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Studies on Gluconeogenesis and Protein Synthesis In Isolated Hepatocytes (38832)

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It is well known that the hormonal and nutritional status of the organism can bring about over-all changes in hepatic carbohydrate and protein synthesis. Such studies have been previously reported using intact animals, liver slices, or perfused liver. Isolated hepatocytes which retain most of their *in vivo* metabolic activity afford an extremely valuable tissue preparation for the study of complex intracellular carbohydrate and protein biosynthetic processes. There have been several procedures published (1–4) for the isolation of rat liver cells for biochemical studies. By modifying these procedures (5) we have been able to isolate large quantities of rat hepatocytes retaining their *in vivo* metabolic characteristics (6–8). In the present studies, we report studies on gluconeogenesis, oxygen consumption, and the incorporation of various amino acids into protein under different experimental conditions using isolated hepatocytes.

Materials and Methods. Chemicals. All organic chemicals were of reagent quality. Biochemical reagents were purchased from Sigma Chemical Company, St. Louis, MO. Radioactive isotopes were purchased from INC, Irvine, CA.

Animals. Fed (180–200 g) or 18 to 24-hr fasted (180–200 g) male Cox rats were used for all studies reported here. All experimental animals were maintained on water and Purina Laboratory Chow fed ad lib., unless otherwise noted. Two methods of feeding were used as it was previously observed (9) that liver glycogen was significantly increased in animals that had easy access to food when it was placed in a dish on the floor as compared to those rats that had to obtain food from suspended wire baskets.

Isolation of hepatocytes. Normal fed and fasted rats were anesthetized with Na-pentobarbital and the liver was rapidly removed and placed in a Miller Perfusion apparatus, perfused for 15 min with 100 ml of Hanks calcium-free buffer containing 1.5% albumin (Sigma Fraction V) and 10 mg each of streptomycin and penicillin G. After this initial perfusion, collagenase (20 mg, Sigma Type 1, 130 units/mg) was added and perfusion was continued for 10–15 min. The liver was removed, finely minced, and bubbled gently with 95% O_2 and 5% CO_2 for 1 min. Cells were isolated as described previously (5, 10) and were brought to a final volume of 30–40 ml. Isolated hepatocytes were used immediately for metabolic studies reported here.

Incubation of hepatocytes. A 1-ml aliquot of the final cell suspension (55–75 mg) was incubated in 3 ml of Umbreit Ringer bicarbonate buffer (25 m*M*) in stoppered 1-oz plastic vials (Nalgene 2002) with various substrates at 37°C and at 90 oscillations/min. The vials were gassed with 95% O_2 and 5% CO_2 for 5 min at time zero and after each 2 hr of incubation. At the end of the incubation period the reaction was stopped by the addition of trichloracetic acid (TCA) for the determination of the incorporation of labeled amino acids into protein (11). Glycogen was determined by the method of Good *et al.* (12). Protein was estimated by the method of Lowry *et al.* (13) after the incubation mixture was sonicated. Medium glucose was assayed by the glucose oxidase method (14). Oxygen consumption of isolated hepatocytes was determined with a Gilson Respirometer using Krebs–Ringer phosphate buffer as media, air as the gas phase, and 0.1 *N* KOH as the trapping agent.

Results and Discussion. Studies on gluconeogenesis. Data showing the effects of increasing concentrations of fructose, lactate, pyruvate, glycerol, galactose, alanine, and succinate on gluconeogenesis in isolated

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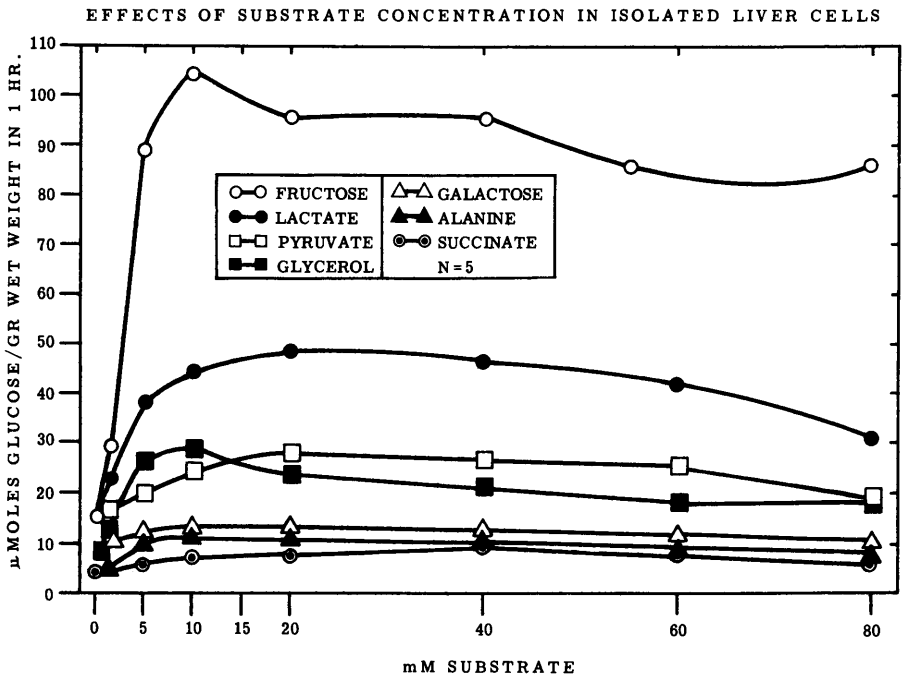

