

Hormonal Regulations of Glucose-6-Phosphate Dehydrogenase and Lipogenesis in Primary Cultures of Rat Hepatocytes¹

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In primary cultured monolayer hepatocytes of adult rats, insulin (1×10^{-8} M) induced glucose-6-phosphate dehydrogenase [EC 1.1.1.49, G6PDH] several fold in 48 h. It also induced lipogenesis, measured as [14 C]acetate incorporation in 2 h, in these cells. Of the various lipids, triglycerides and phospholipids were induced markedly, while cholesterol and its esters were not induced. The increase of G6PDH and lipogenesis were parallel. Glucagon, dibutyryl cyclic AMP, triiodothyronine, dexamethasone, epinephrine, isoproterenol, and dibutyryl cyclic GMP were also tested under similar conditions, but none of them caused significant induction of G6PDH or lipogenesis.

Use of anti-G6PDH serum showed that induction of G6PDH by insulin was due to increase in the amount of enzyme protein. Insulin was found to increase the rate of synthesis of G6PDH about 3-fold. SDS-polyacrylamide gel electrophoresis of the immunoprecipitable protein revealed that besides G6PDH another radioactive fraction (Mr 37,000) was increased by insulin. This suggests that complete synthesis of G6PDH protein is slowed down in primary cultured hepatocytes and that an apparent nascent peptide of the enzyme accumulates.

Although on long-term treatment (48 h), glucagon and dibutyryl cyclic AMP had no effect on lipogenesis, when added with [14 C]acetate for 2 h they strongly inhibited lipogenesis. Significant inhibition of lipogenesis by short-term treatment with glucagon was seen even in cells with a high capacity for lipogenesis induced by long-term treatment with insulin. Insulin again stimulated lipogenesis in short-term treatment, but its effect was slight.

It is concluded from these results that insulin exerts long-term stimulation of lipogenesis by inducing enzymes related to lipogenesis including G6PDH as well as causing slight stimulation by enhancing supply of substrate for lipogenesis. Glucagon seems to play a minor role in long-term control, but it causes short-term inhibition of lipogenesis.

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Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; Bt₂cAMP, dibutyryl cyclic AMP; Bt₂cGMP, dibutyryl cyclic GMP.

The biosynthesis of long chain fatty acids is regulated by not only the nutritional state, but also the hormonal state (1-3). Long-term regulation of fatty acid synthesis involves changes in the amounts of lipogenic enzymes (3-6), while short-term control of lipogenesis involves changes in the supply of substrates (7-10). Glucose-6-phosphate dehydrogenase [EC 1.1.1.49, G6PDH¹] is a key enzyme in lipogenesis and its activity is reduced during starvation and increased by feeding a high carbohydrate, fat-free diet (11-13). Hormonal factors have been implicated as regulators of this nutritional induction of G6PDH (11, 14-18) and insulin is thought to be the main inducer under physiological conditions (14, 15, 19). However, it is still uncertain whether this hormone is the only regulator of enzyme activity (20-22). The exact relation between changes of G6PDH and lipogenic activities and the long-term and short-term hormonal controls of lipogenesis are also unknown.

The complexity of conditions *in vivo* makes it difficult to obtain clear cut results on the mechanisms of enzyme induction and metabolic regulation caused by the nutritional state and hormones. However, we recently showed that primary cultures of adult rat hepatocytes were a suitable *in vitro* system for studies on hormonal and nutritional regulation of enzymes and metabolic activities in the liver (23-35). Using these cells, we showed that insulin induced G6PDH and malic enzyme (35). Recently Winberry *et al.* also reported induction of G6PDH by insulin in cultured liver cells (36). But neither study provided information on the relation between induction of G6PDH and the regulation of lipogenesis. Therefore, in this work using primary cultured hepatocytes of adult rats, we examined the hormonal regulation of G6PDH and its relation with short- and long-term control of lipogenesis.

EXPERIMENTAL PROCEDURE

Materials—The materials used for cell isolation and culture were as reported previously (23). Insulin, glucagon, bt_2cAMP and bt_2cGMP were obtained from Sigma Chemical Co. (St. Louis); dexamethasone was from Schering AG (Berlin); NADP and glucose-6-phosphate were from Oriental Yeast Co. (Osaka); actinomycin D was from

Schwarzmann (New York); puromycin was from Maker Chemicals Ltd. (Jerusalem); [$1-^{14}C$]acetate (57.6 mCi/mmol) and L-[4,5- 3H]leucine (55 Ci/mmol) were from the Radiochemical Centre (Amersham) and markers for determination of molecular weight were from Pharmacia (Uppsala). Other chemicals were from Wako Pure Chemicals Co. (Osaka).

Cell Isolation and Monolayer Culture—Parenchymal hepatocytes were isolated from adult male Wistar strain rats weighing 150 to 200 g which had been given laboratory chow *ad libitum*, by *in situ* perfusion of the liver with collagenase (23). The cells were suspended at 5×10^5 cells/ml in Williams medium E containing 5% calf serum and 10^{-6} M dexamethasone, and cultured at a density of 1×10^5 cells/cm² in Falcon plastic dishes in a humidified chamber at 37°C under 5% CO₂ and 30% O₂ in air. The medium was changed every day.

