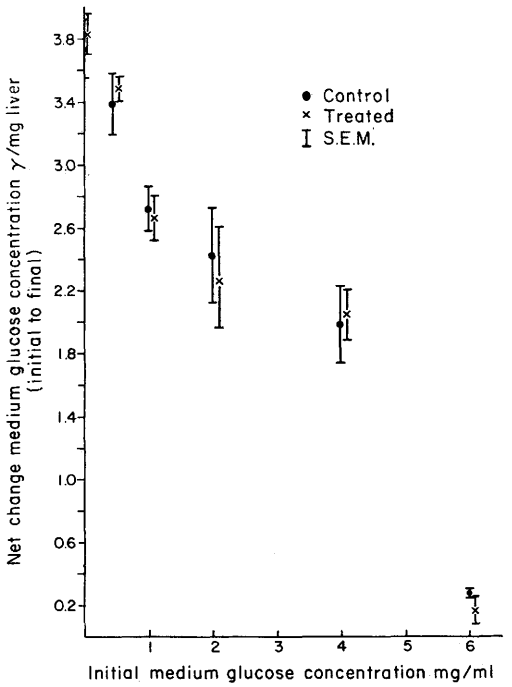


FIG. 1. Net change in medium glucose concentration resulting from release of glucose from the liver slice. Tissue of 11 control and 12 insulin treated rats incubated for 2 hr in media containing an initial glucose concentration of 0.4 to 6.0 mg/ml. Each point represents the mean of pooled data from these animals.

In some experiments liver slices were removed from the incubation medium at the end of 2 hours; glycogen was isolated according to the method of Seifter *et al* and its radioactivity determined(15).

*Results. I. Endogenous glucose release.* The liver slice releases glucose into the medium throughout the 2-hour incubation period, and the specific activity of the glucose to which it is exposed is thus decreased. Since these investigations were concerned with the effects of medium glucose concentration, it was necessary to determine whether the release of endogenous glucose from the liver slice was affected by prior treatment with insulin. Fig. 1 indicates that glucose release is dependent upon the initial glucose concentration in the medium. Insulin pretreatment does not affect this relationship. At each level of initial medium glucose studied, there was no significant difference in the net

TABLE I. Observed Data.

| Animal | Medium glucose concentration | $CO_2$ | Total lipid | Triglyceride | Fatty acid | $CO_2$/T.L. | $CO_2$/T.G. | F.A./T.L. |
|---|---|---|---|---|---|---|---|---|
| | | CPM/g | | | | | | |
| | | | | Control | | | | |
| 119 | "low" | 1,182 | 611 | | 225 | 1.93 | | .368 |
| | "high" | 9,732 | 3,321 | | 509 | 2.93 | | .153 |
| 121 | "low" | 6,002 | 3,276 | | 1,043 | 1.83 | | .318 |
| | "high" | 44,210 | 20,180 | | 2,542 | 2.19 | | .126 |
| 156 | "low" | 8,103 | 3,683 | 810 | | 2.20 | 10.00 | |
| | "high" | 63,612 | 20,520 | 4,142 | | 3.11 | 15.36 | |
| | | | | Insulin-treated | | | | |
| 129 | "low" | 2,602 | 1,481 | | 410 | 1.76 | | .277 |
| | "high" | 20,850 | 17,412 | | 9,081 | 1.20 | | .522 |
| 137 | "low" | 8,576 | 5,732 | | | 1.50 | | |
| | "high" | 89,840 | 77,744 | | | 1.16 | | |
| 161 | "low" | 10,034 | 5,017 | 913 | | 2.00 | 10.99 | |
| | "high" | 42,285 | 38,794 | 9,250 | | 1.09 | 4.57 | |

Values are expressed as CPM/g liver/hr and are means of triplicate experiments for each animal. These data represent 6 of 36 animals studied.

