Insulin Regulation of Hepatic Glycogen Synthase and Phosphorylase†

Lee A. Witters*,‡ and Joseph Avruch

ABSTRACT: The relative roles of insulin and glucose in the regulation of hepatic glycogen synthase and phosphorylase were studied in hepatocytes from fed rats. Elevation of extracellular glucose led to a rapid decrease in phosphorylase *a* activity followed by a slower increase in glycogen synthase I activity. A reciprocal and coordinate relationship between phosphorylase inactivation and synthase activation in response to glucose was observed; following initial glucose-induced inactivation of phosphorylase, there was a highly significant linear inverse relationship between residual phosphorylase activity and glycogen synthase activation. Insulin led to a further decrease in phosphorylase activity and a 30–50% additional increase in glycogen synthase activity over that caused by glucose. The effects of insulin required the presence of glucose and served to augment acute glucose stimulation of glycogen synthase and inhibition of phosphorylase. Insulin did not perturb the reciprocal and coordinate relationship between phosphorylase inactivation and synthase activation in response to glucose. The results suggest that the ability of insulin to activate hepatic glycogen synthase can be entirely accounted for by its ability to inactivate phosphorylase.

In the transition between the fed state and periods of short fasting, the net hepatic glucose output is determined by the reciprocal activities of glycogen synthase (UDPglucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) and glycogen phosphorylase (1,4-α-D-glucan-orthophosphate α-glucosyltransferase, EC 2.4.1.1). The regulation of the activation and inactivation of both of these enzymes has been the object of intense investigation, particularly with regard to the roles of various hormones, including insulin, glucagon, and catecholamines, and of glucose.

Glucose has been recognized to be an important regulator of hepatic glucose output. This observation extends from the initial studies of Soskin et al. (1938) who noted that the level of glycemia controls, in part, net hepatic glucose uptake or output. This concept has been considerably extended, principally by the investigations of Hers and Stalmans (Hers, 1976; Stalmans, 1976). These investigators have indicated a major role for glucose in the regulation of glycogen synthase and phosphorylase in the liver.

Insulin has also been implicated as a major regulator of hepatic glucose output. The studies of Mortimore (1963) and Jefferson et al. (1968) demonstrated that, in the isolated perfused liver, insulin acts to decrease hepatic glucose output. Miller & Larner (1973) have reported that insulin acts to promote glycogen synthesis by a direct activation of glycogen synthase. The study of insulin activation of hepatic glycogen synthase has been undertaken both in vivo (Bishop & Larner, 1967; Gold, 1970; Bishop et al., 1971; Curnow et al., 1975; van der Werve et al., 1977) and in the isolated perfused liver (Miller and Larner, 1973). However, both the quantitative contribution of insulin stimulation to glycogen synthase activation and the locus of insulin action remain unclear. Furthermore, there are few reports of insulin modulation of hepatic phosphorylase activity (van der Werve et al., 1977; Curnow et al., 1975).

Herein we report studies of the relative roles of insulin and glucose in the minute-to-minute regulation of hepatic glycogen synthase and glycogen phosphorylase in the isolated rat hepatocyte.

Materials and Methods

Chemicals. Crude collagenase (type I, lot no. CLS45E059) was obtained from Worthington Biochemical Co. Rats were obtained from the Charles River Breeding Laboratories. Bovine serum albumin (lot no. 55C-0151), rabbit liver glycogen, uridine diphosphate glucose, glucose 1-phosphate, and glucose 6-phosphate were purchased from Sigma. Monocomponent pork insulin was a generous gift from Dr. J. Schlichtkrull of the Novo Research Institute. Minimal essential medium concentrated amino acids and vitamins were obtained from Grand Island Biological Co. Uridine diphosphate [U-^{14}C]-glucose and α-D-[U-^{14}C]glucose 1-phosphate were purchased from New England Nuclear.

Experimental Procedures. Hepatocytes were isolated by collagenase perfusion of the isolated livers from male Sprague-Dawley rats fed standard laboratory chow ad libitum (Witters et al., 1976). Following isolation and washing, the cells were suspended in minimal essential (ME)[1] medium with 5.5 mM glucose, 25 mM Hepes buffer, and 1% bovine serum albumin at a cell density of 5.0 to 10.0 $\times$ 10^6/mL. Cell viability, as assessed by trypan blue exclusion, was routinely 85–95% and was maintained throughout the experiments performed. As judged by electron microscopy[2] Kuppfer cells constituted less than 4% of the cell population. Freshly isolated cells were incubated at 37 °C under 95% O_2/5% CO_2 for 30 min and were then reisolated by low-speed centrifugation (50 $\times$ *g* for 2 min). The cells were resuspended into fresh medium without glucose and incubated for an additional 15 min prior to beginning experiments.

