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Biochemistry

Stimulation of hepatic glycogen synthesis by amino acids

(rat hepatocytes/gluconeogenesis/glutamine/amino acid metabolism)

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ABSTRACT **Hepatocytes isolated from livers of fasted rats form little glycogen from glucose or lactate at concentrations below 20 mM. Glycogen is formed in substantial quantities at a glucose concentration of 60 mM. In the presence of 10 mM glucose, 20–30% as much glycogen as glucose is formed from fructose, sorbitol, or dihydroxyacetone. The addition of either glutamine, alanine, or asparagine stimulates the formation of glycogen from lactate 10- to 40-fold. The formation of glucose and glycogen is then about equal, and glycogen deposition in hepatocytes is similar to rates attained *in vivo* after fasted rats are refed. The amino acids stimulate 1.5- to 2-fold glycogen synthesis from fructose, and 2- to 4-fold synthesis from dihydroxyacetone. Ammonium chloride is about one-half as effective as amino acids in stimulating glycogen synthesis when glucose with lactate are substrates. It increased glycogen synthesis 25–50% from fructose but inhibited synthesis from dihydroxyacetone plus glucose.**

Isolated rat hepatocytes form glucose from numerous substrates at a rate similar to that *in vivo*, and such cells have served in recent years as a major preparation for the study of gluconeogenesis. However, attempts to obtain hepatocytes capable of efficient glycogen synthesis have not been successful. We set out to develop a hepatocyte preparation capable of synthesis, from common gluconeogenic substrates at moderate concentrations, similar to that obtained *in vivo* (1–2 μmol of glucose equivalents per min/g) within 1 hr after refeeding fasted rats. We report here that certain amino acids stimulate glycogen formation from lactate to this extent. Our findings suggest that certain amino acids or their derivatives may have a major role in the regulation of glycogen synthesis.

METHODS

Male rats of the Sprague-Dawley strain which weighed 180–220 g were fasted for 24 hr. The rats were used for hepatocyte preparation without further treatment, or they were injected intraperitoneally with a solution, 1 ml/100 g of body weight, of either 5% glucose plus 5% fructose, or 1 mg/100 g of body weight of prednisolone succinate. They were anesthesized with nembutal and operated on 30 min after the injection of sugar and 1 hr after that of prednisolone.

Hepatocytes were prepared as previously described (1) except that the bile duct and adjacent vein were not ligated prior to cannulation of the portal vein. The yield from a 200 g rat was 6–8 ml of washed and lightly packed cells. Between 0.04 and 0.06 ml of cells were incubated in 3 ml of Krebs bicarbonate buffer, usually for 1 hr, in an atmosphere of 95% O_2–5% CO_2, at 38°. Incubations were terminated by injecting perchloric acid to a 3% final concentration. Each substrate combination was incubated in duplicate, and results between them nearly always agreed to within 10%.

Enzymatic procedures were used to determine glucose, fructose and lactate (1), urea and ammonia (2), glutamate (3), glutamine (4), asparagine and aspartate (5), and alanine (6) spectrophotometrically. Glycogen was determined according to Lust *et al.* (7). To 0.5 ml of the perchloric acid extract (in small tubes), we added 0.25 ml of sodium hydroxide of sufficient molarity to bring the concentration to about 0.1 M in alkali. The covered tubes were boiled for 10 min (which destroyed the glucose and over 99% of the fructose). To the cooled solution, we added 0.25 ml of 1:1 mixture of 0.3 M acetic acid and 2 M acetate buffer at pH 4.5, containing 1 mg of glucosidase (Sigma Co., St. Louis, Mo.). The tubes were incubated for 2 hr at 45°, and the glucose was determined spectrophotometrically. Results were identical with thoe obtained after alcoholic gycogen precipitation. The procedure is simple and quick and permits a reliable assay of less than 1 μg of glycogen, an amount not quantitatively precipitated.

A set of samples were used as previously described (1) for the determination of protein and initial glycogen content. The glycogen content, as glucose equivalents, and all other determinations are expressed per 100 mg of cell protein. With fasted rats 130–160 mg of this protein represents 1 ml of washed packed cells, but the protein content of liver of fasted rats is 20–23%. Accordingly our results can be converted roughly to a base of 1 g wet weight of liver by multiplying by 2.

