

# In Vitro Reversal of the Fasting State of Liver Metabolism in the Rat

## REEVALUATION OF THE ROLES OF INSULIN AND GLUCOSE

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**ABSTRACT** Studies were conducted to determine whether the direction of hepatic carbohydrate and lipid metabolism in the rat could be switched simultaneously from a "fasted" to a "fed" profile in vitro. When incubated for 2 h under appropriate conditions hepatocytes from fasted animals could be induced to synthesize glycogen at in vivo rates. There was concomitant marked elevation of the tissue malonyl-coenzyme A level, acceleration of fatty acid synthesis, and suppression of fatty acid oxidation and ketogenesis. In agreement with reports from some laboratories, but contrary to popular belief, glucose was not taken up efficiently by the cells and was thus a poor substrate for either glycogen synthesis or lipogenesis. The best precursor for glycogen formation was fructose, whereas lactate (pyruvate) was most efficient in lipogenesis. In both cases the addition of glucose to the gluconeogenic substrates was stimulatory, the highest rates being obtained with the further inclusion of glutamine. Insulin was neither necessary for, nor did it stimulate, glycogen deposition or fatty acid synthesis under favorable substrate conditions. Glucagon at physiological concentrations inhibited both glycogen formation and fatty acid synthesis. Insulin readily reversed the effects of glucagon in the submaximal range of its concentration curve.

The following conclusions were drawn. First, the fasted-to-fed transition of hepatic carbohydrate and lipid metabolism can be accomplished in vitro over a time frame similar to that operative in vivo. Second, reversal appears to be a substrate-driven phenomenon, in that insulin is not required. Third, unless an unidentified factor (present in portal blood during feeding) facilitates the uptake of glucose by liver it seems

unlikely that glucose is the immediate precursor for liver glycogen or fat synthesis in vivo. A likely candidate for the primary substrate in both processes is lactate, which is rapidly formed from glucose by the small intestine and peripheral tissues. Fructose and amino acids may also contribute. Fourth, the requirement for insulin in the reversal of the fasting state of liver metabolism in vivo can best be explained by its ability to offset the catabolic actions of glucagon.

### INTRODUCTION

In the transition from the fed to the fasted state, the liver switches from an organ of glucose storage and fatty acid synthesis to one of glucose release, fatty acid oxidation, and ketone body production. Available evidence (recently reviewed [1]) indicates that these metabolic adaptations are triggered primarily by elevation of the circulating [glucagon]:[insulin] ratio. Although changes in both hormones are clearly important, glucagon appears to have the dominant role in altering hepatic metabolism because a modest excess of glucagon will rapidly convert the liver from an anabolic to a catabolic mode, even in the face of hyperinsulinemia (2). Moreover, the changes that occur as a consequence of food deprivation in vivo can be reproduced in vitro by treating isolated hepatocytes from fed rats with glucagon. The key events are (a) activation of glycogenolysis and gluconeogenesis, (b) inhibition of fatty acid synthesis, and (c) stimulation of fatty acid oxidation and ketogenesis. The changes in lipid metabolism are orchestrated by a fall in the tissue malonyl-coenzyme A (CoA) level (1). In the intact rat these events commence ~6 h after the last feeding and are complete some 4–6 h later (3), but with hepatocytes in vitro only minutes are required.

When fasted rats are refeed, hepatic metabolism reverts to the "fed" pattern over a several-hour period

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(3). It is widely assumed that this reversal is mediated by a direct action of insulin on the liver, and that the primary substrate utilized for reinitiation of glycogen and fatty acid synthesis is glucose (e.g., 4). On the other hand, we have been impressed with reports that effects of insulin on the liver *in vitro*, even in preparations from fed animals, are small (e.g., 4–8). In addition, a number of studies have suggested that glucose *per se* is a poor substrate for glycogen synthesis in hepatocytes and perfused livers from fasted rats (9–12).

As was noted above, the metabolism of hepatocytes from fed animals can be rapidly converted to a fasting profile under *in vitro* conditions. To our knowledge the reverse transition (i.e., fasted to fed) has not been accomplished. The present investigation demonstrates such reversal in isolated hepatocytes without hormonal addition. With appropriate substrates, glycogen synthesis was restored, malonyl-CoA concentrations were elevated, lipogenesis was stimulated, and fatty acid oxidation was turned off. The addition of insulin had little direct effect on any of these parameters, although it readily neutralized the catabolic drive initiated by glucagon. By itself, glucose, even in supraphysiological concentrations, was unable to cause reversal of fasting metabolism; a gluconeogenic precursor and an amino acid were also required. These results, if applicable to the *in vivo* situation, raise questions about conventional interpretations of the physiological and biochemical mechanisms whereby the fasted state is reversed.

## METHODS

**Animals.** Male Sprague-Dawley rats weighing ~140 g were used. They were maintained on a diet of high sucrose and low fat, as described previously (13), and housed in a room with lighting from 3:00 p.m. to 3:00 a.m. Experiments began at 9:00 a.m. on fasted animals deprived of food for 18 h before use. In some studies the fast was ended by allowing *ad lib.* feeding for specified periods of time.

***In vivo* experiments.** To determine the *in vivo* rate of hepatic glycogen replenishment after a fast, rats that had been deprived of food for 18 h were refed in a dark, quiet room. At various time intervals groups of animals were anesthetized with pentobarbital; a sample of portal blood was taken into a heparinized syringe for analysis of plasma glucose, fructose, and lactate; and the liver was rapidly frozen in liquid N<sub>2</sub> for subsequent determination of glycogen levels.

