THE ANATOMICAL RECORD 248:406–412 (1997)

Localization of Glycogen Phosphorylase Activity in Liver of Fasted Normal and Adrenalectomized Rats and in Fasted Adrenalectomized Rats After Injection of Dexamethasone

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ABSTRACT ***Background:*** **The intralobular distribution of activity of glycogen phosphorylase (GP), a key enzyme in the breakdown of glycogen, was evaluated to determine changes during early glycogen synthesis. Hepatic GP activity was localized in normal and adrenalectomized (ADX) rats after fasting overnight and in fasted ADX rats stimulated to synthesize glycogen by administration of dexamethasone (DEX) 2–8 h prior to sacrifice.**

Methods: **Cryostat sections were incubated in medium containing appropriate substrate for demonstration of GP activity as indicated by glycogen synthesized by the enzyme during incubation.**

Results: **In sections from fasted normal rats, GP activity in hepatocytes varied from undetectable to substantial amounts with no notable periportal to pericentral gradient evident. In contrast, GP activity in sections from adrenalectomized fasted rats was concentrated in discrete aggregates in random hepatocytes throughout lobules. Two hours after DEX injection, GP enzyme activity occurred as single aggregates or in a dispersed pattern in many hepatocytes. By 4 h after DEX administration, most cells displayed GP enzyme activity, the concentration of which appeared to be greater in pericentral cells than in periportal cells. Eight hours after injection of DEX, GP enzyme activity had increased and appeared more evenly distributed throughout the lobules.**

Conclusions: **These results suggest that GP activity became concentrated in limited regions of selected hepatocytes in fasted ADX rats. DEX stimulation of glycogen synthesis in these rats resulted in increased GP activity that was concentrated in pericentral cells after 4 h. After 8 h, activity increased and was more evenly distributed throughout the lobules. The increase in GP enzyme activity concurrent with overall glycogen synthesis suggests that the enzyme may participate in glycogen turnover.**

Key words: liver glycogen; glycogen phosphorylase; histochemistry; adrenalectomy-fasting-dexamethasone

Glycogen phosphorylase (EC 2.4.1.1) is a key enzyme in the production of glucose from the breakdown of glycogen. It exists in an active "*a*" form that is phosphorylated on a serine residue and in an inactive form, phosphorylase *b*, that lacks phosphorylation at this site (Hers, 1990). The intralobular distribution patterns of glycogen phosphorylase (GP) enzyme activity in the liver have been of interest to investigators of metabolic zonation (e.g., Jungermann, 1988; Jungermann and Katz, 1989; Katz, 1989; Gebhardt, 1992). Although several authors have evaluated the distribution of GP enzyme activity in fed or fasted animals, changes in the patterns of GP activity have not been followed in a sequential manner during early glycogen synthesis.

Previously, Cardell (1977) had established that overnight fasting of adrenalectomized rats reduced liver glycogen content to minimal levels and caused a virtual absence of glycogen β particles in sections viewed by electron microscopy. Administration of a pharmacological dose of dexamethasone to these animals resulted in a pronounced stimulation of hepatic glycogen synthesis for several hours after a 1–2 h lag period (Michaels et

Contract grant sponsor: U.S.P.H.; contract grant number: OK27097.
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Received 8 August 1996; accepted 28 January 1997.

al., 1984). Therefore, by using this model system the intralobular distribution of GP enzyme activity may be observed as the liver glycogen content changes from minimal levels to a substantial glycogen load by the action of glycogen synthase. We have shown previously by light and electron microscopic radioautography (Michaels et al., 1984) that during the period of glycogen synthesis, ^{3}H-labeled glycogen precursor was incorporated into newly formed glycogen in hepatocytes throughout the liver lobules. Progressively, more hepatocytes became heavily labeled as glycogen accumulated. Moreover, we have shown changes in the distribution patterns of glycogen synthase activity during early glycogen synthesis (Michaels and Cardell, 1993) that reflected the patterns of incorporation of ^{3}H-labeled carbohydrate precursor.