RESULTS

Glycogen content of hepatocytes from fasted rats ranged from 0.1–5 μmol/100 mg of protein. We first thought that this low content restricted glycogen deposition because of limited primer sites, and therefore injected one group of rats intraperitoneally with 5% glucose plus 5% fructose. Glycogen content of the liver increased within half an hour to 30–50 μmol/g per liver.

Most of this was lost in the preparation of the cells and the glycogen content of the hepatocytes usually ranged from below 0.1 to 10 μmol of glucose equivalents per 100 mg of protein. Prednisolone treatment (for 1 hr) in the absence of food had little effect on the glycogen content. There was no significant difference in the results between the treated and untreated rats. Our findings obtained with the three groups although reported separately, are essentially the same.

The quantity of glucose and glycogen formed from a number of different substrates is recorded in Table 1. No glycogen was formed at 10 mM glucose and only a little at 20 mM. However, there was considerable glycogen deposition at higher concentrations confirming the observations of Seglen (8). Seglen (8) and Walli *et al.* (9, 10) observed significant glycogen synthesis in perfused liver and hepatocytes from glucose plus fructose and we confirm their findings in hepatocytes. Sorbitol and dihydroxyacetone were similar to fructose. The quantity of glycogen formed was about one-fourth that of glucose formed from these compounds. Xylitol also served as glycogen precursor, but synthesis from this compound was more variable. There was no significant glycogen synthesis from lactate, either as sole substrate (not shown) or in the presence of glucose (Table 1). Apparently glycogen synthesis occurs from compounds entering carbohydrate metabolism pathway at the point of

Table 1. Glucose and glycogen synthesis by hepatocytes*

Incubation addition (mM)	Glucose + fructose injected			Prednisolone injected		
	No. of rats	Δ Glucose	Δ Glycogen	No. of rats	Δ Glucose	Δ Glycogen
Glucose (10)	4	—	−0.9 (−2.6 to +1.0)	4	—	−0.2 (−1.3 to +1.4)
Glucose (20)	9	—	4.1 (2.5–7.8)	7	—	7.8 (3.1–11)
Glucose (60)	—	—		4	—	23 (17–30)
Glucose (10) plus fructose (10)	7	66 (54–85)	15 (11–19)	5	86 (76–90)	20 (16–23)
Glucose (10) plus sorbitol (10)	5	52 (48–61)	16 (13–18)	—	—	—
Glucose (10) plus dihydroxyacetone (20)	7	65 (49–79)	13 (10–17)	3	75 (66–83)	21 (18–25)
Glucose (10) plus xylitol (10)	4	64 (56–78)	6.6 (3.3–8.6)	2	63, 68	18, 21
Glucose (10) plus lactate (20)	7	40 (31–50)	1.1 (−1.4 to +3.8)	5	58 (56–61)	3.1 (1.6–8.3)
Glucose (10) plus alanine (20)	—	—	—	5	19 (14–27)	10 (5.2–13)

Rats were fasted for 20–24 hr and were either injected intraperitoneally with prednisolone or 5% glucose plus 5% fructose (invert sugar) half an hour before the isolation of hepatocytes. Cells, equivalent to 18–27 mg of protein, were incubated in 3 ml of Krebs bicarbonate buffer with the indicated substrate for 1 hr. Results (mean and range) are expressed as μmol of glucose or glucose equivalents (for glycogen) per 100 mg of cell protein per hr. Note that fructose and sorbitol were usually depleted after 1 hr.

* In a group of nine rats, control glycogen values were 3.3 (0.7–9.0) μmol of glucose equivalents per 100 mg of cell protein per hr for the glucose plus fructose injected rats, and 4.2 (0.1–6.4) μmol of glucose equivalents per 100 mg of cell protein per hr for the seven rats injected with prednisolone.

triose-P or above, but not at the point of pyruvate. However, glycerol and glyceraldehyde were inferior to dihydroxyacetone as substrates for glycogen synthesis (not shown). Without the addition of glucose, the formation of glycogen from fructose or dihydroxyacetone was one-half or less than when glucose was initially added (not shown).