**Preparation of hepatocytes.** Hepatocytes were prepared essentially as described by Zuhlten et al. (14). Briefly, livers were initially perfused for a period of 8 min with 100 ml of recirculating, highly oxygenated Krebs bicarbonate buffer, pH 7.4 (see below), lacking calcium and containing 4.5 mM NH<sub>4</sub>Cl together with 20 mM glucose. 50 mg (~6,600 U) of collagenase (type II, Worthington Biochemical Corp., Freehold, N. J.) together with 2.7 mg of Worthington soybean trypsin inhibitor (to minimize trypsin activity present in the crude collagenase) was then added and perfusion was continued for an additional 30 min. At this time the digested liver was removed and suspended at room temperature in 50 ml of normal Krebs bicarbonate buffer containing 20 mM

glucose. After gentle manual agitation, the suspension was filtered through nylon mesh and centrifuged at room temperature in a 250-ml container (50 g for 2 min). The parenchymal cells were resuspended in the same buffer, and the centrifugation step repeated. Finally, the cells were resuspended in normal Krebs bicarbonate buffer (without added glucose) to a density of 100 mg/ml wet wt. As judged by trypan blue exclusion and their ability to retain lactate dehydrogenase, the hepatocyte preparations were generally 85–90% intact.

**Incubation of hepatocytes.** All incubations were carried out in siliconized 25-ml conical flasks. These were gassed with 95% O<sub>2</sub>:5% CO<sub>2</sub>, stoppered, and shaken at 37°C at a rate of 80 oscillations/min. The gassing procedure was repeated at 30-min intervals throughout the incubation. For all studies the flasks contained 100 mg wet wt of hepatocytes, 2% fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), and various additions (described in the text) in a total volume of 2.5 ml of Krebs bicarbonate buffer, pH 7.4. When used, the initial concentrations of glucose, fructose, lactate-pyruvate, and glutamine were ordinarily 20, 5, 10:1, and 10 mM, respectively. To prevent substrate depletion, further additions of fructose and lactate-pyruvate were made at 30, 60, and 90 min, and of glutamine at 60 min, in the same concentrations as those added initially. In the experiment shown in Table II, physiological concentrations of substrates were added as detailed in the legend. Because of their rapid degradation by liver, insulin and glucagon were added at zero time and every 30 min thereafter.

Fatty acid synthesis (<sup>3</sup>H<sub>2</sub>O incorporation into fatty acids), fatty acid oxidation (conversion of [1-<sup>14</sup>C]oleate into total acid-soluble products, ketone bodies, and CO<sub>2</sub>), and tissue malonyl-CoA levels were determined as described previously (15, 16). Glycogen was measured by the method of Chan and Exton (17).

**Liver perfusion.** Livers were perfused in recirculating fashion (18) with 100 ml of Krebs bicarbonate buffer, pH 7.4, containing 2% fatty acid-free albumin. The medium was continuously exposed to a mixture of 95% O<sub>2</sub>:5% CO<sub>2</sub> in a revolving drum and, in addition, was passed through an oxygenation chamber consisting of 50 ft. of silastic tubing exposed to 100% O<sub>2</sub> at a pressure of 20 mm. Flow rate was 14 ml/min. Substrate additions are described in the text. At the end of perfusion the liver was frozen in liquid N<sub>2</sub> and analyzed for glycogen content (17).

## RESULTS

**Hepatic glycogen synthesis in fasted-refed rats.** As seen in Fig. 1, refeeding of fasted rats resulted in a major increase in liver glycogen content. The value rose from 2.5 to 38 mg/g wet wt by the 3-h time point and reached a stable level of about 44 mg/g wet wt after 5 h. By comparison, the value for fed animals *ad lib.* was ~55 mg/g wet wt. The concentration of glucose in portal plasma increased from 75 mg/dl (4.2 mM) at zero time to 180 mg/dl (10 mM) at 1 h and remained in the region of 200 mg/dl (11 mM) for the remainder of the experiment. A similar profile was noted for the portal lactate concentration, which rose from an initial value of 1.6 to ~5 mM after 1 h. Portal fructose (derived from dietary sucrose) was undetectable at zero time but was present at a concentration of 25–30 mg/dl (1.4–1.7 mM) between 1 and 4 h of refeeding.

These observations, coupled with our previous

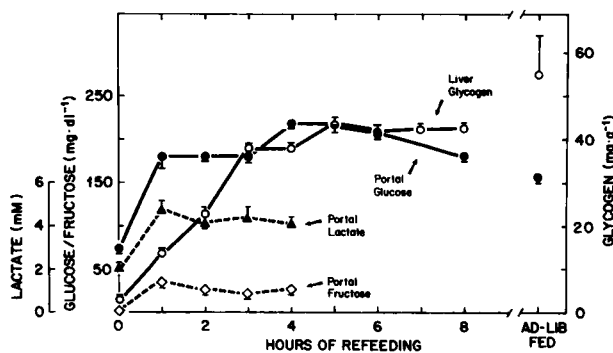


FIGURE 1 Effects of refeeding fasted rats. Rats fasted for 18 h were allowed to eat ad lib. At the indicated times groups of animals were anesthetized, samples of portal blood were taken for plasma analyses, and livers were analyzed for glycogen. Values are means  $\pm$  SEM for 6–12 animals at each time point.

studies on the direction of hepatic carbohydrate and fatty acid metabolism in different nutritional states (3, 15, 16), clearly established that in the rat the transition from a fasted to a fed metabolic profile is essentially complete after  $\sim 3$  h of refeeding. The next series of experiments sought to determine whether this conversion could be achieved in the isolated hepatocytes.