change in the medium glucose concentration between those animals pretreated with insulin and those not so treated. However, since endogenous glucose release is dependent upon the initial concentration of glucose in the medium, decrease of the specific activity of the medium will be greatest at the "low" level. Furthermore, because the slice is continuously releasing glucose into the buffer throughout the period of incubation, the specific activity to which it is exposed is also continuously changing. In order to determine absolute rates of glucose incorporation, it would be necessary to know the rate at which glucose is released into the buffer, and this information is not available. Therefore this report is concerned only with evaluation of the effects of medium glucose concentration and insulin pretreatment on the relative incorporation of glucose-6$^{14}$C into $CO_2$ as compared to lipid. Since insulin treatment did not affect endogenous glucose release, an estimate of the ability of liver slices from any given rat to incorporate glucose into $CO_2$ or lipid can be obtained. In this manner, the influence of medium glucose concentration and insulin pretreatment on this system may be evaluated.

*II. Metabolic studies.* The ability of liver slices to incorporate glucose-6$^{14}$C into $CO_2$ and lipid varied widely between animals. Table I indicates the range of activity in slices from a representative sample of the animals studied. Furthermore, even though there was great variability in activity between animals, the relative amount of label recovered in the parameters measured was quite similar, *i.e.*, $CO_2$/lipid, $CO_2$/triglyceride, fatty acid/lipid. The following discussion, therefore, deals with the influence of glucose and insulin on the amount of glucose-6$^{14}$C the liver slice incorporates into $CO_2$ relative to lipid. In this way comparisons between animals can be made.

The effect of insulin pretreatment on the relative proportion of labelled glucose recovered in the various fractions is seen in Table II and Fig. 2. When liver slices from control rats were incubated in "low" glucose medium, the mean ratio of $^{14}$C recovered as $CO_2$/total lipid was equal to 1.77. Although increased amounts of radioactive glucose were recovered in both fractions following incubation in the "high" glucose medium, relatively more was recovered as $CO_2$, *i.e.*, the $CO_2$/total lipid ratio was greater. However, this increase (1.77 to 2.24) was not statistically significant.

In contrast, insulin pretreatment had the opposite effect. The $CO_2$/total lipid ratio decreased when liver slices were incubated in a "high" as compared to a "low" glucose

TABLE II. Derived Data.

| Ratio | Medium glucose conc | Control: Mean ± S.D. ( ) = n | Control: p value§ | Insulin-treated: Mean ± S.D. ( ) = n | Insulin-treated: p value§ | p value‖ Control *vs* treated |
|---|---|---|---|---|---|---|
| $CO_2$/Lipid* | "low" | 1.77 ± .64 (16) | n.s. | 1.48 ± .30 (16) | <.05 | n.s. |
| | "high" | 2.24 ± 1.01 (16) | | 1.14 ± .25 (16) | | <.05 |
| $CO_2$/Triglyceride† | "low" | 14.21 ± 8.93 ( 4) | n.s. | 9.07 ± 3.83 ( 5) | n.s. | n.s. |
| | "high" | 15.49 ± 8.47 ( 4) | | 5.64 ± 1.43 ( 5) | | <.05 |
| Fatty acid/Lipid‡ | "low" | .38 ± .01 ( 6) | <.05 | .34 ± .14 ( 7) | n.s. | n.s. |
| | "high" | .23 ± .13 ( 6) | | .48 ± .14 ( 7) | | <.05 |

* $CO_2$/Total lipid = CPM/g liver/hr incorporated into $CO_2$/Lipid.
† $CO_2$/Triglyceride = CPM/g liver/hr incorporated into $CO_2$/Triglyceride.
‡ Fatty acid/Total lipid = CPM/g liver/hr incorporated into Fatty acid/Total lipid.
§ Comparison between "high" and "low" glucose concentration.
‖ Comparison between control and insulin pretreatment.
n.s. = not significant at the .05 level.

