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Glucocorticoid Regulation of Rat Liver Urate Oxidase

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Abstract Urate oxidase, an enzyme involved in purine catabolism, comprises the crystalline core of rat liver peroxisomes. An affinity-purified monospecific antibody was developed to study the expression of urate oxidase protein levels. Immunoreactive urate oxidase was not detectable in prenatal liver; however, it is present at low levels after birth until approximately day 15 (postnatal age); expression sharply increases just prior to day 20, after which the enzyme is maintained at adult levels. This pattern of expression was similar to that of another peroxisomal enzyme, catalase; these developmental increases reflect the increase in peroxisomal number. Administration of exogenous glucocorticoid hormone to 10-day-old rats resulted in a precocious rise (2.5-fold) in urate oxidase levels. Adrenalectomy at 10 days of age did not cause decreased levels in the fourth week of life. In adult animals, while exogenous glucocorticoid administration did not influence urate oxidase levels, adrenalectomy at 60 days of age decreased urate oxidase levels to 40 percent of control levels. Subsequent administration of exogenous glucocorticoid hormone restored urate oxidase to normal levels. Parallel studies of catalase levels indicate that this glucocorticoid-sensitive response is not generalized for all peroxisomal proteins. Our results suggest that peroxisomes proliferate during early postnatal development, but after this process is complete, the biogenesis of individual peroxisomal proteins may be independently regulated.

Key words: hepatic development, peroxisomes, monospecific antibody, urate oxidase, rat liver, glucocorticoids

Urate oxidase (urate: O_2 oxidoreductase, E.C. 1.7.3.3) is a cuproprotein which participates in purine base catabolism, degrading urate to produce allantoid, hydrogen peroxide, and carbon dioxide. Urate oxidase is present in the liver of most vertebrates, including several genera of New and Old World monkeys, but absent in humans and other hominoids [1]. In rat liver, urate oxidase is localized to a specialized subcellular structure, the peroxisomal crystalline core [2]. Urate oxidase is synthesized on free polyribosomes [3] and the polypeptide inserted into preexisting peroxisomes without any post-translational modifications [4]. Urate oxidase is a tetramer composed of four identical polypeptides; the subunit size deduced from the cDNA sequence is estimated to be 35 kDa [5].

The expression of urate oxidase and other peroxisomal proteins has been examined using hypolipidemic agents, such as clofibrate, its analog ciprofibrate, and phthalate ester plasticizers; these are well-documented proliferators of liver peroxisomes [6,7,8] although the mechanism involved has not been elucidated [9]. The enzymes of the peroxisomal fatty acid β-oxidation system have been induced by in vivo administration of these chemicals [10]. The mRNAs for the three peroxisomal β-oxidation enzymes increased over twentyfold following plasticizer administration; however, the same study demonstrated only a twofold increase in urate oxidase and catalase mRNAs [11]. Peroxisomal proliferators can differentially induce peroxisomal enzymes and membrane proteins [10,12].

Thyroid hormones induce peroxisomal proliferation [13,14]. Following thyroxine treatment of adult male rats, activities of catalase and D-amino acid oxidase were increased, while urate oxidase levels decreased. Prolonged administration of thyroxine exacerbated these trends [13]. Thyroxine-treated rats were also demonstrated

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to have 2.5-fold increased peroxisomal palmitoyl-CoA oxidation [15]. Consistent with these studies, thyroidectomy alters liver peroxisomal enzymes [16].

While the effects of peroxisomal proliferators and thyroid hormones on urate oxidase expression have been investigated, to date no definitive studies evaluating the regulatory elements responsible for the expression of urate oxidase have been reported. Initial evidence obtained in the present study suggested that urate oxidase is differentially expressed in rat liver during postnatal development. An abrupt increase in the level of urate oxidase occurs during the third week of life. Regulation of this differential developmental expression corresponds to changes in the hormonal status of corticosterone and thyroxine. Because glucocorticoids had not previously been explored as modulators of liver urate oxidase, their role as possible effectors of urate oxidase expression was investigated in this study in both immature and adult rats by hormone administration and adrenalectomy. We report here that the expression of rat liver urate oxidase is regulated in a specific manner by glucocorticoids.