In the present study we used the model of the adrenalectomized overnight-fasted rat stimulated with dexamethasone to determine patterns of intralobular distribution of glycogen phosphorylase activity at several time intervals both prior to and after stimulation of glycogen synthesis. Enzyme activity of GP was localized in cryostat sections of rat liver by a histochemical method (Lindberg and Palkama, 1972) based on that proposed by Takeuchi and Kuriaki (1955). In this method, the incubation conditions are such that instead of breaking down glycogen, as GP does under physiological conditions, the enzyme is utilized to produce new glycogen by addition of glucose-1-phosphate to pre-existing glycogen.

MATERIALS AND METHODS

Eighteen young male Wistar rats weighing approximately 100–150 g at the time of sacrifice were used in this study. Fifteen of the rats were adrenalectomized (ADX) 7 to 10 days before the experiment and all of the animals were fed ad libitum until the night prior to experimentation. The ADX rats were allowed continual access to water containing 0.75% NaCl. Rats were provided with a 12:12 h light-dark cycle. They were housed singly in wire-bottomed cages the night prior to the experiment and fasted in order to deplete their glycogen reserves to minimal levels. The following morning three fasted ADX rats and the three fasted normal rats were sacrificed without further treatment. The remaining twelve fasted ADX rats were separated into groups of four and injected with 2 mg of dexamethasone (DEX), 2, 4, and 8 h prior to sacrifice.

The liver was removed following laparotomy of each anesthetized rat. One lobe was removed, diced into 3 mm cubes and plunged into a bath of isopentane (2-methyl butane) that was contained in a 300 ml steel cup suspended and cooled in a bath of liquid nitrogen. After freezing for 30 seconds to a minute the tissue was placed on dry ice and then maintained at −70°C until sectioned on a cryostat. Frozen sections 10 μm thick were mounted by brief thawing onto glass slides that had been subbed with gelatin chrome alum.

The sections were dried for 10 to 30 min and then incubated for 1 or 3 h (1 h for Figs. shown) at 37°C in medium as described by Eranko and Palkama (1961) and modified by Lindberg and Palkama (1972) to evaluate the distribution of glycogen phosphorylase activity. For each incubation, control slides were incubated in medium without substrate. The incubation mixture consisted of: 100 mg glucose 1-phosphate, 10 mg adenosine 5-phosphate, 2 mg glycogen, 18 mg sodium fluoride, 900 mg polyvinyl pyrrolidone, 2 units insulin (regular iletin I), 2 ml ethanol, and 10 ml 0.1 M acetate buffer at pH 5.9. Glycogen was omitted from some incubations with little change in reaction product.

Newly formed glycogen was the indicator of the glycogen phosphorylase enzyme activity that occurred during incubation in the medium described. The glycogen was made visible by staining with iodine or by the periodic acid-Schiff technique (PAS). Iodine is regarded as differentially staining short and long carbohydrate chains that occur in starch and glycogen (Swanson, 1948). In our experiments, most control sections showed no iodine staining. However, minimal staining, that was very pale, was evident in some of the sections from rats that had been stimulated with DEX for 4 or 8 h. Some sections were stained with iodine alone, whereas other iodine-stained sections were counter-stained. For counter-staining, sections were washed in H_2O after incubation then stained 30 seconds in an aqueous 1:4 dilution of light green S yellowish (Color Index #42095) prepared from a stock solution that consisted of 200 mg light green S yellowish, 0.2 ml glacial acetic acid and 100 ml H_2O. Sections were rinsed in H_2O and stained 5 min in iodine solution consisting of 1 gm iodine, 2 gm potassium iodide in 100 ml H_2O. The sections were drained and rinsed in the following solutions: 95% alcohol—2 min; 100% alcohol—2 min; xylene—2 min; xylene—5 min; each solution contained 0.1% iodine. Sections were then mounted in Histoclad that contained 0.2% iodine.

The second method used for visualizing the glycogen that represented GP activity was to stain the sections by the period acid-Schiff (PAS) technique. All glycogen within hepatocytes, whether newly formed by the enzyme during incubation or pre-existing prior to incubation, was stained by the PAS method. In the ADX rats that received no DEX and in those that were injected with DEX 2 h prior to sacrifice, glycogen content of hepatocytes was minimal prior to incubation for GP activity. Therefore, after incubation of sections in medium that contained substrate, virtually all glycogen present in the hepatocytes was formed by activity of the enzyme during incubation. However, 4 and 8 h after administration of DEX, a substantial amount of glycogen was present in hepatocytes prior to incubation in medium, therefore PAS staining was less useful for evaluating phosphorylase activity in liver sections from these rats and is not included here.