Experiments were initiated by the addition of cell suspension

† From the Diabetes Unit, Medical Services, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114. *Received September 2, 1977.* Dr. Avruch is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by a grant from the American Diabetes Association and by Grants no. AM 19270 and AM 17776 from the National Institutes of Health (NIAMDD).

‡ Address correspondence to this author at the Diabetes Unit, Massachusetts General Hospital, Boston, Massachusetts 02114.

[1] Abbreviations used: ME medium, minimum essential medium; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SEM, standard error of the mean.

[2] Kindly performed in the laboratory of Dr. Leonard Jarrett.

0006-2960/78/0417-0406$01.00/0

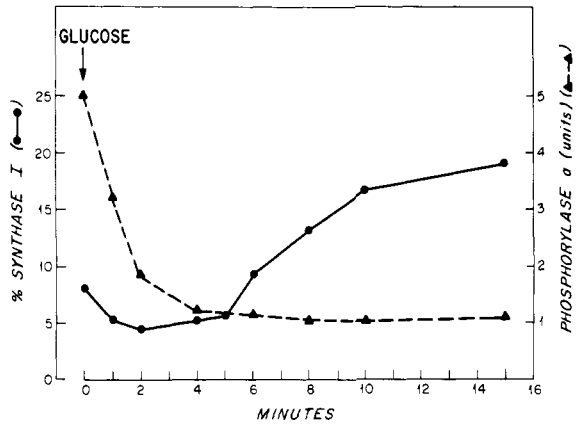

FIGURE 1: Percent glycogen synthase I and phosphorylase *a* with 30 mM glucose. The extracellular glucose concentration was increased acutely from 0 to 30 mM glucose. Aliquots of the cell suspension were withdrawn at various time points and processed for measurements of glycogen phosphorylase *a*, glycogen synthase I, and total glycogen synthase (data not shown).

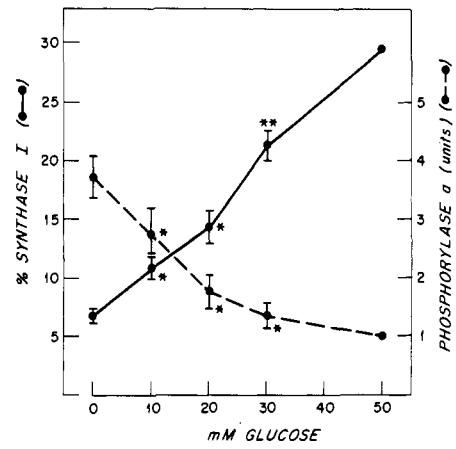

FIGURE 2: Glucose dose-response; percent synthase I and phosphorylase *a*. Cells were incubated in different glucose concentrations for 15 min; enzyme activities were measured as described in Materials and Methods. The results (±SEM) represent five experiments with different cell preparations. The alteration in enzyme activity at each glucose concentration was compared statistically by Student's *t* test with the activity observed at the next lower glucose concentration; * indicates $p < 0.025$ and ** $p < 0.01$.

to vessels containing either glucose or glucose plus insulin. At selected time points an aliquot of cell suspension was removed and immediately frozen in dry ice/acetone, after the addition of 0.1 volume of sucrose (0.25 M final concentration), NaF (150 mM), and EDTA (5 mM). Subsequently, the suspension was rapidly thawed and immediately homogenized by brief sonication (75 W s, Sonifer Cell Disruptor, Branson Instruments). The homogenate was diluted into buffers appropriate for the assays of glycogen synthase and glycogen phosphorylase.