METHODS

Preparation of Anti-Urate Oxidase Antibody (AbUOx)

A Percoll density gradient fraction (density = 1.111) obtained from subcellular fractionation of rat liver homogenates was used to prepare immune sera. This preparation was osmotically lysed and the insoluble pellet recovered after centrifugation was used as antigen for antibody production. Free crystalline cores (composed of urate oxidase) from disrupted peroxisomes were enriched in this pellet. Rabbits were immunized by standard protocols to produce a polyspecific, polyclonal antibody. From immune sera, an immunoglobulin fraction was prepared by chromatography on CM Affigel Blue resin (BioRad), followed by ammonium sulphate precipitation.

Affinity-purified antibody to urate oxidase was obtained by antigen affinity chromatography. Rat liver urate oxidase, purified and solubilized according to Watanabe et al. [17], was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). The column was equilibrated in 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl, polyclonal antibody applied, and the column washed with the equilibration buffer. The affinity-purified antibody (AbUOx) was eluted from the column with 0.2 M glycine, pH 2.8, 0.5 M NaCl, immediately neutralized with 2 M Tris, pH 8.0, and dialyzed against 0.15 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4.

Additional Antibodies

Mouse monoclonal anti-actin antibody was obtained from ICN Immunochemicals (Lisle, IL). Sheep anti-catalase antibody was purchased from Biodesign International (Kennebunkport, ME).

Immunoblotting

Sodium dodecyl sulphate (SDS)-polyacrylamide gels were performed according to Laemmli [18]. For immunoblot analysis, proteins, separated on SDS gels, were electrophoretically transferred to nitrocellulose [19]. Gels were transferred in 25 mM Tris, 192 mM glycine, 20% methanol for 5–6 hours at 200 mA, 25°C. After blotting, nitrocellulose sheets were stained briefly with 0.1% Ponceau S (Sigma), 1.5% trichloroacetic acid, and 1.5% sulfosalicylic acid to determine the efficiency of transfer, then destained with distilled water.

For immunological detection, nitrocellulose blots were blocked with 3% nonfat dry milk (NFDM) in 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl (Tris buffered saline [TBS]) for 1 hour at room temperature, then incubated at 4°C overnight with primary antibody diluted in 1% NFDM-TBS. The blots were rinsed 3 times with 1% NFDM-TBS and incubated with affinity-purified peroxidase-conjugated second antibody (BioRad) for 1 hour at room temperature. After a 5 minute rinse with 0.05% Tween in TBS, the blots were rinsed twice for 5 minutes each with TBS. A color development solution was prepared by mixing 20 ml of cold methanol containing 60 mg 4-chloro-1-napthol into a solution of 0.018% hydrogen peroxide in 100 ml room temperature TBS. This reagent was incubated with the blots at room temperature until purple-grey precipitates appeared on the surface of the nitrocellulose. Development was stopped by rinsing blots three times in distilled water.

Densitometric Analysis of Immunoblots

For quantification of bands on immunoblots, nitrocellulose sheets were scanned directly using a Zeineh Soft Laser scanning densitometer with a tungsten light source. The signals detected were recorded as peaks on a chart re-

corder. The peaks were triangulated and the area of the resulting triangle calculated. These absolute values were compared on each blot to a reference sample and expressed as the percent of the reference value. Statistical significance of immunoblot results was assessed using Student's t-test [20,21].

Immunoelectron Microscopy

Rat liver specimens (approximately 1 mm^3 in size) were fixed for 1 hour with 1% paraformaldehyde, 1% glutaraldehyde in 50 mM sodium cacodylate, pH 7.2, 50 mM KCl, 2.5 mM $MgCl_2$. After fixation, samples were rinsed five times, 5 minutes each, with the cacodylate buffer on ice. The specimens were then dehydrated stepwise in dimethylformamide and embedded in Lowicryl K4M [22]. Lowicryl-embedded specimens were sectioned using a diamond knife and 80 nm sections mounted on 150-mesh nickel grids.