Amylase Incubation

Tissue sections were incubated in a solution containing one percent amylase. Removal of the evidence of GP activity by amylase confirmed that this product was glycogen. The solution for amylase incubation consisted

Abbreviations

P	Portal canal
C	Central vein
ADX	Adrenalectomized
DEX	Dexamethasone
PAS	Periodic acid-Schiff
GP	Glycogen phosphorylase

of 1 gm malt amylase derived from barley in 100 ml phosphate buffered saline. Sections were incubated at 37°C for 15 min to 1 h and then stained with iodine or PAS to determine if glycogen was present.

RESULTS

The focus of the current study was to detect and localize changes in hepatic GP enzyme activity in normal and adrenalectomized (ADX) rats after fasting overnight and in fasted ADX rats after stimulation of glycogen synthesis by administration of dexamethasone (DEX) 2 to 8 h prior to sacrifice. Cryostat sections from rat livers were incubated in medium containing glucose 1-phosphate as substrate for glycogen phosphorylase. The formation of new glycogen by the enzyme was used to indicate sites of GP enzyme activity. These sites of activity were identified and localized either by iodine staining or by the periodic acid-Schiff reaction.

In cryostat sections from the normal fasted rats, GP enzyme activity was found in many hepatocytes throughout the lobules (Figs. 1 and 2). In some hepatocytes cytoplasmic GP activity was abundant, often outlining the nucleus of cells, but many other hepatocytes showed little or no enzyme activity. Within the cells, the enzyme reaction product was finely and rather evenly dispersed. There was no distinct pattern of concentration of enzyme activity within the lobules, although many pericentral cells were positive for GP activity.

The distribution of GP enzyme activity was very different in the sections from adrenalectomized rats that were fasted overnight before sacrifice. Under these conditions (Figs. 3 and 4) the activity of the enzyme remained sparse, but widely distributed throughout the liver lobules and again there was no definitive intralobular distribution pattern for the concentration of activity. However, within the hepatocytes, that were positive for GP enzyme activity, it appeared as concentrated intracellular foci, instead of being distributed throughout the cytoplasm. In many hepatocytes more than one site of activity was present and in many other cells GP enzyme activity was lacking. In order to confirm the observation that the iodine-stained large aggregates, formed by enzyme activity, were in fact glycogen, some sections were stained with periodic acid-Schiff. The patterns of staining in these sections (Fig. 4) were similar to those observed after iodine staining, i.e., foci of GP enzyme activity appeared in random hepatocytes throughout the liver lobules. Further confirmation of this product as glycogen was provided by the removal of these large aggregates after exposure of sections to amylase (not shown). Glycogen was a constituent of the incubation medium (with and without substrate), however even when glycogen was omitted from the medium, the large aggregates of glycogen, resulting from the activity of GP, continued to occur in the liver sections from the fasted ADX animals.