Glycogen synthase was assayed by the method of Thomas et al. (1968). The initial cell homogenate was diluted 1:1 into a second buffer to achieve a final concentration of Tris-Cl, 50 mM, pH 7.80, NaF, 150 mM, EDTA, 5 mM, and sucrose, 0.25 M. Glycogen synthase I was determined in the presence of Na_2SO_4 (10 mM) and total synthase activity (D + I) in the presence of 10 mM glucose 6-phosphate. Total synthase activity which was unchanged throughout all experiments was 0.861 ± 0.052 (SEM) units where 1 unit equals 1 μmol of uridine diphosphate glucose incorporated into glycogen per min per g weight of pelleted hepatocytes. The results are expressed as percent synthase I (I activity/total activity × 100).

Glycogen phosphorylase was assayed by the method of Stalmans & Hers (1975) as adapted to the filter paper method. The initial cell homogenate was diluted 1 volume to 3 volumes into a buffer to achieve a final concentration of β-glycerophosphate, 100 mM (pH 6.50), sodium fluoride, 150 mM, EDTA, 5 mM, and sucrose, 0.25 M. The phosphorylase *a* reaction contained caffeine at 0.5 mM; under these conditions, phosphorylase *b* is totally inactive (data not shown). Results are expressed as units of phosphorylase *a* activity, where one unit equals one μmol of glucose 1-phosphate incorporated into glycogen per min per g of pelleted hepatocytes.

In the present report, the term activation of glycogen synthase refers to an increase in glycogen synthase I activity and inactivation of phosphorylase to a decrease in phosphorylase *a* activity.

Studies were carried out to assess the influence of the residual glucose, amino acids, and vitamins from the ME medium on the activities of both glycogen synthase and phosphorylase *a*. Direct comparison of homogenates prepared by the above method to homogenates obtained by initial pelleting of the hepatocytes with subsequent resuspension and homogenization into the appropriate buffer yielded identical values for percent synthase I and phosphorylase *a*. The quick freeze technique of cell suspension permits rapid sampling during a minute-to-minute time course with measurement of the activities of both enzymes from a single cell homogenate.

Statistical analyses were performed using Student's *t* test, by linear regression analysis and by analysis of variance.

Results

Glucose Regulation of Glycogen Synthase and Phosphorylase. When the glucose concentration of the cell suspension is acutely increased from 0 to 30 mM (Figure 1) the first change demonstrable is a rapid fall in phosphorylase *a* activity commencing within the first minute and reaching an apparently stable plateau between 2 and 4 min. Synthase I increased more slowly, with a rise in percent synthase commencing at 5–6 min, and reaching peak level at 15 min. Values returned to baseline by 30 min (data not shown). A similar temporal sequence of phosphorylase inactivation followed by synthase activation in response to glucose has been observed at all glucose concentrations studied between 10 and 50 mM.

The dose-response relationship between glucose concentrations and percent synthase I (Figure 2) demonstrates a linear increase in percent synthase I between 10 and 50 mM glucose. In contrast, phosphorylase *a* levels decline in a curvilinear pattern toward a level of one unit in response to increasing glucose concentration.

Comparison of percent synthase I with the simultaneous value of phosphorylase *a* (Figure 3) at all time points between 0 and 15 min in response to 0 and 30 mM glucose reveals two important relationships between phosphorylase *a* and percent synthase I. First, there appears to be a distinct "threshold" of phosphorylase *a* activity between 1 and 2 units at which synthase activation commences. In hepatocytes from fed rats, no activation of synthase in response to glucose is achieved if phosphorylase *a* activity is not lowered to these "threshold" levels. Second, below "threshold" levels of phosphorylase *a*, there is a highly significant linear inverse relationship between residual phosphorylase *a* activity and synthase activation. Thus, below a certain "threshold" level of phosphorylase *a*, small changes in absolute phosphorylase *a* activity are accompanied by large changes in synthase I activity.

Insulin Regulation of Glycogen Synthase and Phospho-

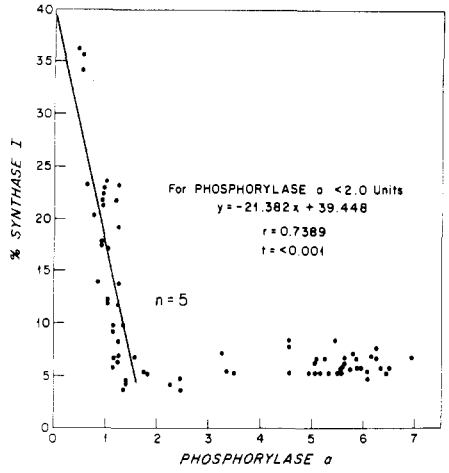

FIGURE 3: Percent synthase I; relationship to phosphorylase *a*. Cells were incubated in either 0 or 30 mM glucose and aliquots removed at 0, 2, 5, 10, and 15 min in five experiments. All values of percent synthase I are plotted against the simultaneous values of phosphorylase *a* determined in the same homogenate.