Alanine was a less satisfactory precursor of glucose than lactate, but was a better precursor for glycogen with the ratio glycogen/glucose much higher from alanine than from lactate (Table 1). This observation led to a closer study of amino acids. We found that the addition of certain amino acids, most notably glutamine, had a profound effect on glycogen formation. In Table 2 the effects of NH_4Cl and glutamine on carbohydrate synthesis from several substrates are shown. Glutamine stimulated markedly glycogen synthesis from all substrates, but the most dramatic was the effect with lactate plus glucose. The synthesis increased from near zero in the absence of glutamine to some 20–30 μmol of glucose per 100 mg of cell protein per hr in its presence. Glutamine increased glycogen formation about 3-fold from glucose, and glucose plus dihydroxyacetone; glutamine increased glycogen formation nearly 2-fold from fructose plus glucose. Ammonium chloride markedly stimulated glycogen synthesis from glucose plus lactate to a level one-third to one-half that with glutamine but the stimulation

Table 2. Effect of ammonium chloride and glutamine on glycogen synthesis by hepatocytes from fasted rats

Incubation addition (mM)	Supplement	No. of rats	Δ Glucose	Δ Glycogen	Δ Glycogen* (%)
Glucose (10)	—	5	—	1.1 (−0.5 to +2.0)	
	Glutamine		—	6.8 (3.2–10)	460 (385–544)
Glucose (20)	—	3	—	6.7 (3.5–11)	
	NH_4Cl	3	—	2.7 (−0.8 to +7.2)	39 (23–67)
	Glutamine	3	—	19 (12–23)	310 (213–337)
Glucose (10)	—	6	79 (71–85)	14 (9.5–20)	
+ Dihydroxyacetone (10)	NH_4Cl	4	75 (68–80)	8.2 (6.5–11)	49 (46–54)
	Glutamine	6	58 (51–68)	39 (24–50)	295 (200–526)
Glucose (10)	—	10	82 (62–95)	14 (8.2–22)	
+ Fructose (10)	NH_4Cl	6	68 (56–84)	19 (12–30)	139 (125–157)
	Glutamine	10	61 (53–70)	23 (13–33)	170 (140–239)
Glucose (10)	—	12	47 (33–56)	0.4 (−10 to +6.3)	—
+ Lactate (18)	NH_4Cl	10	52 (36–62)	8.0 (6.4–12)	2000 (203–633)
+ Pyruvate (2)	Glutamine	12	35 (24–40)	24 (18–30)	3000 (516–2090)

Hepatocytes equivalent to 17–29 mg of protein were incubated in 3 ml of Krebs bicarbonate buffer for 1 hr with added substrates and either 10 mM NH_4Cl or 10 mM glutamine. Mean and (range) are reported as μmol of glucose or glucose equivalents (for glycogen) per 100 mg of cell protein per hr.

* Percent increase shown only when there was net synthesis in the controls.

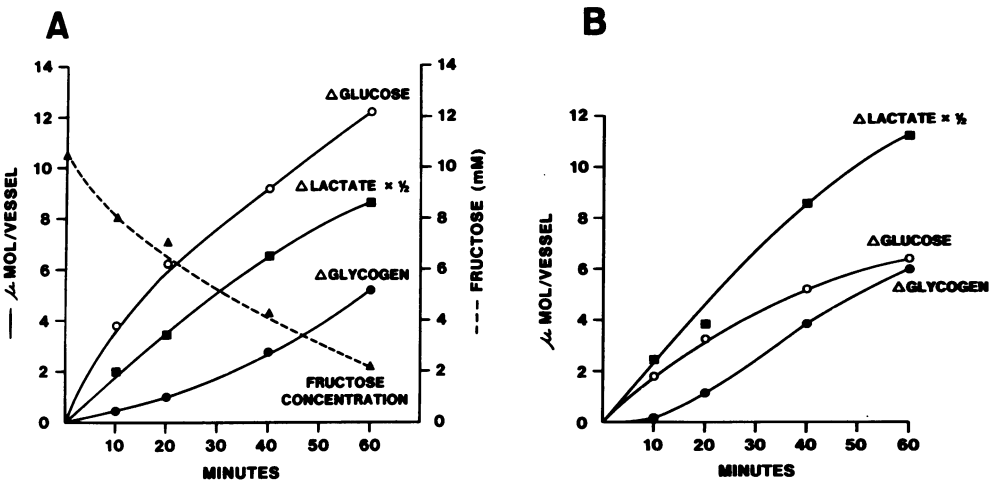

FIG. 1. Hepatocytes from a fasted rat equivalent to 25 mg of cell protein were incubated in a 4 ml volume with either (A) glucose, fructose, and glutamine, 10 mM each; or (B) glucose 10 mM, lactate 15 mM, and 10 mM glutamine. Results shown as μmol per flask. In A, lactate formation, and in B, lactate uptake is shown.

from fructose plus glucose was by only 25–50%. NH_4Cl however inhibited glycogen formation from glucose and dihydroxyacetone.