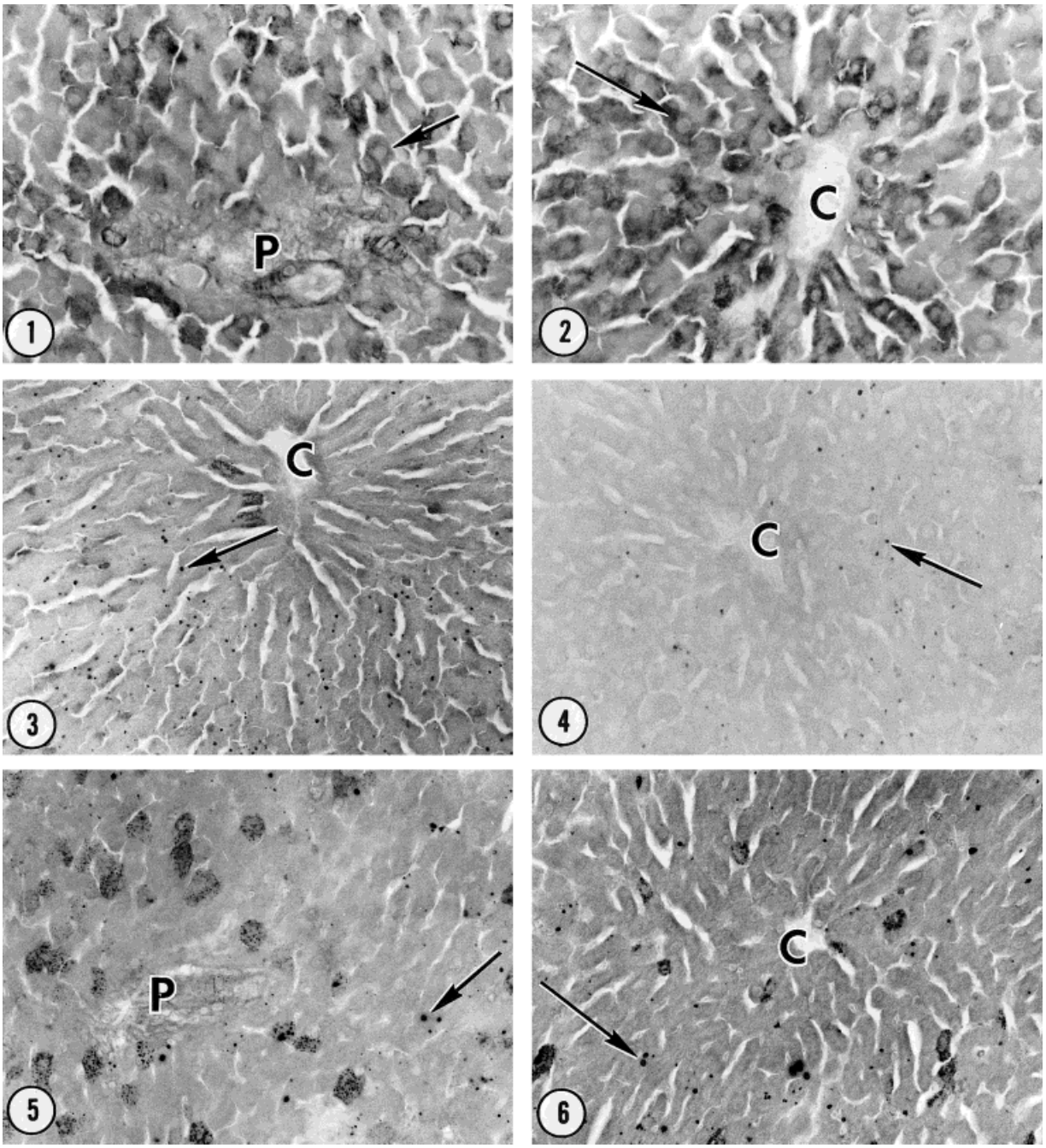
In the sections from the adrenalectomized rats that were fasted overnight and then stimulated to synthesize glycogen by injection of DEX 2 to 8 h prior to sacrifice, the patterns of distribution of GP enzyme activity were altered as glycogen synthesis was stimulated. Two hours after DEX treatment (Figs. 5–8) GP enzyme activity was limited to a few hepatocytes dispersed throughout the lobules. In some hepatocytes, enzyme activity appeared granular throughout the cytoplasm, whereas other cells exhibited large foci of reaction product similar to those observed prior to stimulation of glycogen synthesis. Within the liver lobules, concentrations of GP enzyme activity were not appreciably higher in either pericentral or periportal regions. Staining similar sections with periodic acid-Schiff (Figs. 7 and 8) corroborated these observations.

In the ADX rats that were fasted overnight and injected with DEX 4 h prior to sacrifice, sections revealed GP enzyme activity in the majority of hepatocytes throughout the lobules (Fig. 9). The distribution of sites of GP activity gave the hepatocyte cytoplasm a coarsely granular appearance. In contrast to the sections observed from the other time intervals, in which significant intralobular differences in the concentration of GP reaction product were not detected, a higher concentration of enzyme activity occurred near the pericentral regions. Almost all pericentral hepatocytes were positive for GP enzyme activity, whereas near the portal regions of the lobules some hepatocytes showed little or no enzyme activity.

In the sections from the ADX rats that were fasted overnight and injected with DEX 8 h prior to sacrifice, virtually all hepatocytes showed evidence of GP activity (Figs. 10 and 11). The enzyme activity was relatively evenly distributed throughout the lobules, in both periportal and pericentral regions and the GP reaction product was granular. The granules appeared to be coarser in the periportal hepatocytes and finer in the pericentral cells. In hepatocytes from both the 4 h and 8 h time periods, substantial quantities of glycogen had been formed within the cytoplasm prior to the histochemical procedures, therefore staining by the periodic acid-Schiff method was not appropriate for confirming the presence of glycogen, newly formed by GP activity.