Assay of G6PDH Activity—The cells were harvested with a rubber policeman and homogenized in 1 ml of 20 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM dithiothreitol and 5 mM EDTA in a Polytron homogenizer for 1.5 min. The homogenate was centrifuged at $30,000 \times g$ for 30 min and the resulting supernatant was used as the enzyme preparation for assay of G6PDH. G6PDH activity was measured by the method of Glock and McLean (37). One unit of G6PDH was defined as the amount forming 1 μ mol of NADPH/min at 24°C. Protein was measured by the method of Lowry *et al.* (38). Enzyme activity was expressed in munit/mg protein.

Assay of Fatty Acid Synthesis—Monolayers of hepatocytes in 35 mm dishes were incubated with 1 ml of culture medium containing 5 mM sodium [$1-^{14}C$]acetate (1 μ Ci/ml) for 2 h. The cells were washed twice with phosphate-buffered saline solution and then solubilized in 1 ml of 0.5 N KOH at 37°C. The resulting solution was transferred to test tubes containing 1 ml of 20% KOH in methanol and placed in a boiling water bath for 1 h. Non-saponifiable lipids were extracted with petroleum ether, and then the aqueous phase was acidified by addition of 0.5 ml of 60% H₂SO₄. Fatty acids were extracted into petroleum ether, the extract was evaporated under N₂ and its radioactivity was measured.

Identification of Labeled Lipids by Thin Layer Chromatography—Cultured hepatocytes were incubated with [^{14}C]acetate, washed twice with phosphate-buffered saline solution and solubilized in 1 ml of 0.5 N KOH at 37°C. The lipids in the solution were extracted twice with 2 ml of ethylether. The extracts were combined, evaporated to dryness under N_2 and dissolved in a small volume of ethylether. This concentrated extract was spotted on Silica Gel 60 plates (Merck, Darmstadt) and developed with *n*-hexane-ethylether-glacial acetic acid = 70 : 20 : 1. Triglycerides, cholesterol and its esters and phosphatidylcholine were added as standards. Spots were located with iodine vapour, cut out and counted in a toluene scintillator.

Purification of G6PDH—For immunochemical studies on synthesis of G6PDH protein, the enzyme was purified from the liver of rats by the methods of Matsuda and Yugari (39) and Dao *et al.* (40) with modifications for simplification and to give a higher yield. Eleven adult male Wistar strain rats were starved for 2 days and then fed on a high carbohydrate, fat-free diet composed of 70% sucrose, 23% casein, 1% vitamin mixture, 4% salt mixture, 0.01% choline chloride and 2% cellulose powder for 3 days. Then their livers (157 g wet weight) were homogenized in 3 volumes of 20 mM potassium phosphate buffer (pH 7.5) containing 5 mM EDTA and 10 mM 2-mercaptoethanol [buffer A] in a Polytron homogenizer for 1 min and then in a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 105,000 $\times g$ for 60 min, and the resulting supernatant was fractionated with ammonium sulfate. The precipitate at 35–55% saturation was dissolved in a small volume of buffer A and dialyzed against 100 volumes of buffer A for 4 h in a cold room (4°C) with one change of the outer buffer.

The dialysate was applied to a column (2.5 \times 30 cm) of DEAE-cellulose (DE-52, Whatman, Springfield Mill) previously equilibrated with buffer A. The column was washed with buffer A, and then with buffer A containing 50 mM KCl. The enzyme was eluted with buffer A containing 0.2 M KCl and concentrated by precipitation with ammonium sulfate. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 6.5) containing 5 mM MgCl_2 , 0.5 mM EDTA, and 0.01 mM 2-mercaptoethanol [buffer B], and applied to a column (1.5 \times 15 cm) of Blue-Sepharose CL6B (Pharmacia Fine Chemicals Co., Uppsala) previously equilibrated with buffer B. The column was washed with buffer B, then with buffer B containing 20 mM KCl. The enzyme was eluted with buffer B containing 1 mM NADP.

The purified enzyme had a specific activity of 148 units/mg protein and gave a single band on SDS-polyacrylamide gel electrophoresis (data not shown). The overall recovery of G6PDH activity in this procedure was about 30%. Results of a typical purification are shown in Table I.

Preparation of G6PDH Antiserum—Antiserum against pure rat liver G6PDH was prepared by the procedure of Winberry and Holten (41). Purified enzyme (4 mg) was emulsified in an equal volume of complete Freund's adjuvant (Difco, Michigan), and injected into a foot-pad and the back of a female white rabbit weighing 2.5 kg. A booster injection of 3 mg of purified enzyme emulsified in an equal volume of incomplete adjuvant (Difco, Michigan) was given 4 weeks later and 1 week after the second booster injection, blood was collected from the carotid artery and the serum was separated and stored at -70°C.

Immunochemical Analysis—Standard procedures were used for the Ouchterlony double diffusion test (42) and immunoelectrophoresis (43).

TABLE I. Purification of G6PDH from rat liver.