FIG. 1. Effect of substrate concentration on glucose production in isolated hepatocytes. Approximately 70 mg of isolated hepatocytes from fasted (24 hr) rats were incubated with various concentrations of fructose, lactate, pyruvate, glycerol, galactose, alanine, or succinate in 3 ml of Umbreit-Ringer medium for 1 hr. Each point is the mean of five observations. Standard error of the mean at each point is within 10% of the mean.

hepatocytes are presented in Fig. 1. Increased gluconeogenesis is observed in hepatocytes incubated for 1 hr with increasing substrate concentrations from 0 to 10 m*M* with all substrates studied. Increasing the various substrate concentrations above 10 m*M* did not further stimulate gluconeogenesis. We have not been able to repeat our earlier observation (5) that increasing concentrations of lactate above 20 m*M* further stimulates gluconeogenesis above 50 μmoles/g/hr in isolated hepatocytes. This discrepancy was due to glucose contamination in the lactate used in our earlier experiments. In the present study all substrates were checked for glucose contamination at all substrate concentrations used. No significant amount of glucose or interference of the assay by other contaminants was observed. Fructose was the best gluconeogenic substrate tested causing an increase in net glucose production of 100 μmoles/g/hr. Pyruvate and glycerol stimulated net glucose production to 30 μmoles/g/hr while galactose and alanine produced a net increase of 12 μmoles/g/hr. Succinate caused an increase of 5 μmoles/g/hr.

In these and our previous studies (6) with isolated hepatocytes we have observed that the ability of fructose to stimulate net glucose synthesis (50 μmoles/g/hr in fed, 100 in 18- to 24-hr fasted, and 160 in cells from diabetic rats) has always been about twice that observed with lactate (10 μmoles/g/hr in fed, 50 in 18- to 24-hr fasted, and 90 in cells from diabetic rats). These data suggest that the rate of gluconeogenesis can be controlled by the carbon flow through pyruvate carboxylase (PC) and phosphoenol-pyruvate-carboxykinase (PEPCK). Greater increases (6) in gluconeogenesis were observed in cells from diabetic rats with substrates which enter through PC and PEPCK (e.g., alanine 14- to 34-fold, lactate 7-fold, and pyruvate 6-fold) than with fructose (2- to 3-fold) which enters through triose phosphate. These increases in gluconeogenic rate correlate well with increases previously (15, 16) observed in the gluconeogenic enzymes (PC and PEPCK 3- to 4-fold and fructose-1,6-diphosphatase and glucose-6-phosphatase, 1- to 2-fold) in rat livers under similar conditions. A site of control at these enzymes