DISCUSSION

In the current study, the intralobular distribution of GP activity was observed in normal and adrenalectomized rats that were fasted overnight and in adrenalectomized rats that were fasted overnight and then stimulated to synthesize glycogen by administration of dexamethasone 2 to 8 h prior to sacrifice. Overall, it was evident that when glycogen synthesis was stimulated, GP activity increased within the lobules and progressively more hepatocytes displayed GP enzyme activity in spite of the fact that the enzyme is associated with glycogen breakdown (e.g., Hers, 1990). The distribution patterns of enzyme activity that were observed in the fasted ADX animals prior to glycogen stimulation were similar to those observed for glycogen synthase enzyme activity (Michaels and Cardell, 1993). In livers from fasted adrenalectomized rats that had been shown previously to contain minimal amounts of glycogen (Cardell, 1975), GP enzyme activity was confined to dense aggregates or "hot spots" in randomly dispersed hepatocytes throughout the lobules. Incubation of the sections in the medium used in the current study apparently induced the localized GP to synthesize reaction product, i.e. glycogen, the identity of which was confirmed either by staining with PAS or removing this product with amylase, prior to staining. Previously, we have shown (Cardell et al., 1985) that after stimulation of glycogen synthesis with dexamethasone, hepato-

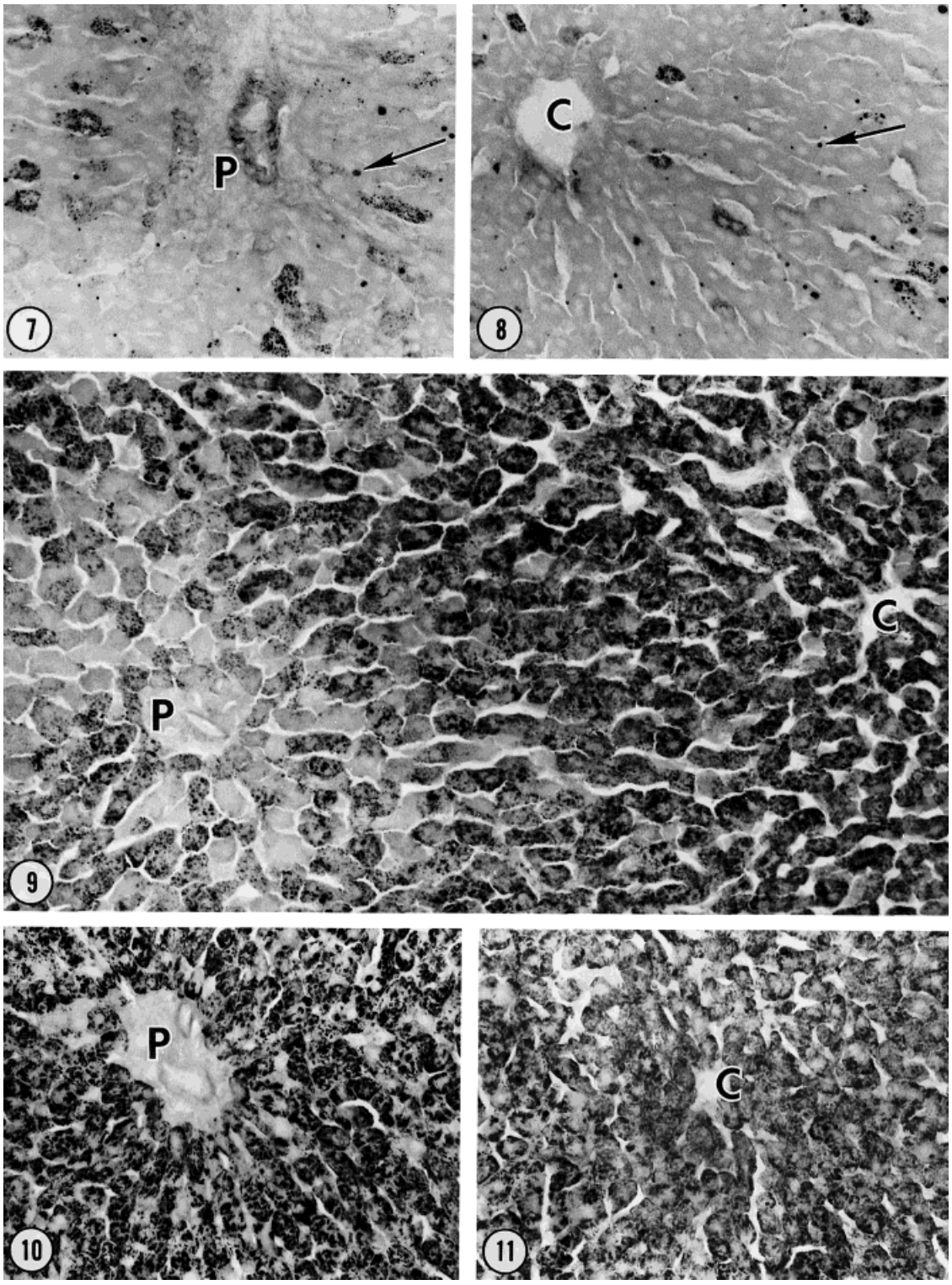


Figs. 1, 2. These sections from liver of fasted normal rats demonstrate that glycogen phosphorylase enzyme activity in hepatocytes (arrows) is limited and dispersed in both periportal **(Fig. 1)** and pericentral **(Fig. 2)** regions. Many hepatocytes show little or no GP enzyme activity. All sections unless otherwise noted were stained with iodine and counter-stained with light green S yellowish. ×215.

Figs. 3, 4. These micrographs show pericentral regions of hepatocytes of fasted ADX rats. Aggregates of glycogen formed by GP enzyme activity are distributed in hepatocytes throughout the lobules (arrows). **Fig. 3:** Section was stained as indicated above. **Fig. 4:** Section was stained by the PAS method and not counter-stained. ×215.