The kinetics of glycogen and glucose synthesis from lactate and fructose in the presence of glucose and glutamine are shown in Fig. 1. Glucose synthesis began at once but there is a lag period for glycogen synthesis which is especially pronounced with lactate. This was not eliminated by preincubation in 10 mM glucose. The lag does not appear to be due to the need to form sufficient primer. We found no correlation between glycogen synthesis and initial glycogen content in the range from 0.1 to 20 μmol/100 mg of protein.

In the period between 20 and 40 min, the rate of glycogen synthesis from lactate (Fig. 1B) was over 1.5 μmol/g of liver per min, higher than the rate of glucose synthesis. Glycogen synthesis continued at high rates even when lactate concentration decreased to 3–4 mM (not shown). With fructose, the synthesis of glucose always exceeded that of glycogen. There was also a considerable lactate accumulation (Fig. 1A) which was subsequently utilized (not shown).

In Table 3, the ability of several amino acids to stimulate glycogen synthesis from glucose plus lactate is compared. Glutamine, asparagine and alanine were, essentially, equally effective. Serine, proline, arginine, and lysine had a smaller effect, and leucine, glutamate, and aspartate did not stimulate glycogen synthesis. Very little ammonia or urea was formed from the dicarboxylic acids. They apparently do not penetrate into the cells whereas the cell is permeable to the other amino acids and to the amides.

The amino acids that stimulate glycogen formation are also precursors for carbohydrates and possibly, at least in part, the stimulation may indicate their roles as additional substrates. We therefore examined in more detail the metabolism and interconversion of alanine, glutamine, and asparagine. The amides were incubated either as sole substrate, and together with glucose and with glucose plus lactate (Table 4). There was very

Table 3. Effect of amino acids on glycogen synthesis from lactate by hepatocytes

Incubation additions	Injection: None		Glucose + fructose		Prednisolone		Prednisolone	
	Δ Glucose	Δ Glycogen	Δ Glucose	Δ Glycogen	Δ Glucose	Δ Glycogen	Δ Glucose	Δ Glycogen
None	38	5.8	51	−9.8	40	3.0	48	1.2
NH_4Cl	34	15	62	−6.4	41	6.8	49	4.6
Glutamine	27	21	33	24	34	22	40	23
Asparagine	25	22	—	—	—	—	45	19
Alanine	21	24	48	13	42	18	49	21
Serine		—	48	6.3	30	14	—	—
Proline		—	44	8.0	32	14	—	—
Leucine		—	—	−7.5	38	2.2	50	0.7
Glutamate		—	—	—			50	3.5
Aspartate		—	—	—			52	1.2
Arginine		—	—	—			47	9.5
Lysine		—	—	—			53	8.5

Rats were fasted for 24 hr and were either untreated or injected with 5% glucose + 5% fructose, or prednisolone for 1 hr before operation. Hepatocytes equivalent to 17 to 26 mg of protein, were incubated for 1 hr in 3 ml of Krebs bicarbonate buffer containing 10 mM glucose, and 20 mM lactate. The concentration of NH_4Cl and amino acids was 10 mM. Results are expressed as μmol of glucose or glucose equivalents (for glycogen) per 100 mg of cell protein per hr. Each of the four experiments represents results with a single rat. Initial glycogen levels were 0.55 (none), 27 (glucose + fructose), 0.23 (prednisolone), and 0.35 (prednisolone).

Table 4. Glycogen synthesis and amino acid metabolism by rat hepatocytes

Substrates (mM)	Δ Glucose* (a)	Δ Glycogen* (b)	Δ Carbohydrate* (a) + (b)	Δ Lactate* (c)	Δ NH_3† (d)	Δ Urea† (e)	Δ Glutamine* (f)	Δ Glutamate* (g)	Δ Asparagine* (h)	Δ Alanine* (i)	PEP and pyruvate from amino acids*‡ (I)	(II)
Glucose (10)	3.4	0.3	3.7	5.1								
Glucose (20)	−14	4.9	−9.1	16								
Lactate (20)	37	0	37	−82								
Alanine (10)	23	0.6	23	11	2	95	3.3	6.7		−102	97	92
Glutamine (10)	22	4.5	27	4.8	93	86	−114	37		N.D.	71	—
Asparagine (10)	16	−0.1	16	10	49	90	2.5	1.5	−71	N.D.	68	—
Glucose (10) plus:												
NH_4Cl (10)	0	0.2	0	2.5	−69	68	2.0			N.D.		
Alanine (10)	13	6.2	19	14	15§	90	5.1	5.1		−99	92	89
Glutamine (10)	13	13	26	8.1	101	78	119	35		N.D.	72	—
Asparagine (10)	5.2	2.8	8.0	13	37	99	3.0	3.6	−71	N.D.	61	—
Glucose (10) plus lactate (20) plus:	31	3.3	34	−82	—	—	—	—				
NH_4Cl (10)	43	12	55	−178	−117	88	6.3	5.2		11		
Alanine (10)	34	20	54	−92	1.2	54	5.1	15		−75	55	50
Glutamine (10)	24	22	46	−105	63	41	−108	33		68	35	7
Asparagine (10)	39	21	60	−135	36	61	6.6	12	−73§	22	31	32