Step	Activity (unit/ml)	Volume (ml)	Total activity (unit)	Protein (mg/ml)	Specific activity (unit/mg protein)	Purity (fold)	Recovery (%)
Extract	10.6	490	5,168	25.8	0.41	1.00	100
Ammonium sulfate	106.8	37.5	4,003	178	0.61	1.61	77.5
DEAE-cellulose	8.0	294	2,337	1.12	7.10	17.3	45.2
Blue Sepharose CL6B	44.0	33.5	1,474	0.30	148	358	28.5

The antibody produced in the rabbit was specific for rat liver G6PDH, as indicated by the formation of single precipitin lines on immunochemical analyses with both rat liver extract and purified enzyme and fusion of these lines (Fig. 1).

For measurement of supernatant enzyme activity after immunoprecipitation, cells harvested from 5–20 dishes of 10-cm diameter were homogenized in 1 ml of phosphate-buffered saline solution in a Polytron homogenizer for 1.5 min. The homogenate was treated with antiserum to G6PDH, first at 37°C for 60 min and then overnight at 4°C. The resulting precipitate was removed by centrifugation and remaining activity in the clear supernatant was determined. The supernatant after treatment with non-immune serum in a similar way showed almost 100% recovery of activity.

Measurement of the Rate of Enzyme Synthesis

—Cells were incubated with hormones for 24 h

and then in Williams medium E supplemented with [³H]leucine (10 μM, 1 mCi/μmol) for 5 h. Then the cells were harvested, homogenized and centrifuged at 30,000 × g. The resulting supernatant was mixed with 2 units of unlabeled, partially purified G6PDH (5 units/mg protein) as a carrier and applied to a DEAE-cellulose column (0.5 × 1 cm) equilibrated with buffer A. The column was washed with buffer A containing 0.05 M KCl and then enzyme was eluted with 1 ml of buffer A containing 0.3 M KCl. Enzyme recovery was usually about 80%. A 0.2 ml aliquot of the eluate was incubated with 10 μl of control rabbit serum as described above. The resulting non-specific precipitate was removed by centrifugation, and the supernatant was incubated with 10 μl of anti-G6PDH serum. The immunoprecipitate was collected, washed twice with ice-cold phosphate-buffered saline solution by centrifugation at 4°C and subjected to SDS-gel electrophoresis as described below.

Disc Gel Electrophoresis—The washed immunoprecipitate was solubilized by heating in 100 μl of 0.0625 M Tris-HCl buffer (pH 8.0) containing 2% SDS and 5% 2-mercaptoethanol, and subjected to electrophoresis in 10% SDS-polyacrylamide gel as described by Laemmli (44). After electrophoresis, the gel was cut into 1-mm slices, and protein in each slice was extracted with 0.2 ml of 60% perchloric acid and 0.4 ml of 30% hydrogen peroxide by heating for 3 h at 60°C in a sealed scintillation vial. Then 10 ml of scintillation liquid was added and the solutions were kept at 4°C for 24 h to reduce chemiluminescence and then counted.

RESULTS

Induction of G6PDH by Insulin—Freshly isolated hepatocytes of adult rats fed on laboratory chow *ad libitum* contained 18 munits of G6PDH/mg protein. This initial activity was maintained for at least 5 days during primary culture. As shown in Fig. 2, addition of 10⁻⁸ M insulin to the culture medium caused a marked increase in the enzyme activity. The activity reached a maximum of about 5 times the initial value in 2 days after addition of insulin. When insulin was added at the start of the culture, G6PDH activity did not increase significantly during the 1st day. We have

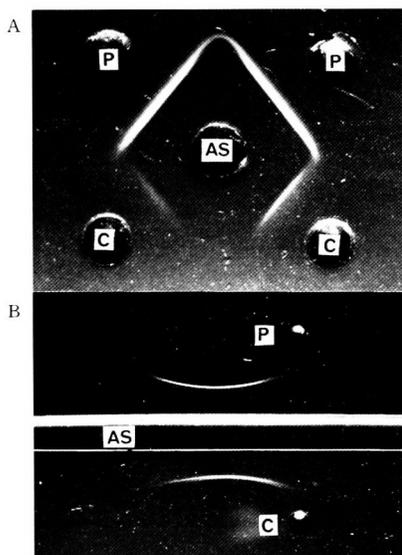


Fig. 1. Immunochemical analyses of purified and crude preparations of G6PDH with anti-serum. A. Ouchterlony double diffusion analysis. Antiserum (5 μl) was placed in the center well (AS). C; Concentrated crude liver extract (0.32 units/well), P; Purified G6PDH (0.47 units/well). B. Immunoelectrophoresis. Electrophoresis was carried out on agar-agarose gel in Veronal buffer (pH 8.6, I=0.05) at 150 volts for 2 h.