**Effects of insulin and glucose on glycogen synthesis *in vitro*.** There have been a number of reports that insulin activates hepatic glycogen synthase, thereby promoting the conversion of glucose into glycogen (6, 19–21). For this reason we examined the effects of insulin plus glucose on glycogen synthesis in hepatocytes from fasted rats. As shown in Table I, rates of glycogen formation from glucose were low, and insulin added in supraphysiological levels (2,000  $\mu$ U/ml) had no stimulatory effect. Because it had

TABLE I  
Effects of Insulin on Glycogen Synthesis in  
Hepatocytes from Fasted Rats

Medium	Insulin	Glycogen
		mg $\cdot$ g cells $^{-1}$
Krebs bicarbonate buffer	–	4.6
	+	4.2
Eagle's minimal essential medium	–	2.1
	+	2.1

Hepatocytes from 18-h fasted rats were incubated for 2 h as described under Methods. Glucose was present at a concentration of 20 mM at the start of the experiment. Insulin was added at a concentration of 2,000  $\mu$ U/ml at zero time and at 30, 60, and 90 min. Values are means of two experiments and represent glycogen content of cells after 2 h of incubation. The glycogen level at zero time was 2 mg  $\cdot$  g cells $^{-1}$ .

been previously reported that insulin effects were more marked when cells were incubated in Eagle's minimal essential medium than in Krebs' bicarbonate buffer (19), we repeated the experiment using the former medium. Net glycogen synthesis was not observed and insulin did not stimulate the process.

In view of the cited reports that glucose was a poor substrate for glycogen synthesis (9–12), we next evaluated glycogen deposition in hepatocytes from fasted rats incubated with various substrates over a period of 2 h (longer incubations were not performed to avoid the possibility of cell deterioration; Fig. 2). As predicted from the experiment of Table I, glucose at a concentration of 20 mM functioned poorly as a substrate for glycogen synthesis, and measurement of glucose in the medium at zero time and 120 min showed little, if any, uptake of the hexose (data not shown). By contrast, fructose supported significant rates of glycogen formation, particularly during the 2nd h, and a distinct synergism was noted when glucose and fructose were added together. Glutamine, used as a representative of the amino acids taken up by the liver in the fasting state, was essentially without effect when

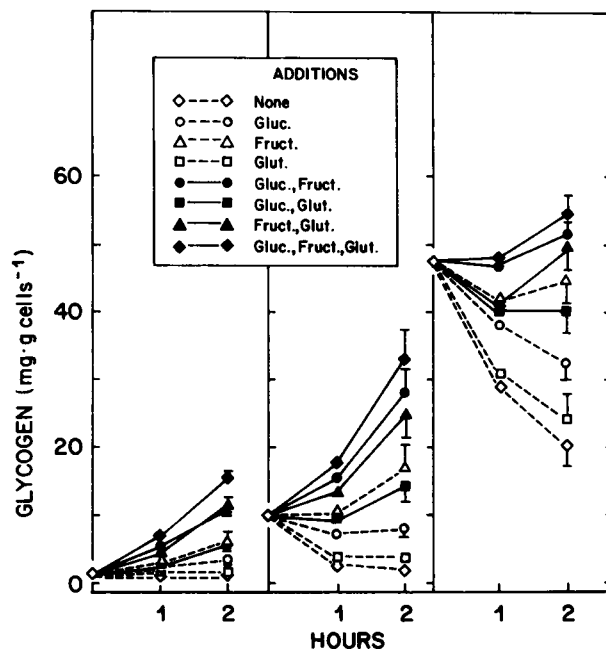


FIGURE 2 Glycogen synthesis in hepatocytes. Hepatocytes were prepared from rats that had been fasted for 18 h (panel A), fasted for 18 h and refed for 2 h (panel B), or from non-fasted animals (panel C). The cells (100 mg wet wt) were incubated with the indicated substrates as described under Methods and analyzed for glycogen content at the indicated times. The initial concentrations of glucose (Gluc.), fructose (Fruct.), and glutamine (Glut.) were 20, 5, and 10 mM, respectively. Further additions of fructose (5 mM) were made at 30, 60, and 90 min, and of glutamine (10 mM) at 60 min. Values are means  $\pm$  SEM for three experiments.

present alone, but invariably stimulated glycogen synthesis when added with fructose or with glucose plus fructose.<sup>1</sup> Highest rates of glycogen formation were always seen when the three substrates were present simultaneously.<sup>2</sup> In experiments not shown it was observed that a combination of lactate and pyruvate (10 and 1 mM, respectively) could effectively substitute for fructose, although a mixture of the C<sub>3</sub> compounds, glucose, and glutamine did not give as high a rate as the fructose-glucose-glutamine combination. Addition of lactate-pyruvate together with glucose, glutamine, and fructose provided no further stimulation.

Qualitatively similar results were obtained with hepatocytes isolated from rats that had been fasted and refed for 2 h (Fig. 2B) or from animals fed ad lib. (Fig. 2C). In both cases there was loss of glycogen when the cells were incubated without substrates. Little protection against this loss was afforded by either glutamine or lactate-pyruvate. Although glucose was more protective, it too failed to maintain the initial glycogen level. In contrast, the net loss of glycogen was converted into a significant gain with those substrate combinations that had proved effective in fasted cells. We assume that the minimal additional deposition of glycogen seen with favorable substrate additions in cells from rats fed ad lib. (Fig. 2C) was related to their much higher initial glycogen stores. In experiments not shown, insulin had no stimulatory effect with any substrate or substrate combination.