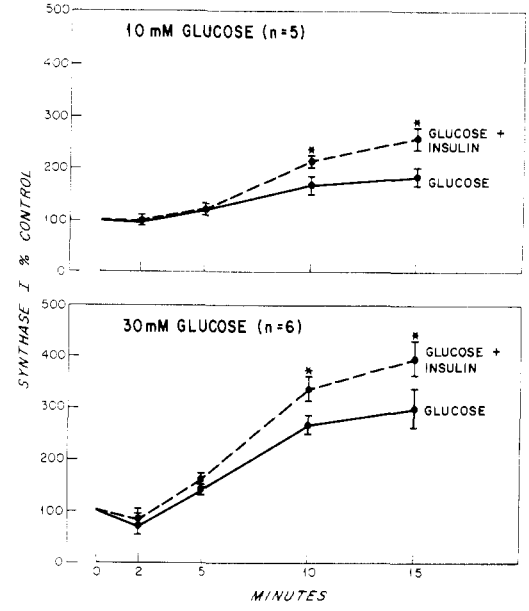

FIGURE 4: Insulin stimulation of percent synthase I; time course. At zero time, the extracellular glucose concentration of cell suspension was increased from 0 to 10 mM (upper panel) or 30 mM (lower panel) with or without insulin (1 mU/mL) introduced simultaneously; glycogen synthase I and total glycogen synthase (unchanged; not shown) were measured as described in Materials and Methods. Results are expressed as a percentage of zero glucose control (±SEM) at each time point. The change in percent synthase I with glucose plus insulin to that with glucose alone was compared by Student's *t* test; * indicates $p < 0.02$ relative to glucose alone.

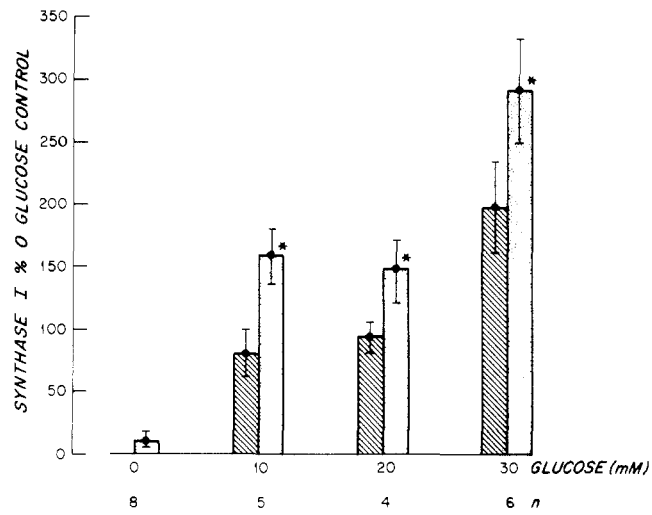

FIGURE 5: Insulin stimulation of percent synthase I; relationship to glucose. Cells incubated in the absence of glucose were introduced into vessels to bring the final glucose concentration to 0, 10, 20, or 30 mM with or without insulin (1 mU/mL). Aliquots were taken at 15 min for assay of glycogen synthase I and total glycogen synthase (unchanged; not shown); results are expressed as percentage of zero glucose control (±SEM). The response of glucose plus insulin (stippled bars) to glucose alone (hatched bars) at each glucose concentration was compared by Student's *t* test; * indicates $p < 0.02$ relative to glucose alone.

rylase. When insulin is introduced with glucose, a further increase in percent synthase I is observed as compared with that elicited by glucose alone. The time course of insulin stimulation of glycogen synthase (Figure 4) is identical at 10 and 30 mM glucose with insulin stimulation emerging between 5 and 10 min, and peaking at 10 and 15 min. All values returned to baseline by 30 min (data not shown). Half-maximal activation of glycogen synthase by insulin (at 30 mM glucose) occurs at an insulin concentration of 50–100 μU/mL (data not shown). These levels are consistent with insulin levels achieved in the portal circulation under physiologic conditions.