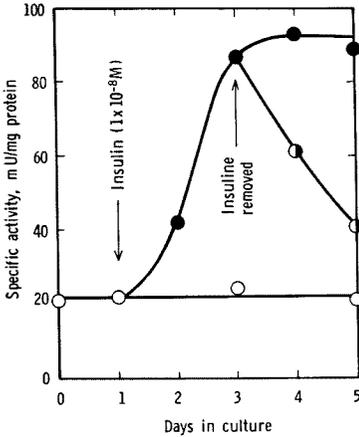


Fig. 2. Induction of G6PDH by insulin in primary cultured rat hepatocytes. Hepatocytes were cultured as described in "EXPERIMENTAL PROCEDURE" and insulin (1×10^{-8} M) was added in fresh medium 1 day after plating. Some cultures were washed twice with serum-free Williams medium E 48 h later and then maintained in fresh insulin-free medium. Values are means for duplicate experiments. ○, without insulin; ●, with insulin; ◐, insulin removed after 48 h.

shown that freshly isolated hepatocytes have impaired functions (23, 25, 30) and that they have only about half as many insulin receptors as intact liver cells (30). This reduced number of insulin receptors on isolated hepatocytes may be why insulin did not cause an increase in the enzyme activity in the 1st day of culture. Since the impaired functions and reduced number of insulin receptor of the cells are restored after one day of culture, insulin was added 20–24 h after the start of culture. The activity induced by insulin decreased toward the initial level when insulin was removed from the culture medium. In all experiments in this work dexamethasone (1×10^{-6} M) was added to cultures to maintain viable cells. Therefore, we next examined whether glucocorticoid is necessary for the effect of insulin. In this experiment we also used serum-free medium, because glucocorticoid may be present in serum, although at very low concentration. Figure 3 shows the dose-dependence of G6PDH induction on insulin with or without dexamethasone in serum-free medium. Induction of G6PDH was significant with a physiological concentration of about 10^{-9} M

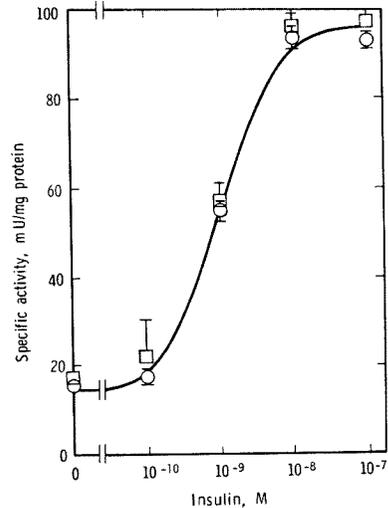


Fig. 3. Dose-response curves to insulin of G6PDH activity with or without dexamethasone. After culture for 1 day, the cells were incubated for 48 h in serum-free medium containing various concentrations of insulin with (○) or without (□) dexamethasone (1×10^{-6} M). Values are means \pm S.D. for 6 experiments.

insulin and the induction increased with the concentration of insulin up to 10^{-8} M whether dexamethasone was present or not. Serum-free medium did not affect the induction by insulin. Thus, stimulation of G6PDH activity by insulin is not dependent on the permissive effect of glucocorticoid or on any other factor in serum.

The effects of other hormones with or without insulin were examined. Neither glucagon nor bt_2cAMP suppressed the induction of G6PDH by insulin (Table II). Similarly, triiodothyronine, adrenergic hormones and bt_2cGMP had no effects on G6PDH activity with or without insulin.

The induction of G6PDH was completely inhibited by addition of cycloheximide (1×10^{-5} M) or actinomycin D ($0.3 \mu\text{g/ml}$), indicating that it was due to the synthesis of new protein (data not shown).

Induction of Synthesis of G6PDH Protein by Insulin—The titer of antibody specific to G6PDH, as described in "EXPERIMENTAL PROCEDURE," or the amount of enzyme precipitated by a known amount of antiserum was determined

TABLE II. Effects of hormones on G6PDH activity in primary cultured adult rat hepatocytes. Culture conditions were as described in Fig. 2, except that various hormones were added as indicated and then the hepatocytes were cultured for 48 h. Values are means \pm S.D. for 3-5 experiments.

Hormones	Concentration (M)	G6PDH activity (munit/mg protein)
None		20.0 \pm 3.20
Insulin	1×10^{-8}	73.1 \pm 5.55
Glucagon	1×10^{-7}	18.2 \pm 4.20
Insulin + glucagon		66.0 \pm 3.90
Insulin + bt ₂ cAMP	1×10^{-4}	75.9 \pm 3.32
None		17.3 \pm 4.25
Insulin	1×10^{-8}	88.1 \pm 5.55
Triiodothyronine	1×10^{-7}	20.6 \pm 2.88
Epinephrine	1×10^{-4}	23.1 \pm 5.03
Isoproterenol	1×10^{-4}	19.7 \pm 2.28
Bt ₂ cGMP	1×10^{-4}	20.9 \pm 3.15
Insulin + triiodothyronine		79.5 \pm 6.00
Insulin + epinephrine		70.2 \pm 3.62
Insulin + isoproterenol		89.7 \pm 4.83
Insulin + bt ₂ cGMP		70.0 \pm 4.07

from the data in Fig. 4. Results showed that 2.5 μ l of antiserum neutralized 0.47 units of G6PDH in homogenates of primary cultured hepatocytes. Assuming that the specific activity of homogeneous G6PDH is 148 units/mg protein, it was calculated that 1 ml of antiserum could precipitate about 1.27 mg of enzyme protein. Figure 4 also shows that the increase in activity induced by insulin was due to increase in the amount of enzyme protein, because the slopes of the curves for untreated- and insulin-treated cells were the same, but considerably more antiserum was required to neutralize the high activity in insulin-treated cells than the low activity in untreated cells. The increase in G6PDH activity was well correlated with the increase in antigenic activity, that is the amount of antiserum required to neutralize the enzyme.