The substrate concentrations used in the experiments of Fig. 2 were supraphysiological in order to assure maximal rates of glycogen formation and to prevent substrate depletion during the experiment. We next repeated the study using glucose and fructose concentrations equivalent to those measured in the portal vein after refeeding (Fig. 1). Glutamine was not measured, but 0.5 mM was considered physiological (22, 23). As shown in Table II glycogen synthesis was essentially unaffected by any substrate at 1 h, but by 2 h the stimulatory effects of the glucose-fructose and glucose-fructose-glutamine combinations were clearly evident. Comparison with the experiments of Fig. 2 indicated that rates of glycogen synthesis were not maximal (2-h values for glucose-fructose-glutamine: physiological substrate concentrations, 8.2 mg per g cells; supraphysiological substrate concentrations, 18.0 mg per g cells).

<sup>1</sup> Since fructose is rapidly converted into glucose by these cells (data not shown) it is not possible to assess its effects independent of glucose. It is probable that rates of glycogen synthesis obtained with fructose were facilitated by the accumulation of glucose in the medium.

<sup>2</sup> In experiments not shown the stimulatory effect of glutamine on glycogen synthesis could be reproduced with alanine, as previously noted by Katz et al. (11).

TABLE II  
*Glycogen Synthesis with Physiological  
Substrate Concentrations*

Substrate	Glycogen	
	1 h	2 h
Glucose	4.3	4.2
Fructose	4.0	5.0
Glutamine	3.2	4.1
Glucose, fructose	4.6	6.9
Glucose, glutamine	3.3	4.1
Fructose, glutamine	3.7	5.1
Glucose, fructose, glutamine	4.4	8.2

Hepatocytes from 18-h fasted rats were incubated as described under the legend for Fig. 2, except that initial substrate concentrations were: glucose (10 mM), fructose (2 mM), and glutamine (0.5 mM). Further additions of fructose and glutamine were made in the same concentrations at 30, 60, and 90 min.

*Glycogen synthesis in the perfused liver.* It might be argued that the failure to observe an effect of insulin on glycogen synthesis in isolated hepatocytes stemmed from damage to insulin receptors during collagenase digestion of the liver (the insulin receptor on adipocyte cell membranes is known to be sensitive to proteolytic enzymes, and the collagenase used in the preparation of hepatocytes contained some trypsin activity). Although we felt this to be an unlikely explanation (since we always included soybean trypsin inhibitor during the preparation of hepatocytes), some of the studies described in Fig. 2A were repeated using intact perfused livers from fasted rats in which the insulin receptor was presumably intact. As seen from Table III, when present in the perfusion fluid at a concentration of 10 mM, glucose was neither taken up by the liver nor utilized for glycogen synthesis. Even 20 mM glucose caused only modest glycogen deposition, yet at either glucose concentration the simultaneous presence of fructose and glutamine resulted in clear-cut stimulation of glycogen formation. In no circumstance did insulin have a stimulatory effect.

*Fatty acid metabolism in hepatocytes.* An analogous series of experiments focusing on the processes of fatty acid synthesis and oxidation was next undertaken. As expected, in the absence of added substrates, there was essentially no fatty acid synthesis in hepatocytes from fasted rats, whereas basal rates were substantial in cells from fed animals (Fig. 3). Refeeding for 2 h produced only a marginal increase in basal rates of lipogenesis. That the difference in rates reflected primarily the difference in endogenous substrate availability (glycogen) rather than a starvation-induced loss of enzyme activity is seen from the effects of added substrates on cells from 18-h fasted

TABLE III  
Glycogen Synthesis in Perfused Livers from Fasted Rats

	Fructose and glutamine	Insulin	Perfusate glucose concentration			Liver glycogen at 120 min
			0 min	60 min	120 min	
			mg·dl <sup>-1</sup>			mg·g wet wt <sup>-1</sup>
Experiment 1	—	—	178±4	176±3	181±4	1.6±0.7
	—	+	176±3	187±4	181±6	1.8±0.4
	+	—	—	—	—	10.6±1.6
	+	+	—	—	—	9.1±1.1
Experiment 2	—	—	374±4	366±9	338±10	5.5±1.2
	—	+	367±5	368±6	351±5	4.3±0.5
	+	—	—	—	—	12.0±1.0
	+	+	—	—	—	10.0±0.9

Livers from 18-h fasted rats were perfused with recirculating medium for 2-h as described under Methods. The initial perfusate glucose concentration was 10 and 20 mM in experiment 1 and 2, respectively. When present, fructose, glutamine, and insulin were added in concentrations of 5 mM, 10 mM, and 2,000  $\mu$ U/ml, respectively, at zero time and at intervals thereafter as in the experiments of Fig. 2 and Table I. Initial glycogen levels were generally in the region of 2–3 mg/g wet wt of liver. Values are means  $\pm$  SEM for three to six perfusions.

rats and those refed for 2 h. In both cases glucose or glutamine when added alone produced a modest stimulation of lipogenesis, but this was less than that seen with fructose, which was in turn less effective than lactate-pyruvate. Maximal rates were seen

with a combination of glucose, glutamine, and lactate-pyruvate. The substrate triad of glucose, fructose, and glutamine, which was maximally effective in glycogen synthesis, yielded rates only one-third of those seen with glucose, lactate-pyruvate, and glutamine (data not shown). Moreover, fructose added to the latter combination was inhibitory (data not shown).<sup>3</sup>

The high, intermediate, and low capacities, respectively, of lactate, fructose, and glucose to support hepatic fatty acid synthesis were previously reported by Clark et al. (24) for rat hepatocytes and by Salmon et al. (25) for the perfused mouse liver. In both studies fed animals were used. The important findings here were that the impaired lipogenesis characteristic of the fasted state could be reversed *in vitro* by the addition of substrate, and that with the appropriate combinations, rates approached those seen in cells from fed animals. The reason for the stimulatory effect of glutamine is not known. Since glycogen was accumulating during the stimulatory period for lipogenesis, it is conceivable that glutamine was acting secondarily through this process. It has been suggested that glycogen plays a regulatory role in fatty acid synthesis apart from its capacity to serve as a carbon source for fat formation (25).