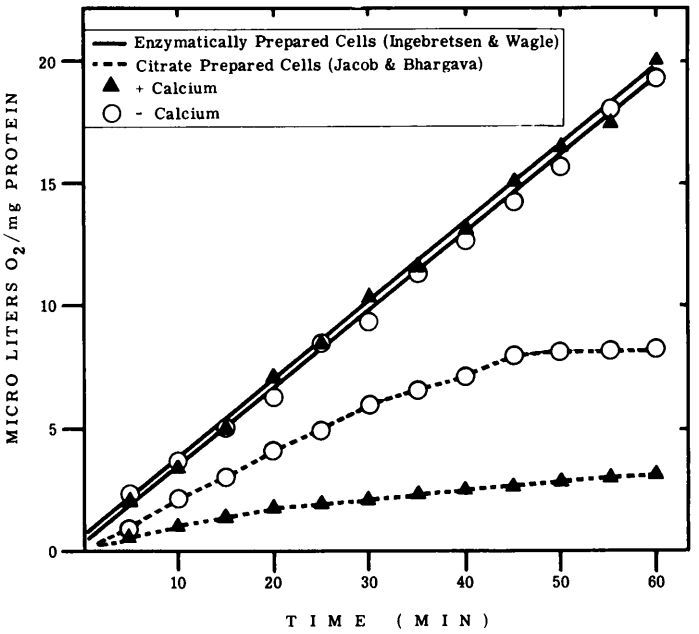

FIG. 2. Effect of calcium on endogenous respiration of isolated hepatocytes. Respiration was studied by using Gilson respirometer in the presence of, and absence of 1 m*M* calcium. Approximately 70 mg of cells were used.

seems reasonable since carbon from both amino acids and other noncarbohydrate sources enter through PC and PEPCK thus allowing better control over substrate flow through the citric acid cycle for gluconeogenesis.

Studies on oxygen consumption. The results of oxygen consumption studies under various conditions are presented in Fig. 2. Unlike cells isolated by the Jacob and Bhargava method (17), the cells isolated by the present method showed linear rates of endogenous respiration for 60 min. Calcium had no effect on enzymatically prepared cells but greatly inhibited respiration in citrate-prepared cells. Liver cells isolated by mechanical or citrate methods (18, 19) have been shown to exhibit lower respiration rates than do liver slices. It has been previously pointed out that the leakage of calcium from the incubation medium into the cell interferes with mitochondrial respiration (20). These studies further indicate that cells isolated by enzymatic methods are metabolically active and have intact cell membranes.

Studies on net protein synthesis. Results of the studies on net protein synthesis by isolated rat hepatocytes are summarized in Table I. Total protein content of the isolated cells was slightly lower than that observed in whole liver. This difference is probably due to the removal of connective and vascular tissue. Glycogen content was also lower in isolated cells. This was due to glycogenolysis occurring during the surgical and perfusion period. Glycogen levels were maintained for 2–6 hr when the cells were incubated with glucose, lactate, an amino acid mixture, and insulin. There was a decrease in total protein content when the cells were incubated for 4 hr without any substrate. This decrease was probably due to proteolysis. When the cells were incubated with an amino acid mixture, glucose, lactate, and insulin, a small net increase in protein content was observed. The difference in protein content between these two conditions represents a rate of net protein synthesis of approximately 3 mg/g/hr. Recently (21, 22) it has been calculated that the *in vivo* rate of protein synthesis in liver is 7–8 mg/g/hr. The observed rate of net protein synthesis by isolated hepatocytes in the present study is approximately 30–40% of this calculated *in vivo* rate.

TABLE I. EFFECT OF AMINO ACIDS, GLUCOSE, LACTATE, AND INSULIN ON NET PROTEIN SYNTHESIS BY ISOLATED RAT HEPATOCYTES.

Tissue	Protein content (mg/100 mg wet wt.)	Glycogen content (μmoles/g)	Net protein change	Net protein synthesized (mg/g/hr)
Whole liver	16.5 ± 1.1	320 ± 41	—	—
Isolated hepatocytes (unincubated)	13.6 ± 0.4	195 ± 23	—	—
Isolated hepatocytes incubated (4 hr) with no substrates	12.8 ± 0.4	20 ± 1.8	—	—
Isolated hepatocytes incubated[a] (4 hr) with amino acid mixture, glucose, lactate, and insulin	14.0 ± 0.3	80 ± 14	1.2	3.0

[a] Approximately 100 mg of isolated liver cells were incubated in 3 ml of Umbreit-Ringer bicarbonate buffer containing 5.5 m*M* glucose, 5 m*M* lactate, and 5.0 m*M* of each of the following amino acids: isoleucine, leucine, valine, phenylalanine, tryptophane, tyrosine, glycine, serine, methionine, arginine, lysine, threonine, histidine, cystine, proline, alanine, asparatic, glutamic acid, and 100 mU of insulin.

TABLE II. INCORPORATION OF ^{14}C-LEUCINE INTO ISOLATED HEPATOCYTES[a] CONTAINING HIGH AND LOW GLYCOGEN.