The interaction of insulin and glucose in the activation of glycogen synthase was studied at four different glucose concentrations (Figure 5). While glucose is a potent activator of glycogen synthase, insulin contributes approximately one-third (20 and 30 mM glucose) to one-half (10 mM glucose) of total synthase activation. However, analysis of eight experiments performed at zero glucose reveals no significant difference between insulin and control values for glycogen synthase I in hepatocytes from fed rats. Thus, insulin substantially augments the activation of glycogen synthase only in the presence of a concomitant stimulation by glucose.

Because of the reciprocal relationship between phosphorylase inactivation and synthase activation in response to glucose alone (Figures 1 and 3), the effect of insulin on phosphorylase inactivation and its possible relation to the insulin stimulation of synthase were evaluated. Glucose produces a rapid and profound inactivation of phosphorylase; when the same glucose pulse is given with insulin, a further inactivation of phosphorylase due to insulin is observed. Though the absolute change in phosphorylase *a* activity due to insulin is small in magnitude, insulin-induced inactivation of phosphorylase is observed at both 10 and 30 mM glucose (Figure 6) and is highly significant in both instances. However, in the absence of glucose, insulin has no discernible effect on phosphorylase *a*.

Insulin inactivation of phosphorylase at 10 and 30 mM glucose achieves significance after 10 and 15 min of hormone exposure (Figure 7). This time course is similar to that of insulin-induced activation of glycogen synthase (Figure 4).

Thus, a reciprocal relationship between phosphorylase inactivation and synthase activation as seen in response to glucose alone is still observed in the presence of insulin plus glucose. In order to evaluate the effect of insulin on this reciprocal relationship, we compared by analysis of variance the slopes of the regression lines generated in the presence of glucose alone (Figure 3) to that observed with glucose plus insulin at all time points in six experiments. Such a comparison indicates that insulin does not significantly alter the relationship between the residual phosphorylase *a* and synthase I (glucose alone: $y = -19.031 \times +35.453$; $r = 0.6760$; $p < 0.0025$; insulin plus

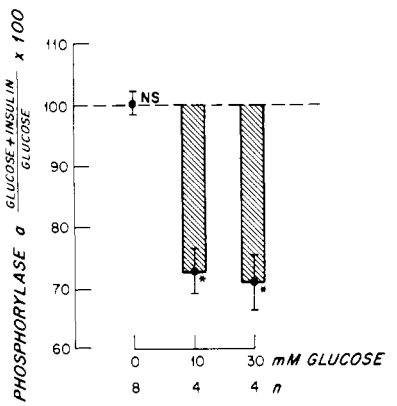

FIGURE 6: Insulin inactivation of phosphorylase *a*; relationship to glucose. Cells incubated in the absence of glucose were introduced into vessels to bring the final glucose concentration to 0, 10, or 30 mM with or without insulin (1 mU/mL). Aliquots were taken at 15 min for assay of phosphorylase *a* as described in Materials and Methods. The results are expressed as the ratio of phosphorylase *a* activity in the presence of glucose plus insulin to that in glucose alone (× 100) (±SEM) at each glucose concentration and the comparison evaluated by Student's *t* test; * indicates $p < 0.02$ relative to glucose control and NS, nonsignificance.

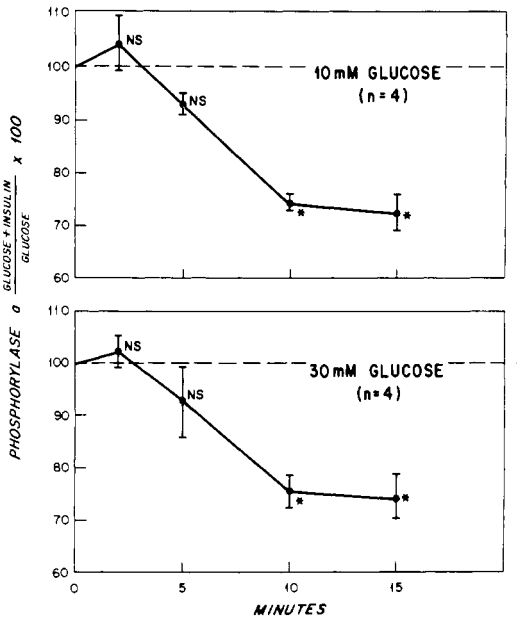

FIGURE 7: Effect of insulin on phosphorylase *a*; time course. The extracellular glucose concentration of cell suspension was increased from 0 to 10 mM (upper panel) or 30 mM (lower panel) with or without insulin (1 mU/mL) introduced simultaneously. Phosphorylase *a* was measured as described in Materials and Methods. Results are expressed as percentage of glucose control ±SEM. The change in phosphorylase *a* with glucose plus insulin to that with glucose alone was compared by Student's *t* test; * indicates $p < 0.02$ relative to control and NS, nonsignificance.