The influence of insulin on fatty acid synthesis is shown in Table IV. Irrespective of the substrate used there was little effect of the hormone in hepatocytes

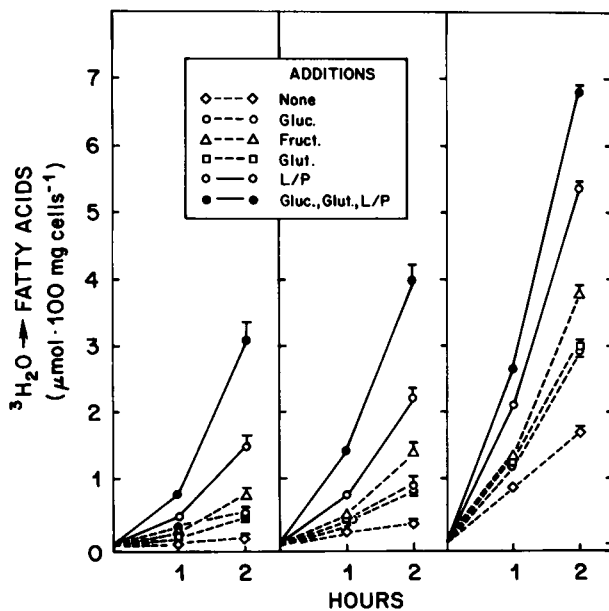


FIGURE 3 Fatty acid synthesis in hepatocytes. 100 mg of hepatocytes from 18-h fasted rats (panel A), 18-h fasted and 2-h refed rats (panel B), or rats fed ad lib. (panel C) were incubated with the indicated substrates and  $^3\text{H}_2\text{O}$  (1 mCi/flask) as described under Methods. L/P stands for lactate-pyruvate; other abbreviations are given in the legend to Fig. 2. Values are means  $\pm$  SEM for three experiments.

<sup>3</sup> The inhibitory effect of fructose in the presence of glucose, glutamine, and lactate-pyruvate might stem from depletion of cellular ATP. Such a mechanism was postulated by Clark et al. (24) to account for the inhibition of hepatic lipogenesis caused by fructose concentrations  $>5$  mM.

**TABLE IV**  
*Effects of Insulin on Fatty Acid Synthesis in Hepatocytes*

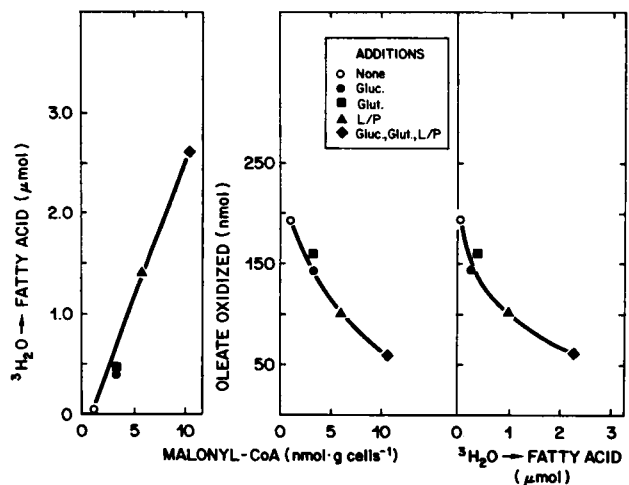
State of animal	Substrate	nmol $^3\text{H}_2\text{O} \rightarrow$ fatty acid		
		-Insulin	+Insulin	% Stimulation
Fasted 18 h	Glucose	247 $\pm$ 34	314 $\pm$ 52	26 $\pm$ 5
	Glutamine	342 $\pm$ 67	457 $\pm$ 89	34 $\pm$ 0.3
	Lactate/pyruvate	1,084 $\pm$ 153	1,270 $\pm$ 203	24 $\pm$ 5
	Glucose, glutamine, lactate-pyruvate	2,017 $\pm$ 56	2,388 $\pm$ 8	18 $\pm$ 5
Fed ad lib.	Glucose	1,933 $\pm$ 307	3,129 $\pm$ 407	63 $\pm$ 6
	Glutamine	1,305 $\pm$ 334	1,984 $\pm$ 414	56 $\pm$ 7
	Lactate-pyruvate	2,747 $\pm$ 198	3,342 $\pm$ 261	22 $\pm$ 1
	Glucose, glutamine, lactate-pyruvate	3,875 $\pm$ 347	3,763 $\pm$ 173	0

Hepatocytes from 18-h-fasted or ad lib.-fed rats were incubated as described in Fig. 3. Fatty acid synthetic rates are given for the 60–120-min period. Values are means $\pm$ SEM for three experiments in each group.

from fasted rats, the stimulation being generally in the order of 25%. Although there appeared to be a somewhat greater responsiveness in cells from fed animals, the maximal stimulation observed was 63% in the presence of glucose alone, and no effect of the hormone could be detected when a full complement of substrates was used. This is to be compared with an 8-fold stimulation by glutamine and lactate-pyruvate when added to glucose alone (Table IV) and a 30-fold stimulation above rates seen in the absence of exogenous substrate in fasted cells (Fig. 3).