Hepatocytes from a fasted rat equivalent to 19 mg of cell protein were incubated in 3 ml of Krebs bicarbonate buffer with the different substrate combinations. Initial glycogen content was 0.1 μmol of glucose equivalents/per 100 mg of cell protein. Essentially similar results were obtained with four fasted, two untreated, and two injected rats with glucose + fructose (see *Materials and Methods*). There was no aspartate formed with glutamine and alanine; between 3 and 10 μmol formed in the presence of asparagine. The values of aspartate are not presented, but were used in the calculations.

* μmol/100 mg of cell protein.

† μatoms of N/100 mg of cell protein.

‡ The maximal contribution of the amino acids to phosphoenolpyruvate (PEP) and/or pyruvate was calculated by two methods. In method (I), the contribution was calculated from the nitrogen in urea and ammonia. For the amides, this was corrected for the nitrogen retained in the corresponding amino acid. In method (II), it was calculated from the decrease in substrate less the sum of the products in columns (f) to (i). The calculation according to method (I) is for alanine (d) + (e); for glutamine 0.5 (d) + (e) − (g); for asparagine 0.5 × (d) + (e) − aspartate.

§ Ten micromoles of aspartate was also formed.

little ammonia accumulation from alanine, with the liberated nitrogen nearly all accunted for as urea, whereas both urea and ammonia were formed from the amides. There is considerable formation of glutamate from glutamine, as well as formation of glutamine and glutamate from the other amino compounds. Very little aspartate was formed even with asparagine as substrate. Glutamine and alanine as sole substrates yield glucose at about half the rate as lactate. Glycogen in the absence of glucose however is formed only from glutamine (Table 4). When added with glucose the same pattern persists and glycogen synthesis, while low, is much higher from glutamine than from the other amino acids.

The effect of ammonium chloride on the metabolism of lactate is striking. When added to lactate with glucose, it doubles lactate uptake, increases the formation of glucose, and greatly stimulates glycogen deposition (Table 4). The formation of labeled alanine, glutamic acid, and of glutamine was demonstrated by paper chromatography with cells incubated with [^{14}C]lactate. In contrast, when ammonia was added with glucose as the only substrate, there was very little amino acid synthesis, and nearly all the ammonia uptake could be accounted as urea (Table 4). The lack of formation of amino acids may account for the failure of ammonia to stimulate glycogen synthesis from glucose.

Maximal glycogen synthesis requires the presence of three components—glucose, lactate, (or other precursors as fructose or dihydroxyacetone), and amino acids. Alanine, glutamine, and asparagine, with glucose + lactate, are equally effective for glycogen formation, but glucose synthesis is considerably higher with asparagine (Table 4). The reason for this is not clear.

From the yield of urea and ammonia and the amino acid uptake and production, the contribution of the amino acid carbon to the synthesis of carbohydrate plus pyruvate oxidation can be calculated. In four experiments similar to those of Table 4, glutamine contribution to carbon metabolized beyond the 3 carbon stage was small as compared to lactate. By one method of calculation the maximal contribution of glutamine carbon ranged from 12 to 25% (method I of Table 4), and by another (method II) from near zero to 10%. The contribution of asparagine was similar to that of glutamine. The contribution by alanine was higher, as much as one-half from lactate. The synthesis of urea (three ATP per nitrogen) requires nearly as much energy as carbohydrate synthesis. With glutamine and asparagine, much of the ATP can be derived from the oxidation of the five and four carbon dicarboxylic acids to the level of oxalacetate, but with alanine the extra energy must derive from the oxidation of pyruvate and acetyl-CoA.