To determine whether the increase in G6PDH was due to increase in the rate of synthesis of enzyme protein, we cultured hepatocytes in medium containing [³H]leucine for 5 h from 24 h after addition of insulin. The labeled G6PDH from these cells was immunoprecipitated with the

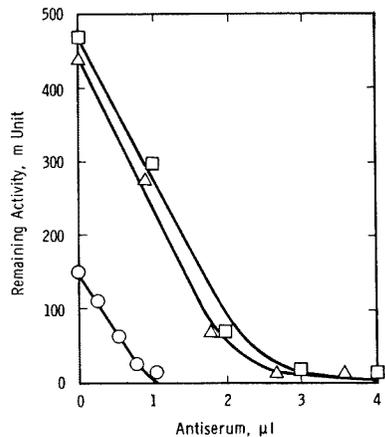


Fig. 4. Immunotitration of G6PDH in cell supernatants from hepatocytes treated with various hormones. Increasing volumes of anti-serum were added to extracts of cells treated with dexamethasone (1×10^{-6} M) (0.15 units of G6PDH) (○), cells treated with insulin (1×10^{-8} M) (0.46 units) (□), and cells treated with insulin and glucagon (1×10^{-7} M) (0.44 units) (Δ).

specific antibody, and the resulting antigen-antibody complexes were subjected to SDS-polyacrylamide gel electrophoresis. Figure 5 shows that the radioactivity was recovered on the gel in two main peaks, the first of which (Mr 56,000) corresponded to G6PDH. There was another broad radioactive peak, of which the highest radioactivity was found in the fraction of Mr 37,000. Material in this peak also increased in cells treated

with insulin. The radioactivities in this peak from control and insulin-treated cells were proportional to those in the respective G6PDH fractions. Therefore, this peak of Mr 37,000 may be that of immunoprecipitable nascent peptide of G6PDH released from polysomes. As shown in Table III, the observed 3-fold increase in enzyme activity was due to increase in the relative rate of synthesis of enzyme protein, since there was a similar increase in immunoprecipitated radioactivity. The rate of G6PDH synthesis increased about the same whether hepatocytes were incubated with insulin alone or with insulin and glucagon.

Induction of Lipogenesis by Insulin—Besides inducing G6PDH, insulin also induced lipogenesis. Figure 6 shows that the rate of fatty acid synthesis increased about 7-fold in association with induction of G6PDH activity when 1-day cultures of hepatocytes were incubated with 1×10^{-8} M insulin for 2 days. In these experiments, lipogenic activity was assayed by measuring incorporation of [14 C]-acetate into lipids in 2 h in hormone-free, serum-free medium, to avoid short-term effects of hormones. Maximum stimulation of the rate of fatty acid synthesis required at least 30 h of culture with insulin. Thus, this stimulation was due to a long-term effect of insulin on lipogenesis *via* inductions of key lipogenic enzymes in rat hepatocytes. Rat hepatocytes in monolayer cultures synthesized fatty acids from acetate at a rate of 6–9 nmol of acetate incorporated into fatty acyl groups per hour after incubation with insulin for 48 h.

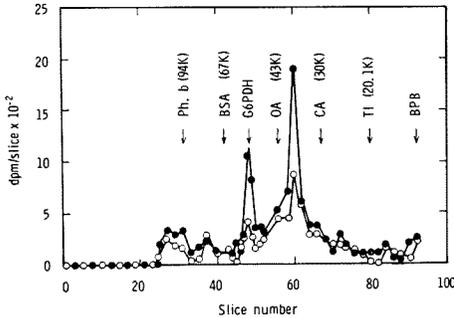


Fig. 5. SDS-polyacrylamide gel electrophoresis of G6PDH immunoprecipitated from untreated and insulin-treated cells. One-day cultures of cells were cultured further with (●) or without (○) insulin (1×10^{-8} M) for 24 h, labeled with [3 H]leucine for 5 h, harvested and treated with anti-serum. Immunoprecipitates were subjected to SDS gel electrophoresis as described in "EXPERIMENTAL PROCEDURE." The following markers of Mr were used: Ph, b, phosphorylase b; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase; TI, trypsin inhibitor; BPB, bromophenol blue.

TABLE III. Effects of insulin and glucagon on the rate of G6PDH synthesis in primary cultured hepatocytes. Experimental conditions were as for Fig. 5. Radioactivity of G6PDH fractions separated by disc gel electrophoresis was measured.