We have previously shown that the pathways of fatty acid synthesis and oxidation in rat liver are inversely related and that reciprocal control of these opposing processes is exerted largely through fluctuation in the cellular content of malonyl-CoA, a potent inhibitor of carnitine acyltransferase I (15, 16, 26). Assuming this concept to be correct, it would be expected that malonyl-CoA levels would change in predictable fashion with the substrate additions. As seen from Fig. 4, when cells from fasted rats were exposed to glucose, lactate-pyruvate, and glutamine, singly and in combination, the expected relationships were found among malonyl-CoA concentration, fatty acid synthesis, and fatty acid oxidation. A comparison of the effects of substrate and insulin on malonyl-CoA levels and fatty acid oxidation in hepatocytes from fasted and fed animals is given in Table V. From experiment 1 it can be seen that inclusion of lipogenic substrates resulted in a striking elevation of malonyl-CoA and a concomitant 71% suppression of  $\beta$ -oxidation (conversion of labeled oleate into total acid-soluble products plus  $\text{CO}_2$ ) in hepatocytes from fasted rats. This was accompanied by an even greater suppression of ketogenesis (95%). The greater sensitivity of ketogenesis stems from the increased provision of oxaloacetate (derived from the added substrates) with attendant

diversion of acetyl-CoA away from the ketogenic pathway and into the tricarboxylic acid cycle (15, 16). Experiment 2 illustrates the failure of insulin to alter either the malonyl-CoA level or the high rate of fatty acid oxidation in these cells, whether added alone or together with the lipogenic substrates. In accord with the fact that hepatocytes from fed animals displayed significant rates of fatty acid synthesis in the absence of



**FIGURE 4** Relationship among fatty acid oxidation, fatty acid synthesis, and tissue malonyl-CoA levels in hepatocytes. 100 mg of hepatocytes from 18-h fasted rats was incubated with the indicated substrates in the presence or absence of  $^3\text{H}_2\text{O}$  as described in Table IV. For measurements of fatty acid oxidation, 0.1 mM albumin-bound [ $1\text{-}^{14}\text{C}$ ]oleate was added at 90 min and its conversion into acid-soluble products plus  $\text{CO}_2$  was determined over the next 30 min. The same concentration of unlabeled oleate was added at 90 min to flasks used for the measurement of fatty acid synthesis (90–120 min) or tissue malonyl-CoA levels (105 min). Values are means of three experiments, all of which yielded similar results.

TABLE V  
*Effects of Lipogenic Substrates on Malonyl-CoA Levels and Fatty Acid Oxidation in Rat Hepatocytes*

	State of animal	Additions	[1- <sup>14</sup> C]Oleate converted into		Malonyl-CoA (105 min)
			Total acid-soluble products + CO <sub>2</sub> (90–120 min)	Ketones (90–120 min)	
			nmol		nmol/g·cells <sup>-1</sup>
Experiment 1	Fasted 18 h (3)	None	228±5.6	204±10.4	1.1±0.4
		Substrates	66±8.5	10±1.9	10.6±2.4
Experiment 2	Fasted 18 h (2)	None	211	184	<1
		Substrates	60	15	11.0
		Insulin	207	190	<1
		Substrates and insulin	52	10	10.3
Experiment 3	Fed ad lib. (2)	None	80	16	2.6
		Substrates	28	5	7.6
		Insulin	67	13	2.6
		Substrates and insulin	27	6	7.1

100 mg of hepatocytes was incubated in the absence or presence of substrates (glucose, glutamine, and lactate-pyruvate) and insulin, as described in Table IV and Fig. 3. Values are means or means±SEM for the number of experiments shown in parentheses.

added substrates (Fig. 3), their basal levels of malonyl-CoA were elevated compared with those of fasted cells, and this was associated with a diminished capacity for fatty acid oxidation and ketogenesis (compare line 1 of experiments 1 and 3). The addition of substrates caused a further increase in the malonyl-CoA concentration, and reduced fatty acid oxidation and ketone production to 12 and 2%, respectively, of the rates seen in fasted cells without lipogenic substrates (compare line 2 of experiment 3 with line 1 of experiment 1). Once again, insulin had little or no effect on malonyl-CoA levels or fatty acid oxidation, which is entirely consistent with its minimal effects on the rate of fatty acid synthesis (Table IV).

*Antagonistic effects of insulin and glucagon on glycogen and fatty acid synthesis in hepatocytes.* The failure of insulin to promote glycogen synthesis or lipogenesis in hepatocytes supplied with optimal substrates raised the question of how the hormone exerts its well-known stimulatory action on both processes in vivo. The data of Table VI suggest that the anabolic action of insulin on liver metabolism is not direct but stems from its ability to antagonize the catabolic effect of glucagon. Thus, the high rate of glycogen synthesis in hepatocytes from fasted rats exposed to glucose, fructose, and glutamine was greatly diminished in a dose-dependent fashion by increasing concentrations of glucagon. At the lowest concentration of glucagon used (nominally 0.057 nM),<sup>4</sup>

its inhibitory effect was completely overcome by the high concentration of insulin employed (nominally 2,000  $\mu$ U/ml), whereas a 66% protection by insulin was observed in the presence of 0.285 nM glucagon. The inhibition by higher levels of glucagon could not be reversed by insulin. A similar pattern was seen when fatty acid synthesis was assessed in hepatocytes from fed animals.

## DISCUSSION

Hepatic carbohydrate and fatty acid metabolism in rodents has been studied on many occasions, but only as isolated phenomena. The present investigation had three goals: first, to examine in parallel the processes of glycogen synthesis, fatty acid synthesis, and fatty acid oxidation in hepatocytes from fasted rats, and to determine whether the change from a "fasted" to a "fed" profile of all three systems could be accomplished in vitro; second, to compare the efficacy of certain representative substrates in producing this metabolic switch; and third, to establish whether insulin is an essential factor. The main observations were as follows. First, the fasted-to-fed transformation of carbohydrate and lipid metabolism could be achieved in the isolated hepatocyte; second, contrary to popular belief, reversal could be accomplished in the absence of insulin; third, glucose was necessary for maximal glycogen and

glucagon concentrations achieved over the nominal levels of 0.057–0.285 nM (200–1,000 pg/ml) were probably within the physiological range for portal blood (39). The average insulin concentration probably remained supraphysiological.