Figs. 5, 6. These micrographs show the distribution of enzyme activity for glycogen phosphorylase in liver from a fasted ADX rat injected with DEX 2 h prior to sacrifice. Hepatocytes in both periportal **(Fig. 5)** and pericentral **(Fig. 6)** regions reveal aggregated (arrows) and dispersed sites of GP enzyme activity. In other hepatocytes GP enzyme activity is not evident ×215.

cytes of adrenalectomized overnight-fasted rats contained cytoplasmic foci that became labeled when injected tritiated-carbohydrate (^{3}H-carbohydrate) precursor was incorporated into glycogen. These foci may correspond to sites of residual concentrations of glycogen and associated glycogen phosphorylase (as well as glycogen synthase, Michaels and Cardell, 1993) that demonstrated enzyme activity.



Figs. 7, 8. These micrographs show the distribution of enzyme activity for glycogen phosphorylase in liver from a fasted ADX rat injected with DEX 2 h prior to sacrifice. Hepatocytes in both periportal **(Fig. 7)** and pericentral **(Fig. 8)** regions reveal aggregated (arrows) and dispersed sites of GP enzyme activity. In other hepatocytes GP enzyme activity is not evident. Sections in Figures 7 and 8 were stained with PAS and counter-stained with light green S yellowish. ×200.

Fig. 9. This micrograph shows liver from a fasted ADX rat injected with DEX 4 h prior to sacrifice. Although many hepatocytes in both periportal and pericentral regions show GP enzyme activity, it is evident that there is a pericentral concentration of enzyme activity at this time interval. ×260.

Figs. 10, 11. These micrographs show sections of liver from a fasted ADX rat injected with DEX 8 hours prior to sacrifice. In virtually all hepatocytes, some GP enzyme activity is found. There are substantial differences in glycogen phosphorylase enzyme activity even between adjacent cells. In periportal cells, the glycogen formed by GP enzyme activity appears as larger aggregates **(Fig. 10)** than in the pericentral hepatocytes in which the glycogen derived from GP enzyme activity exhibits a finer granularity **(Fig. 11)**. ×200.