glucose: $y = -25.561 \times +42.856$; $r = 0.8907$; $p < 0.005$; analysis of variance of differences of slopes: $0.2 < p < 0.3$). Thus, at phosphorylase *a* levels less than 1–2 units, small changes in phosphorylase *a* activity, such as those induced by insulin, are accompanied by large changes in synthase I.

In the fed state, we have been unable to dissociate the two observed effects of insulin, activation of glycogen synthase and inactivation of glycogen phosphorylase. Insulin activation of synthase under all conditions studied is always accompanied by a concomitant inhibition of phosphorylase activity.

Discussion

These studies indicate that both glucose and insulin are major regulators of hepatic glycogen synthase and glycogen phosphorylase in hepatocytes isolated from fed rats. The data further demonstrate that the regulation by glucose and by insulin is interdependent, depending critically on the underlying reciprocal relationship of the activities of glycogen synthase and phosphorylase.

Glucose regulation involves an initial reduction of phosphorylase *a* activity to a "threshold" level at which synthase activation commences. At levels of phosphorylase *a* below this threshold, a strong linear inverse relationship between residual phosphorylase *a* activity and synthase I remains. A role for glucose as an important regulator of phosphorylase was first suggested by Cori et al. (1943) and later amplified in studies of Helmreich and co-workers (1967). The present observations are consistent with the studies of Hers and Stalmans (Stalmans et al., 1974a,b; Hue et al., 1975). Based in part on studies of isolated enzymes in vitro, they have postulated that the level of phosphorylase *a* is a critical regulator of synthase activation, through phosphorylase *a* inhibition of synthase D dephosphorylation. By this model, glucose binding to phosphorylase *a* increases the rate of dephosphorylation of phosphorylase *a* (Stalmans et al., 1974a). As phosphorylase *a* levels fall, the inhibition of synthase D dephosphorylation is relieved with consequent activation to synthase I. While this model has been the most thoroughly studied with regard to the mechanism of glucose action, it does not exclude the possibility that glucose might in addition have direct effects to promote synthase activation independent of changes in phosphorylase. Hizurki & Takeda (1970) have suggested that glucose 6-phosphate might directly promote the dephosphorylation of synthase D to the I form, based on studies of a partially purified synthase from bovine spleen. It is not clear, however, that this effect of glucose 6-phosphate is operative intracellularly in the presence of other effectors of enzyme activity.

The present studies confirm earlier observations on the ability of insulin to activate hepatic glycogen synthase (Miller & Larner, 1973; Bishop & Larner, 1967; Gold, 1970; Bishop, 1970; Bishop et al., 1971; Curnow et al., 1975; van der Werve et al., 1977) but serve to extend and clarify the relative roles of insulin stimulation and glucose stimulation quantitatively, as well as suggest a locus of insulin action. Thus, insulin can exert a quantitatively major effect on synthase activation in comparison with glucose, i.e., a 30–50% augmentation of the response elicited by glucose alone. The demonstration of this effect of insulin may depend critically on the conditions employed. It is to be emphasized that insulin stimulation was observed in hepatocytes from fed rats, required the presence of glucose, and occurred during acute glucose stimulation of synthase.

In addition, these studies demonstrate clearly that insulin can promote the inactivation of glycogen phosphorylase. There are a few published reports of the effects of insulin on hepatic phosphorylase. Curnow and co-workers (1975) have demonstrated insulin-induced inactivation of phosphorylase in the liver of the rhesus monkey studied in vivo. Van der Werve and co-workers (1977) observed a similar inactivation in the rabbit liver in vivo. In the present studies, insulin-induced inactivation of phosphorylase represents a further inactivation beyond that achieved by glucose alone.