<sup>4</sup> Because rates of glucagon and insulin degradation were not determined, the precise concentrations of the hormones present over the 2-h incubation period is unknown. Actual

**TABLE VI**  
*Antagonistic Effects of Insulin and Glucagon on Glycogen and Fatty Acid Synthesis in Hepatocytes*

Glucagon	Insulin	Net glycogen synthesis		Fatty acid synthesis	
		Suppression by glucagon	Protection by insulin	Suppression by glucagon	Protection by insulin
nM	2,000 $\mu$ U/ml	%		%	
0.057	— +	8.7 0	100	24 0	100
0.114	— +	15.2 0.8	95	37 0	100
0.171	— +	23.2 3.2	86	50 10	80
0.285	— +	39.8 13.5	66	60 33	45
1.14	— +	58.5 55.6	5	—	—
2.85	— +	79.5 77.1	3	87 79	9

Glycogen synthesis was measured over a period of 2 h in hepatocytes from fasted rats incubated with glucose, fructose, and glutamine as described in Fig. 2. Fatty acid synthesis was measured over the same time period in hepatocytes from fed animals incubated in the absence of substrates as described in Fig. 3. When present, insulin and glucagon were added at the indicated concentrations at 0, 30, 60, and 90 min. In the representative experiments shown, absolute rates of glycogen and fatty acid synthesis in the absence of hormones were similar to those found in the equivalent incubations of Figs. 2 and 3.

fatty acid synthesis but was not in itself sufficient to reverse fasting metabolism.

The fact that, although glucose was of poor utility for glycogen synthesis, glycogenesis could be stimulated to near-normal rates in hepatocytes and isolated perfused livers from fasted rats by inclusion of a gluconeogenic precursor such as lactate or fructose confirmed earlier observations from the laboratories of Hems et al. (9), Seglen (10), and Katz et al. (11, 12). Katz and colleagues also made the important observation that addition of an amino acid such as glutamine or alanine had a major stimulatory effect on glycogen synthesis, a fact readily confirmed in the present studies. To this is now added the demonstration that fasting-induced changes in lipid metabolism can be similarly reversed by the proper combination of substrates in vitro. Thus, fatty acid synthesis was activated, fatty acid oxidation and ketogenesis were turned off, and malonyl-CoA levels were altered in the expected directions to accomplish these changes (1).

The fact that the substrates added in these experiments were able to accomplish the conversion of

hepatic metabolism from the catabolic direction characteristic of fasting to an anabolic pattern does not mean that these are the precise mediators of reversal after refeeding in vivo. They are doubtless, however, representative of the amino acids and gluconeogenic precursors able to function in this manner in the intact organism. Further, although we used relatively high concentrations of these substrates in most experiments, qualitatively similar results were obtained at physiological levels.

It is not clear why exogenous glucose at physiological concentrations does not function well as a substrate for glycogen synthesis, unlike other precursors of glucose-6-phosphate in the presence of glucose. This paradox of intermediary metabolism, which has been discussed at length by Katz and Rognstad (27), does not seem to have gained general recognition. It is known that when rat liver is exposed to glucose alone in concentrations up to 20 mM a significant fraction of the hexose is converted into glucose-6-phosphate, but that the latter is immediately hydrolyzed via the glucose-6-phosphatase reaction, with



the result that little glycogen is formed and essentially no net uptake of glucose is seen (27). Similarly, in the absence of external glucose, the bulk of the glucose-6-phosphate formed from gluconeogenic precursors also traverses the glucose-6-phosphatase reaction (27). Yet when the liver is exposed to a mixture of glucose and a gluconeogenic precursor, good rates of glycogen formation are observed although the carbon skeleton appears to derive mainly from the precursor (9–12, 27). It thus appears that glucose somehow signals the cell to direct glucose-6-phosphate formed from gluconeogenic intermediates away from the glucose-6-phosphatase reaction and into the pathway of glycogen synthesis while not being used directly itself (10, 12). The reason why addition of glucose is necessary for maximal rates of glycogen synthesis is unclear. The existence of two pools of glucose-6-phosphate in the cells, one regulatory for glycogen synthesis or glucose release (through control of glucose-6-phosphatase) and the other functioning as substrate, is theoretically possible. At high concentrations (~40 mM), glucose becomes more effective as a substrate for glycogen synthesis (12). It is conceivable that under these circumstances exogenous glucose might enter both pools. We have no direct evidence to support these speculations. Another possibility is that the "glucose effect" might stem from the ability of free glucose to cause inactivation of glycogen phosphorylase with concomitant activation of glycogen synthase, as has been proposed by Hers (28). The role of amino acids is even less clear, although the fact that Katz et al. (11) showed similar effects with  $\text{NH}_4\text{Cl}$  suggests that transaminases might be involved.

Glucose also did not support significant rates of fatty acid synthesis in hepatocytes from fasted rats, and was only mildly stimulatory in cells from fed animals displaying high rates of lipogenesis in the absence of added substrates. Similar observations were made by Brunengraber et al. (29), Clark et al. (24), and Salmon et al. (25) in perfused livers and hepatocytes from fed rats and mice. In two of these reports (24, 25) the most efficient precursor for hepatic fatty acid synthesis was shown to be lactate. We confirmed this observation and found that highest rates of lipogenesis were achieved with a mixture of glucose, lactate (pyruvate), and glutamine. In addition, we observed that with this substrate combination the rate of fatty acid synthesis in hepatocytes from fasted rats accelerated with time and after 2 h of incubation approached that seen in cells from fed animals. Accelerated lipogenesis was accompanied by elevation of the tissue malonyl-CoA level and by almost complete suppression of the ability of the cells to synthesize ketone bodies from added oleate. This is precisely the opposite sequence of events to that induced by glucagon in hepatocytes from fed animals (15, 16), and constitutes further

support for the postulated central role of malonyl-CoA in the coordination of hepatic carbohydrate and lipid metabolism (1).