Hormones	G6PDH activity (munit/mg protein)	3 H]Leucine incorporation (dpm/mg protein/h)		Rate of synthesis of G6PDH (G6PDH/soluble protein $\times 10^{-3}$)
		G6PDH	Soluble protein ($\times 10^{-6}$)	
None	18.5	280	2.59	1.08
	24.2	230	1.30	1.76
Insulin (1×10^{-8} M)	37.1	772	3.47	2.23
	48.3	504	1.52	3.32
Insulin + glucagon (1×10^{-7} M)	46.8	1,506	4.15	3.60
	50.9	485	1.45	3.30

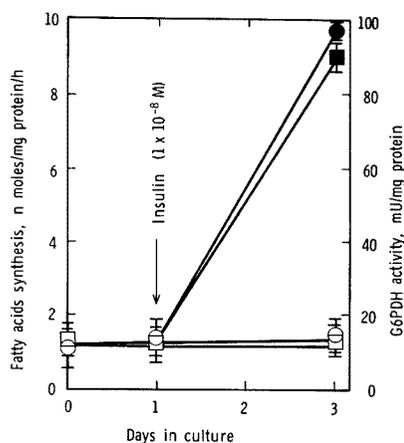


Fig. 6. Stimulation of fatty acid synthesis by insulin. Culture conditions were as for Fig. 2. Fatty acid synthesis was measured as [14 C]acetate incorporation in 2 h without hormones or serum. Values are means \pm S.D. for 6 experiments. \square , G6PDH; \circ , fatty acid synthesis. Closed symbols; with insulin.

TABLE IV. Effects of hormones on the composition of lipids in primary cultured rat hepatocytes. Culture conditions were as for Fig. 3, except that the medium contained serum. Values are means for duplicate experiments.

Hormones	Concentration (M)	[14 C]Acetate incorporation into lipids (dpm/mg cellular protein/h)			
		Triglycerides	Cholesterol	Cholesterol esters	Phospholipids
None		769	266	10.4	512
Dexamethasone	1×10^{-6}	1,115	436	7.4	743
Insulin	1×10^{-8}	1,780	378	6.6	1,187
Insulin + dexamethasone		2,600	430	13.9	1,733

TABLE V. Long-term effects of various hormones on synthesis of fatty acids in primary cultured rat hepatocytes. Culture conditions were as for Fig. 6, except that various hormones were added. Values are means \pm S.D. for 5-7 experiments.

Hormones	Concentration (M)	Synthesis of fatty acids (nmol/mg cell protein/h)
None		1.33 ± 0.41
Insulin	1×10^{-8}	9.02 ± 3.18
Glucagon	1×10^{-7}	1.06 ± 0.88
Triiodothyronine	1×10^{-7}	1.45 ± 0.13
Insulin + glucagon		7.38 ± 1.43
Insulin + triiodothyronine		8.93 ± 1.05

Most of the lipids synthesized from [14 C]-acetate in 2 h by rat hepatocytes in primary culture were triglycerides and phospholipids, as shown in Table IV, insulin stimulated synthesis of both lipids greatly, but had no effect on the syntheses of cholesterol and its esters.

Just as insulin was the only hormone that induced G6PDH, it was the only hormone that induced lipogenic activity: glucagon and triiodothyronine had no effect on fatty acid synthesis when added alone or with insulin (Table V). Dexamethasone slightly enhanced the induction of lipogenesis by insulin, but this may have been because it increased the viability of the cells (23) (Fig. 7).

The close relation between the inductions of G6PDH and lipogenesis is apparent from comparison of their dose-response curves to insulin (Figs. 3 and 7, respectively). This relation is further substantiated by the close correlation between changes in G6PDH and the rate of fatty acid synthesis shown in Fig. 8.

TABLE VI. Long- and short-term effects of insulin and glucagon on fatty acid synthesis in primary cultured hepatocytes. Values are means for duplicate experiments.

Hormones in cultures (long-term, 48 h)	Hormones in assay (short-term, 2 h)	Synthesis of fatty acids (nmol/mg cell protein/h)
Dexamethasone (1×10^{-6} M)	None	1.62
	Insulin (1×10^{-8} M)	2.40
	Glucagon (1×10^{-7} M)	1.08
Dexamethasone + insulin (1×10^{-8} M)	None	7.73
	Insulin	8.12
	Glucagon	2.13
	Bt ₂ cAMP (1×10^{-4} M)	1.80
Dexamethasone + insulin + glucagon (1×10^{-7} M)	None	7.01

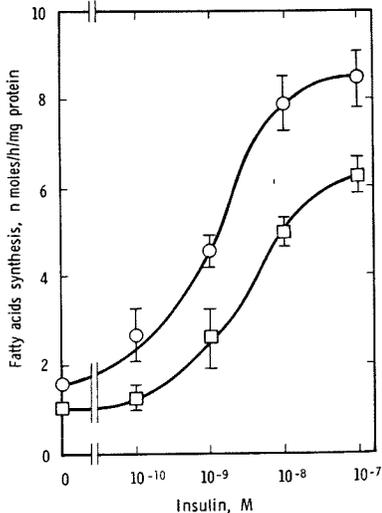


Fig. 7. Dose-response curves to insulin of fatty acid synthesis with or without dexamethasone. Culture conditions were as for Fig. 3, but the medium contained serum. Values are means \pm S.D. for 5 experiments. \circ , with dexamethasone (1×10^{-6} M); \square , without dexamethasone.