Insulin activation of glycogen synthase occurs following acute glucose-induced suppression of phosphorylase activity. This observation is consistent with studies in the rabbit liver in vivo of van der Werve and co-workers (1977) who noted insulin activation of glycogen synthase only when the concomitant levels of phosphorylase *a* were low. The current data

demonstrate, in addition, that insulin promotes a further inactivation of phosphorylase beyond that achieved with glucose. Furthermore, insulin-induced glycogen synthase activation is not dissociable from insulin-induced phosphorylase inactivation. The ability of insulin to further suppress phosphorylase activity at levels of phosphorylase *a* wherein relatively small changes in the latter activity are associated with large changes in synthase I suggests that the ability of insulin to activate hepatic glycogen synthase may be entirely accounted for by this hormone's ability to inactivate phosphorylase. A second action of insulin to independently and simultaneously activate glycogen synthase cannot, however, be excluded.

The effects of insulin on both hepatic glycogen synthase and phosphorylase appear to require the presence of glucose in the incubation medium. Insulin-induced alterations in either glucose entry or in glucose metabolism cannot be excluded as the basis for this observation; this possibility is currently under study. A second possibility is that insulin induces a stable modification in the substrate for glucose action, rendering it more sensitive to ligand-mediated alterations. Changes in covalent enzyme phosphorylation or dephosphorylation induced by insulin are one such possible modification.

In summary, in hepatocytes isolated from fed rats, both insulin and glucose play major regulatory roles in the modulation of glycogen synthase and glycogen phosphorylase. Studies on the hormonal and substrate regulation of glycogen metabolism must consider the apparent obligatory relationship between the activities of glycogen synthase and glycogen phosphorylase. The present studies establish the characteristics of these enzymes in hepatocytes from the fed rat and serve as a basis for comparison with other tissues as well as a variety of other nutritional and disease states.

Acknowledgments

The authors wish to acknowledge the excellent technical assistance of Ms. Lena Alberico and Mr. Jeffrey Bernhard.

References

Bishop, J. S. (1970) *Biochim. Biophys. Acta 208,* 208–218.
Bishop, J. S., & Larner, J. (1967) *J. Biol. Chem. 242,* 1354–1356.
Bishop, J. S., Goldberg, N. D., & Larner, J. (1971) *Am. J. Physiol. 220,* 499–506.
Cori, C. F., Cori, G. T., & Green, A. A. (1943) *J. Biol. Chem. 151,* 39–55.
Curnow, R. T., Rayfield, E. J., George, D. T., et al. (1975) *Am. J. Physiol. 228,* 80–87.
Gold, A. H. (1970) *J. Biol. Chem. 245,* 903–905.
Helmreich, E., Michaelides, M. C., and Cori, C. E. (1967) *Biochemistry 6,* 3695–3710.
Hers, H. G. (1976) *Annu. Rev. Biochem. 45,* 167–189.
Hizurki, S., & Takeda, Y. (1970) *Biochim. Biophys. Acta 212,* 179–181.
Hue, L., Bontemps, F., & Hers, H. G. (1975) *Biochem. J. 152,* 105–114.
Jefferson, L. S., Exton, J. H., Butcher, R. W., et al. (1968) *J. Biol. chem. 243,* 1031–1038.
Miller, T. B., & Larner, J. (1973) *J. Biol. Chem. 248,* 3483–3488.
Mortimore, G. E. (1963) *Am. J. Physiol. 204,* 699–704.
Soskin, S., Essex, H. E., Herrick, J. F., & Mann, F. C. (1938) *Am. J. Physiol. 124,* 558–567.
Stalmans, W. (1976) *Curr. Top. Cell. Regul. 11,* 51–97.
Stalmans, W., & Hers, H. G. (1975) *Eur. J. Biochem 54,* 341–350.
Stalmans, W., Laloux, M., & Hers, H. G. (1974a) *Eur. J. Biochem. 49,* 415–427.
Stalmans, W., de Wulf, H., Hue, L., & Hers, H. G. (1974b) *Eur. J. Biochem. 44,* 127–134.
Thomas, J. A., Schlender, K. K., & Larner, J. (1968) *Anal. Biochem. 25,* 486–499.
van der Werve, G., Stalmans, W., & Hers, H. G. (1977) *Biochem. J. 162,* 143–146.
Witters, L. A., Alberico, L., & Avruch, J. (1976) *Biochem. Biophys. Res. Commun. 69,* 997–1003.