There have been many reports showing that insulin directly activates glycogen synthase in liver and in some instances this was found to be accompanied by an increased incorporation of label from [ $^{14}\text{C}$ ]glucose into glycogen (6, 19–21). Most of these insulin effects have been small, however, and to our knowledge insulin has never been shown to cause a net accumulation of liver glycogen from glucose or any other substrate. This was certainly the case in the present study and in those described by Golden et al. (30) and Hems (31). Stimulatory effects of insulin on fatty acid synthesis in rat hepatocytes have also been reported (4, 32–34), but again were not of great magnitude. In the present study the enhancement of lipogenesis in hepatocytes from fasted rats was no more than 25%, with a 63% stimulation observed in cells from fed animals incubated with glucose alone. No stimulation by insulin could be demonstrated when lactate and glutamine were present. In contrast, large doses of insulin did antagonize the suppressive effects of submaximal doses of glucagon on net glycogen deposition. Similar antagonism was seen between the two hormones on fatty acid synthesis in cells from fed animals, which confirms the observations of Beynen et al. (33, 34). These findings are entirely in keeping with early reports of Exton, Park, and colleagues (35 and the literature cited therein) on the ability of insulin to offset the glucagon-induced rise in hepatic cyclic AMP levels. Thus, while insulin per se clearly promotes the metabolism of glucose in peripheral tissues, its anabolic action on liver, at least in terms of acute control of carbohydrate and fat metabolism, appears to be exerted mainly through its ability to antagonize the catabolic effect of glucagon. A similar viewpoint was recently expressed by Strickland et al. (36).

Finally, the question arises as to how these *in vitro* observations can be rationalized with the well-established fact that under normal circumstances the feeding of a mixed meal results in the active deposition of glycogen and synthesis of fat in the liver, although neither of these processes operates efficiently in the insulin-deficient animal. The most likely explanation is that dietary glucose is not the major immediate precursor for hepatic glycogen synthesis. Rather, it is probable that glucose must first be converted into a gluconeogenic substrate which, in the presence of the high concentration of free glucose that occurs in portal blood after a carbohydrate meal, can then be directed into the pathway of glycogen synthesis. A prime candidate would be lactate, which is rapidly formed from glucose by the intestine (its concentration in portal blood reached 5 mM when rats were feeding)

and peripheral tissues. When the diet contained sucrose, fructose would provide an additional source of gluconeogenic carbon. Amino acids in portal blood would also be contributory. Support for this notion comes from a recent study by Rémésy et al. (37), who examined arterial, portal, and hepatic venous concentrations of glucose in rats feeding on different diets. On a 40% starch diet no net uptake of glucose by the liver was observed, despite active glycogen deposition. Similarly, Assal et al. (38) found that after an oral glucose load rats with portacaval shunts deposited liver glycogen at much slower rates than did control animals, despite marked hyperinsulinemia. The livers of the shunted animals were almost certainly exposed to much lower concentrations of intestinally produced lactate, a situation that would presumably attenuate the rate of glycogen synthesis.

Under normal feeding conditions, fatty acid synthesis in the liver would also be active, but again the immediate precursor for this process would likely be lactate (or fructose) rather than glucose itself, to judge from the relative efficiencies of substrate utilization demonstrated in the hepatocyte.

The requirement for insulin to ensure active glycogen synthesis and lipogenesis by the liver *in vivo* could be explained on the basis of three known functions of the hormone as it acts indirectly on the processes under study. First, in the presence of glucose, insulin suppresses the secretion of glucagon by the pancreatic alpha cell. Second, insulin antagonizes the glycogenolytic and antilipogenic action of glucagon (and possibly catecholamines) on the liver, at least in part through its ability to lower the elevated cyclic AMP levels caused by these counter-regulatory hormones. Third, by stimulating the uptake of glucose in muscle, insulin presumably initiates operation of the Cori cycle and thereby provides the liver with a portion of the lactate required for glycogen and fat synthesis.

The above conclusions have been drawn almost entirely from studies with the rat. To what extent they can be extrapolated to man is not yet clear. Certainly, the opposing effects of insulin and glucagon on liver metabolism and the importance of this bihormonal control for glucose homeostasis in man is now firmly established (39). Yet recent reports on splanchnic glucose balance in humans leave the distinct impression that the bulk of dietary glucose is directly taken up and stored in the liver and that insulin exerts a positive driving effect on this process (40, 41). An alternative interpretation for these findings would be that man, like the rat, converts absorbed glucose into liver glycogen only after prior metabolism to gluconeogenic precursors, and that insulin promotes glycogen deposition primarily by negating the opposing action of glucagon. One caveat should be expressed. If, as has been suggested (41), the intestine produces an unknown factor

promoting the hepatic uptake of glucose, the above formulation could prove incorrect. The failure to show glucose uptake and its poor utilization as immediate precursor for glycogen or fatty acid synthesis *in vitro* might then be due to failure to provide this factor. We have searched for such a regulatory intermediate (or hormone) in portal blood and thus far have failed to identify one.

Under any circumstance the picture emerging from studies with the rat suggests that a reevaluation of long-standing concepts concerning this area of mammalian intermediary metabolism is in order.

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