All ratios derived from individual values for each animal. All data were normalized and analyzed by "t" test and one way analysis of variance with the aid of a Monrobot XI digital computer.

medium and this fall from 1.48 to 1.14 was statistically significant. In other words, when liver slices from insulin pretreated rats were incubated in a "high" glucose medium, they incorporated relatively more glucose into lipid than when they were incubated in a "low" glucose medium. Furthermore, when insulin pretreated slices were compared to normal slices, a significantly greater proportion of glucose-6$^{14}$C was recovered as total lipid than as $CO_2$ following incubation in the "high" glucose medium, *i.e.*, the $CO_2$/total lipid ratio was 1.14 as compared to 2.24. Finally, when the effects of insulin pretreatment and a "high" glucose medium were analyzed separately, there was found to be significant interaction between these two variables(16). Specifically, the effect of insulin pretreatment at the "high" glucose concentration was more than could be accounted for by the combined effects of either acting alone and was therefore dependent upon the glucose concentration at which it was studied. Since liver slices from the same animal were exposed to both "high" and "low" glucose concentrations, the measurement of interaction in these experiments was based upon these paired data.

Similar comparisons between control and insulin pretreated animals with reference to the $CO_2$/triglyceride ratio are also seen in Table II and Fig. 2. The results are very similar to those observed upon analysis of the $CO_2$/total lipid ratio. There was a rise in the $CO_2$/triglyceride ratio in control animals when comparing incubation in "low" with "high" glucose medium, while the ratio fell in insulin pretreated animals. The $CO_2$/

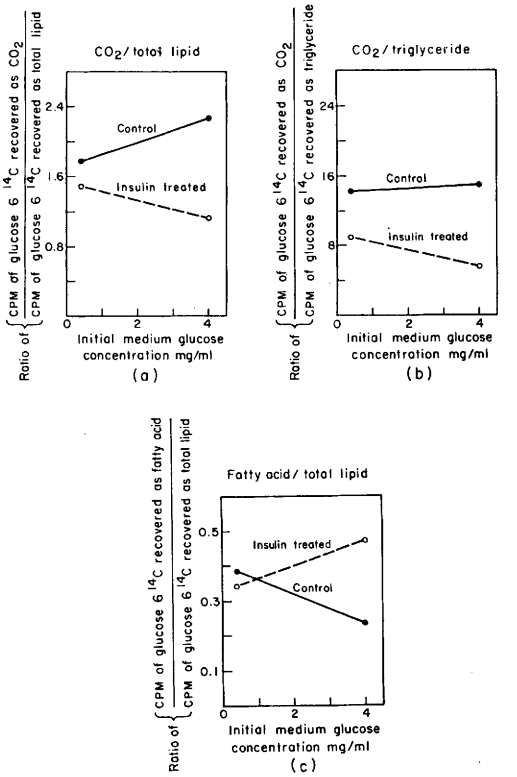


FIG. 2. Effect of varying medium glucose concentration and insulin pretreatment on relative metabolism of glucose by rat liver slices. Points are means of individual values derived from each animal for each parameter.

triglyceride ratio was significantly lower in insulin pretreated animals following incubation in the "high" glucose medium (5.64 compared to 15.49). In other words, there was relatively more glucose-6$^{14}$C recovered as triglyceride from liver slices of insulin pretreated animals as compared to controls.

Glucose-6$^{14}$C could be in either the fatty acid or the glycerol moiety of the lipid, and an attempt was made to distinguish between these two possibilities by estimating the proportion of total radioactivity in the total lipid fraction recovered as fatty acid. This has been expressed as the fatty acid/total lipid ratio, and the results of this analysis are seen in TableII and Fig. 2. In control animals the fatty acid/total lipid ratio fell when slices were incubated in the "high" as compared to the "low" glucose medium. Relatively more glucose-6$^{14}$C was recovered in the non-fatty acid, presumably glycerol, fraction as medium glucose concentration was increased. On the other hand, the fatty acid/total lipid ratio rose when the same comparison was made in the insulin treated animals, indicating a relatively greater increase in recovery of radioactivity in the fatty acid moiety. This is further supported by the fact that this ratio was significantly higher in the insulin pretreated animals (.43) than in controls (.23) following incubation of slices in the "high" glucose medium.

Only a small amount of glucose was found to be incorporated into the glycogen fraction, as would be expected in the liver of fed rats incubated in a "high" sodium, low potassium buffer(17). A significant effect of insulin on the incorporation of glucose-6$^{14}$C into glycogen could not be demonstrated.