Short-Term Hormonal Control of Lipogenesis

—As shown above, the main factor in long-term control of lipogenesis is insulin, probably owing to its inductions of many lipogenic enzymes. Glucagon had no effect in long-term control, but when glucagon was added during the period of assay of lipogenesis, it was found to be inhibitory,

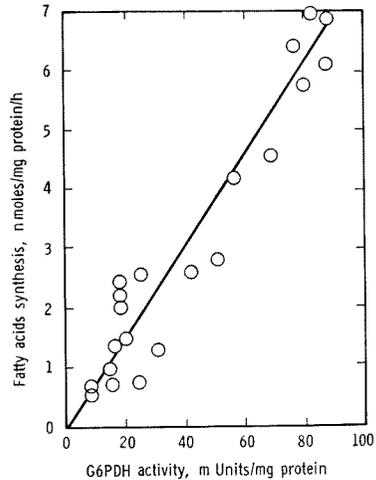


Fig. 8. Correlation between activity of G6PDH and rate of fatty acid synthesis.

whether the cells had been pretreated with insulin to increase lipogenic activity or not (Table VI). The effect of insulin was also inhibited by bt₂cAMP under these conditions. Insulin stimulated lipogenesis slightly on short-term treatment, but its effect was much less than its long-term effect.

DISCUSSION

Liver is the primary site of synthesis of fatty acids in mammals, synthesizing as much as 50% to the total fatty acids of the body (45, 46). Insulin is

known to play a central role in control of lipogenesis in rat liver (47, 48), causing both rapid and long-term stimulation of lipogenesis. Its long-term effect is known to involve increase in the amounts of key lipogenic enzymes (3-6). G6PDH is a key lipogenic enzyme supplying NADPH for lipogenesis. Starvation of animals and then refeeding a high carbohydrate diet caused marked increase in G6PDH activity (11-13, 19-22). This induction of G6PDH has been shown to result from increase in the rate of enzyme synthesis (41, 49), and it seems to be mediated by insulin, because in diabetic rats G6PDH activity did not change appreciably on refeeding unless insulin was injected (14, 19). However, the following findings do not support the idea of this role of insulin: 1) Insulin given without carbohydrate had no effect on the liver G6PDH level (20, 21). 2) G6PDH was induced even when rats were fed on fructose diet, which does not cause release of insulin from the pancreas (22). 3) Increase in liver G6PDH activity was directly proportional to carbohydrate uptake and so the induction by insulin might be attributed to its ability to stimulate carbohydrate consumption (20, 22). Thus the role of insulin in regulation of G6PDH activity *in vivo* is still controversial. However, we and Winberry *et al.* showed that insulin induced G6PDH in primary cultured hepatocytes (35, 36) and in this work we showed that insulin is the only hormone that induces G6PDH. We also showed that induction of G6PDH by insulin was detectable with a physiological concentration of 10^{-9} M insulin and maximal with a concentration of 10^{-8} M. Moreover, we found that the increase resulted from increase in the amount of enzyme protein as a result of increase in the rate of enzyme synthesis.

Permissive effects of glucocorticoid on the actions of insulin on glucokinase and glycogen synthesis were recently demonstrated in hepatocytes cultured in serum-free medium (50-53). Glucocorticoid is also reported to increase the induction of G6PDH in adrenalectomized rats when they are starved and then refed on high carbohydrate diet (18, 54). Dawson and Hales suggested that the effects of glucocorticoid may be mediated by effects on the insulin level (55). Moreover, Winberry and Holten suggested that glucocorticoid plays a role in regulating the level

of G6PDH in the liver, since hydrocortisone slightly stimulated the induction of G6PDH by insulin in primary cultures of rat hepatocytes (41). Our results showed that dexamethasone did not enhance insulin-dependent induction of G6PDH in rat hepatocytes, although it slightly stimulated the induction of G6PDH by insulin when the viability of the hepatocytes in primary culture was poor. Thus, it seems likely that glucocorticoid has no direct effect in the regulation of G6PDH in the liver.

Studies on thyroidectomized rats (56, 57) and primary cultured chick hepatocytes (58-60) have suggested that thyroxine may be involved in the induction of lipogenic enzymes. However, triiodothyronine did not induce G6PDH, or enhance the induction by insulin in this work. However, we cannot conclude from these results that triiodothyronine has no direct effect in regulation of the G6PDH level in the liver, because Spence *et al.* reported that hepatocytes must be prepared from thyroidectomized rats to demonstrate triiodothyronine-dependent increase in glucokinase and ATP-citrate lyase activities (61, 62).