DISCUSSION

It is generally held that liver of glycogen depleted animals removes glucose from the circulation at concentrations above a

threshold of some 7–12 mM, and that the uptake is accounted mainly as glycogen (see ref. 11). However, attempts to demonstrate *in vitro* deposition of appreciable glycogen under similar conditions have been largely unsuccessful.* In perfused liver, substantial net synthesis of glycogen was obtained either at very high concentrations of glucose, or with combinations of glucose and other substrates, when there was active gluconeogenesis and an increase in medium glucose. For example, Glinsman *et al.* (13) used 35 mM glucose; Ruderman and Herrera (14) 20 mM glucose plus 10 mM alanine; Hems and coworkers (15, 16) 20 mM glucose plus a mixture of amino acids and lactate; and Walli and Schimassek (10), glucose plus fructose. In isolated hepatocytes, moderate synthesis occurred at glucose concentrations of 30–40 mM (8) or when the sodium in the medium was replaced by potassium (17). With normal sodium media (17), synthesis was limited even from 40 mM glucose. Better synthesis was obtained from glucose plus fructose (8, 10). Walli and Schimassek (10) conclude "... hepatocytes possess glycogen metabolizing enzymes at activities comparable in perfused liver ... yet even when the concentration of substrates is high the rate of synthesis is less."

We confirm the findings on glycogen synthesis from high glucose concentrations and from glucose plus fructose. Sorbitol and dihydroxyacetone act much as fructose (Table 1), but lactate or pyruvate, the major physiological glycogenic precursors, formed hardly any glycogen. However, we show that in the presence of several amino acids glycogen synthesis was dramatically increased and became equal to that of glucose. Synthesis of 1.5 μmol of glucose equivalents per g/min, similar to maximal rates *in vivo*, was obtained. Apparently, glutamine is the most effective compound, and other amino acids may serve by providing intracellular glutamate and glutamine. Ammonium chloride also markedly stimulates glycogen synthesis from lactate but not from glucose. There is extensive synthesis of amino acids from ammonia and lactate but not with glucose (Table 4). The effect of ammonia indicates that the action of the amino acids is not that of an added carbon substrate.

The mechanisms of the stimulation are yet obscure. The active compound could be glutamine or glutamate, or yet an unknown, most likely nitrogenous compound derived from glutamine(ate).

Two types of mechanism for stimulation appear possible: (*i*) the stimulant may act on one or several of the kinases or phosphatases which constitute part of the cascade of the phosphorylase and synthase system, as to increase the fraction of the synthase in the active dephospho form. (*ii*) The stimulant may be an allosteric ligand, depressing phosphorylase and/or increasing the activity of one or both forms of the synthase. It is generally held that the rate of glycogen synthesis is determined by the fraction of the synthase in the a (active) form. A perfect correlation between the fraction in the a form and glycogen synthesis *in vivo* and *in vitro* has been reported (17, 18). However Hue *et al.* (17) show the maximal activity of the enzyme as 1.5 μmol/min per g of protein while the corresponding rate of glycogen synthesis was 3 μmol/min. Moreover the maximal enzyme rate, as assayed in the extract, is likely to exceed considerably that occurring in the cell.

Some of the contradictions between *in vivo* and *in vitro* studies are resolved if we consider that there are two conditions for glycogen synthesis, which differ in their regulatory mechanism. First, glucose uptake and extensive glycogen deposition proceeds at very high glucose concentrations, as it occurs in portal blood after the ingestion of carbohydrate. Second, glycogen synthesis proceeds simultaneously with that of glucose from precursors such as fructose, glycerol, lactate, or amino acids of dietary or endogenous origin. The distribution of glycogenic flux between glucose and glycogen would depend, among other factors, on the presence of glutamine(ate) or their products.

We have established with hepatocytes of fasted rats that in the absence of amino acids the sole carbohydrate formed from lactate is glucose, but in the presence of amino acids similar amounts of glycogen and glucose are formed. Whether this is also true *in vivo* is unknown, and future studies are required to determine the physiological significance of our findings.

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* Many investigators have used the incorporation of ^{14}C from glucose or other substrates as a measure of hepatic glycogenesis. (For example with hepatocytes, see ref. 12.) ^{14}C incorporation occurs, however, in the absence of net glycogen synthesis and during glycogen breakdown (1), so it is not clear how ^{14}C yields correlate with synthesis. Incorporation of tracer could represent an isotopic exchange catalyzed by phosphorylase between hexose 6-phosphates and glycogen or futile cycling (1).