Period of incubation (hr)	^{14}C-Leucine incorporated into protein (dpm/mg)	
	Low glycogen[b]	High glycogen[c]
1	610 ± 75	630 ± 72
2	1280 ± 110	1350 ± 150
3	1450 ± 160	1880 ± 160
4	1560 ± 195	2680 ± 220
6	1680 ± 200	3650 ± 310
8	2020 ± 250	4870 ± 440
10	2210 ± 280	5600 ± 800

[a] Approximately 55–75 mg of isolated cells were incubated in 3 ml of Umbreit-Ringer bicarbonate buffer containing 5.5 m*M* glucose, 5 m*M* lactate, 100 mU of insulin, and 5 mmoles of each of the amino acids as in Table I, containing 0.5 μCi of ^{14}C-leucine.

[b] Cells prepared from fed rats that received food from suspended wire cage. Initial liver glycogen was in the range of 130 ± 25 μmoles glucose/g. Isolated cells contained 65 ± 15 μmoles glucose/g.

[c] Cells were prepared from well-fed rats (these rats received food in a dish that was placed on the floor of the cage) which had initial glycogen levels in the range of 320 ± 40 μmoles glucose/g liver before perfusion. Isolated cells had glycogen in the range of 220 ± 35 μmoles glucose/g.

Studies on amino acid incorporation into protein by isolated hepatocytes containing high and low glycogen. Incorporation of ^{14}C-leucine into protein is summarized in Table II. It can be seen that ^{14}C-leucine incorporation was linear for 8–10 hr in the presence of glucose, lactate, and amino acids mixture in cell preparations that contained high amounts of glycogen. In cell preparations that contained low glycogen only a small increase in the incorporation of ^{14}C-leucine was observed after 3 hr. It was also observed that more than 50% of the radioactivity incorporated into protein was present in the supernatant fraction. This suggests that newly synthesized protein was being rapidly released into the medium. These results are in agreement with observations obtained using perfused rat liver (23). The incorporation of amino acids into protein presumably represents active protein synthesis by isolated hepatocytes and probably is not due to bacterial growth since both penicillin and streptomycin (750 μg/3 ml) were added to the incubation medium. If antibiotics were not added, large numbers of bacteria were noted at the end of 6 hr of incubation. No bacterial growth was observed when incubation was carried out with antibiotics.

The results presented here demonstrate that isolated hepatocytes linearly incorporate amino acids into protein for at least 8–10 hr. Similar results have been obtained with isolated perfused liver (23) but not with liver cells isolated by other methods. Liver cells isolated by mechanical (24) or citrate methods (24) incorporate amino acids linearly into protein for 30 min. Friedmann and Epstein (25) have shown that tetra-

phenylboron-prepared cells actively incorporate labeled leucine into protein only in the presence of added cofactors of pH 5 enzyme fraction. Liver cells prepared by other enzymatic methods (26, 27) have been shown to incorporate amino acids into protein for 2–3 hr. It is difficult to compare rates of incorporation of amino acids into protein from one system to another because of differences in experimental conditions, specific activity of the labeled amino acid used, and amino acid pool size.

In our previous studies (28) we have observed that in isolated hepatocytes with high content of glycogen, gluconeogenesis is stimulated by glucagon at the concentration of 10^{-12}–10^{-10} *M* and a small change in cyclic AMP levels was observed under these conditions. However, in cells containing low or no glycogen 10^{-10}–10^{-8} *M* concentrations of glucagon was needed to stimulate gluconeogenesis and under these conditions a 10- to 20-fold increase in cyclic AMP levels was observed (28). These changes in cyclic AMP levels may be controlled by intracellular glycogen levels and high glycogen content helps to maintain *in vivo* metabolic characteristics of isolated cells. The ability of isolated hepatocytes to retain *in vivo* metabolic characteristics and to incorporate labeled amino acids into protein for prolonged periods suggest that these cells may prove to be useful in studying factors involved in the regulation of protein synthesis (29) and enzyme induction.

Summary. Glucose production was studied in isolated hepatocytes using various substrates and with increasing substrate concentrations (0–10 m*M*). Fructose was the best gluconeogenic substrate while other substrates studied stimulated net glucose production in the following decreasing order: lactate, pyruvate, glycerol, galactose, alanine, and succinate. Studies on oxygen consumption showed that endogenous respiration was linear for 60 min and was not altered by extracellular calcium. Studies on the incorporation of ^{14}C-leucine into protein was linear for only 3–4 hr in cells containing low glycogen. However, cells containing high glycogen incorporated ^{14}C-leucine into protein linearly for 8–10 hr. About 3 mg of protein per g per hr was synthesized by isolated cells when incubated for 4 hr with amino acids mixture, glucose, lactate, and insulin.

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