Rudack and Holten showed that glucagon or cAMP decreased the G6PDH level in starved rats refed on high carbohydrate diet (16) by reducing the rate of G6PDH synthesis (63). Glucagon and cAMP were also found to inhibit synthesis of malic enzyme in primary cultured chick hepatocytes (59), ATP-citrate lyase in primary cultured rat hepatocytes (62), and fatty acid synthetase *in vivo* (64, 65). However, neither glucagon nor bt_2 cAMP suppressed induction of G6PDH by insulin in the present work. Winberry *et al.* also reported that glucagon had no effect on G6PDH induction in primary cultured rat hepatocytes (36). This does not mean that cultured hepatocytes do not respond at all to glucagon; we have shown that in primary cultured hepatocytes under similar conditions glucagon induced tryptophan oxygenase (28), serine dehydratase (34), tyrosine aminotransferase (32), and lysine-2-oxoglutarate reductase (29), and inhibited the induction of glucokinase by insulin (27). Thus, the present results seem to imply that glucagon and bt_2 cAMP have no direct effect on G6PDH synthesis *in vivo*. Unlike other lipogenic enzymes which are suppressed by glucagon, G6PDH is involved not only in lipogenesis but also in supply of riboside for nucleotides.

Thus hormonal control of G6PDH may be different from those of other lipogenic enzymes.

Immunochemical study showed that induction of G6PDH activity by insulin is due to increased synthesis of enzyme protein and hence to increase in the amount of the enzyme. SDS-polyacrylamide gel electrophoresis showed a large radioactive peak which comigrated with G6PDH. But in addition a second peak of Mr 37,000 was consistently found. The radioactivity in this second peak from both untreated and insulin-treated hepatocytes was proportional to that in G6PDH. This suggests that this second peak was that of nascent G6PDH. Winberry and Holten also separated a peak of nascent radioactive material from the liver of rats fed on high carbohydrate diet (41), but the radioactivity in this fraction from cultured hepatocytes was much higher than that from liver of rats on high carbohydrate diet. The reason why more nascent G6PDH was found in cultured hepatocytes than *in vivo* is unknown. Possibly protein synthesis in these cells is partially blocked (23) and thus incomplete G6PDH protein accumulates.

We did not examine the rate of degradation of this enzyme in these cells. But as shown in Fig. 2, the enzyme decay was fairly slow and hence it is reasonable to conclude that enzyme induction is mainly due to the increase of the rate of synthesis and that there is little, if any, involvement of degradation in these experimental conditions.

Another difference between findings *in vivo* and in cultured hepatocytes is that *in vivo* the specific activity of G6PDH can be induced to about 0.5 units/mg protein, whereas in cultured hepatocytes it is induced to only 0.1 unit/mg protein. The amount of nascent G6PDH in cultured hepatocytes might explain this much lower activity *in vitro* than *in vivo*. Other explanations for the difference between the inductions *in vivo* and *in vitro* are as follows: 1) Some factor(s) other than insulin may participate in the regulation of G6PDH *in vivo*. However, this possibility is unlikely, because of the various factors tested in this work, only insulin induced G6PDH. 2) G6PDH may be induced by fasting-refeeding in non-parenchymal hepatocytes as well. Consistent with this idea, Knook *et al.* reported that non-parenchymal hepatocytes have 5–10 times higher

specific activity of G6PDH than parenchymal hepatocytes of normal adult rats (66). Our preparation of hepatocytes consists mainly of parenchymal cells, because they retain various functions that are specific for parenchymal cells.

Insulin stimulates fatty acid synthesis in primary cultured hepatocytes in two ways, by long-term and short-term stimulations. The long-term stimulation was detectable in hepatocytes after incubation for 1 day with insulin and reached a maximum after 2 days. The rate of fatty acid synthesis varied with the activity of G6PDH in these cultured hepatocytes. This finding is consistent with the idea that in rat hepatocytes, NADPH for lipogenesis is supplied mainly by the hexose monophosphate shunt. It is also suggested that activity of G6PDH regulates lipogenesis in these cells, because glucagon did not inhibit lipogenesis stimulated by insulin, under conditions in which other lipogenic enzymes were suppressed, as described above. In avian liver, however, supply of NADPH for lipogenesis seems to come from the malic enzyme system, because this enzyme has the highest specific activity of all key lipogenic enzymes in avian liver and its activity is increased 100-fold by insulin and triiodothyronine (59). Moreover, lipogenic activity in chick liver, which is much higher than that in rat cells, is markedly stimulated by triiodothyronine as well as insulin. Thus, the regulation of lipogenesis in rat hepatocytes seems to be different from that in avian cells.

Although glucagon seems to play a minor role in long-term control of lipogenesis in rat hepatocytes, it strongly inhibited acetate incorporation into lipids when added for a short period. Even in cells with high lipogenic activity induced by long-term treatment with insulin, glucagon caused short-term suppression of fatty acid synthesis, presumably by inhibiting supply of substrates, since it was shown to inhibit acetyl-CoA carboxylase and the flow of citrate from mitochondria to the cytosol (7–10). Under these conditions insulin had only a slight stimulatory effect, insufficient to counteract that of glucagon. Recently we found that insulin stimulated synthesis of core-proteins of very low density lipoprotein and the secretion of this lipoprotein by primary cultured hepatocytes and that there is a correlation between lipogenesis and synthesis of core-protein

of lipoproteins (unpublished data). Thus, increase in lipoprotein secretion from the liver induced by insulin must result from stimulation of both lipogenesis and synthesis of core-proteins of lipoprotein.

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