*Discussion.* The present studies provide further information concerning the relative participation of glucose and insulin in the metabolism of glucose by the liver of a normal rat.

Chernick, and later Renold and their coworkers, showed that insulin pretreatment of rats for periods of at least 24 hours resulted in greater incorporation of glucose and acetate into $CO_2$ and fatty acid when compared to untreated animals(3,4). These investigations did not deal with the relative incorporation of glucose into its various metabolic pathways or with the relationship between glucose concentration and insulin action on the metabolism of glucose by the liver.

Millstein *et al,* reported that pretreatment of rats with PZI insulin for 3 days allowed hepatic lipogenesis to proceed at a much lower medium glucose concentration than in untreated animals(18). The doses of insulin were massive (36 $\mu$ PZI over 3 days), and resulted in some degree of hypoglycemia. In our studies, administration of large doses of insulin resulted in a decrease in ability of the liver slices to incorporate glucose into lipid, presumably due to hypoglycemia. In addition, the inability to demonstrate lipogenic activity in medium glucose concentrations of less than 4.0 mg/ml is contrary to what we and others have observed. The reason for this discrepancy is not clear.

More recently, Goldman and Cahill have reported that insulin, administered intravenously to rats, reduced the specific activity of hepatic triglyceride glycerol while increasing the incorporation of label into glyceride fatty acid(19). The fall in specific activity of triglyceride glycerol may be related to the acute effects of intravenously administered insulin on hepatic metabolism, *i.e.,* hypoglycemia, reflecting the usefulness of hepatic glycerol as a ready substrate for gluconeogenesis(20).

An increased rate of incorporation of glucose into total liver fatty acids has been demonstrated in strains of obese hyperglycemic mice in which carbohydrate intolerance has been documented and there is good evidence for hyperinsulinism(21,22). Nikkila has produced an elevation of plasma and hepatic triglyceride concentration by prolonged administration of a high carbohydrate diet to adult rats(23).

The following general statements seem appropriate concerning the present studies. Glucose metabolism by liver slices of normal rats was altered by pretreatment with insulin and by exposure of the slice to 2 widely different concentrations of medium glucose. Relatively more glucose-6$^{14}$C was recovered as $CO_2$ and non-fatty acid lipid (presumably glycerol) when liver slices of control animals were ex-

posed to a "high" glucose concentration. Insulin pretreatment resulted in the recovery of relatively more label in lipid fractions, specifically in the fatty acid moiety. The effect of insulin on the liver, *i.e.*, preferential shunting of glucose into lipid pathways, was dependent upon the presence of an increased concentration of glucose. When large amounts of glucose were supplied to the liver of a rat not previously exposed to insulin, this shunting did not occur.

These observations are of interest when considering the relationship between hepatic glucose and lipid metabolism. A better understanding of the mechanisms by which glucose concentration and insulin influence hepatic lipogenesis from glucose may result from ongoing studies concerned with fatty acid and glycerol uptake and release, and esterification. In order to do this, the kinetics of glucose metabolism by liver slices is being investigated.

The present observations may be of some importance in the study of the syndrome of carbohydrate-induced hypertriglyceridemia as it occurs in man. The results are compatible with the hypothesis that elevated levels of insulin and glucose in patients with this syndrome may act in concert on an otherwise normal liver to cause an increase in hepatic triglyceride synthesis. The data do not establish that this is the only, or even the primary, cause of hypertriglyceridemia observed in these patients.

*Summary.* Liver slices from normal fed rats were incubated in physiologic bicarbonate buffer, and the effect of increasing glucose concentration and insulin pretreatment on hepatic glucose metabolism was studied. Exposure of the liver slice to increased levels of glucose resulted in the recovery of more glucose-6$^{14}$C in $CO_2$ relative to lipid pathways. Insulin pretreatment, in the presence of a "high" medium glucose concentration, resulted in a preferential incorporation of glucose-6$^{14}$C into total lipid and triglyceride relative to $CO_2$ and into fatty acid to a greater extent than into glycerol. The effects of insulin were dependent upon the presence of a "high" concentration of glucose in the medium.

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