The intralobular distribution patterns of glycogen phosphorylase activity observed after stimulation of glycogen synthesis were similar to the distribution patterns of glycogen during early glycogen synthesis as reported by Babcock and Cardell (1974). In the present study, a pericentral pattern of distribution of GP enzyme activity (after 4 h DEX treatment) was followed by a more uniform to periportal distribution pattern. The early distribution pattern of glycogen produced by the control-fed rats (Babcock and Cardell, 1974) was also pericentral followed by a more even to periportal distribution pattern. In addition, as glycogen, either metabolically produced in hepatocytes or the result of the activity of GP, accumulated throughout the lobule, the periportal regions showed coarse aggregates, whereas the glycogen in pericentral regions was finely dispersed.

Previous histochemical studies have shown that the intralobular distribution of GP enzyme activity in fasted (Sasse et al., 1975; Frederiks et al., 1987) and in fed rats (Lindberg and Palkama, 1972) was higher periportally than pericentrally. In contrast, Keppens and DeWulf (1988) reported that hepatocytes isolated from either the periportal or pericentral regions had similar glycogenolytic potency. Also, localization of GP protein, identified by immunohistochemistry (Giffin et al., 1993), displayed a pericentral pattern of distribution in livers from both fed and fasted rats. Western blot analysis revealed that within livers of both fed and fasted rats the content of GP protein was about the same (Giffin et al., 1993).

In the current study, it was shown that GP enzyme activity increased concurrently with glycogen synthesis. Other workers have noted also, that activities of both glycogen synthase and phosphorylase followed similar patterns as metabolic conditions were altered. Biochemical assays by Margolis and Curnow (1984) indicated a reduction in total phosphorylase activity in livers of fasted adrenalectomized rats. Two hours after stimulation of glycogen synthesis by injecting these animals with dexamethasone, an increase in total GP activity was found and a further increase occurred 6 h after injection. Enzyme activity of glycogen synthase *a* also increased after dexamethasone treatment. Isolated liver cells have been shown to have a rapid increase in GP activity when exposed to steroid hormones including dexamethasone (Gomez-Munoz et al., 1989).

Other methods have shown a close association between GP activity and sites where GS activity and glycogen were present. Vandaris (1991) determined enzyme activities associated with isolated particles of glycogen separated according to their weight. Glycogen synthase activity was found to be associated with a broad spectrum of glycogen particle sizes, but GP activity, especially in fasted animals, was more concentrated in the smaller particles that may correspond to the fine granular particles observed in the foci described above (Cardell et al., 1985). Roach and coworkers (Cao et al., 1993) have suggested that GP plays a role in modulating the state of glucosylation of glycogenin, thereby determining whether or not glycogenin can serve as a substrate for glycogen synthase. In an in vivo study of incorporation of ^{13}C-glucose into glycogen, using nuclear magnetic resonance spectroscopy, Barnett and coworkers (David et al., 1990) concluded that in both fasted and fed rats, glycogen synthase and phosphorylase were active, resulting in turnover of glycogen as net glycogen synthesis occurred. In addition, glycogen turnover was more notable in the livers of rats that were not fasted prior to the experiment. They found that activity of glycogen phosphorylase was higher after substantial quantities of glycogen were formed. Subsequent experiments using dogs (Barrett et al., 1994) confirmed and extended the earlier study. We have suggested previously (Michaels et al., 1990), that glycogen turnover occurred during long-term stimulation of glycogen synthesis. The increase in enzyme activity, observed in the present study, after 2 to 8 h stimulation of glycogen synthesis, suggests that the enzyme may participate in one or more aspects of glycogen metabolism, such as glycogen turnover and/or glycogenin modification, during this early period of overall glycogen synthesis.

In conclusion, it was found that in fasted adrenalectomized rats, enzyme activity of glycogen phosphorylase was confined to small foci similar to those shown for glycogen synthase (Michaels and Cardell, 1993). Stimulation of glycogen synthesis increased the enzyme activity of both enzymes, however, apparently GP activity transiently exhibited a pericentral pattern of distribution at a time when GS enzyme activity was more concentrated in the periportal region. After longer stimulation of glycogen synthesis, GP enzyme activity appeared more evenly distributed within the lobules. Periportal hepatocytes formed glycogen from GP activity that consisted of large aggregates, whereas the glycogen formed from GP activity in pericentral cells was finely granular.

ACKNOWLEDGMENTS

We thank Mrs. Thelma Shepard for technical assistance and Mr. Paul Esmahan for photographic assistance. This work was supported by U.S.P.H. Grant DK27097.

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