Grids were initially incubated for 5 minutes on drops of 0.2 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.5% Tween 20, 0.1% Triton X-100 (detergent-PBS), then exposed for 1 hour at room temperature to primary antibody diluted (20 μg/ml) in the detergent-PBS buffer. After a brief rinse with the buffer alone, the grids were exposed to colloidal gold-conjugated goat anti-rabbit antibody (Jannsen Life Sciences Products) diluted 1:20 (volume:volume) in buffer for 1 hour, then washed with distilled water. Specimens were post-stained for 5 minutes in 2.0% aqueous uranyl acetate, washed with distilled water and air-dried. Sections were observed using a Philips 410 electron microscope operated at 60kV.

Preparation of Tissue Homogenates

Tissue samples were collected from rats and immediately used for homogenization or quick-frozen on dry ice and stored at −70°C until use. Tissue extracts were prepared by homogenization in 20 volumes of 60°C Laemmli sample buffer, adjusted to 5% SDS and 10 mM EDTA. Extracts were heated to 90°C for 10 minutes, then centrifuged for 20 minutes at 10,000*g* to remove insoluble material [23]. The supernatant material (25 μl/lane) was used for gel electrophoresis and immunoblotting, resulting in equivalent amounts of tissue extract to be electrophoresed in all lanes.

Prevention of Weaning

Rat pups were prevented from weaning by using the 16 + 8 suckling protocol [24] starting on postnatal day 13. Pups were housed with their mothers in cages that provided water but no access to solid food. The dams were removed from these cages during the first 8 hours of the dark cycle (1800 to 0200 hours) and allowed to feed. The dams were then returned to the pups for the remaining 16 hours of each day (0200 to 1800 hours). This schedule was continued through postnatal day 25. Control rat pups were allowed access to solid food ad libitum and were weaned by removal of the dams at 21 days of age. Two pups from each litter were sacrificed on postnatal days 15, 20, 25, and 31, and liver tissue removed.

Hydrocortisone and Aldosterone Administration

Hydrocortisone acetate (Merck, Sharp and Dohme) in saline was administered (50 μg/gram body weight) to ten-day-old rats in a single subcutaneous injection. Control littermates were injected with an equal volume of saline based on weight. The same dose and route of administration was used for hydrocortisone treatment of 60-day-old adult rats. All rats were sacrificed five days after hydrocortisone or saline treatment.

For administration of hormones to sham-adrenalectomized or adrenalectomized adult rats, injections were begun four days post-surgery. Hydrocortisone acetate (50 μg/gram body weight) in saline was administered in a single dose subcutaneously. Aldosterone (Sigma; 50 μg/kilogram body weight) in saline was administered twice a day subcutaneously. All rats were sacrificed on the ninth post-surgical day.

Adrenalectomy and Hypophysectomy of Rats

Ether-anesthetized ten-day-old Sprague-Dawley rats were subjected to bilateral adrenalectomy in the laboratory of Dr. Susan Henning (University of Houston, Houston, TX). After removal of the adrenal glands with forceps, sutures were used to close the incision and the rats were kept in a warm bed for approximately 1.5 hours before being returned to the litter. As a control, sham adrenalectomies were performed on three pups from each litter. Beginning on postnatal day 11 and continuing through day 21, aldosterone in 0.9% NaCl (8 μg/100 grams body weight) was administered daily by subcuta-

neous injection to adrenalectomized rats. Sham-operated rats were given equivalent volumes of saline, based on body weight, as the adrenalectomized rats. In addition to drinking water, the adrenalectomized rats were provided with 0.9% NaCl post-operatively. The pups were given free access to solid food and on day 21, all pups were completely weaned by removal of the mother from the cage. Half of the pups from each litter were sacrificed on postnatal days 23 and 26, and liver samples collected.

Adult rats were either adrenalectomized or sham-adrenalectomized at 60 days of age at Harlan Sprague-Dawley (Indianapolis, IN). Adrenalectomized animals were provided access to 0.9% NaCl. Animals were sacrificed four days post-surgery.

Trunk blood was obtained from adrenalectomized rats as they were sacrificed and the serum assayed for corticosterone to determine the efficiency of the surgical procedure. Corticosterone concentration was determined using a modified competitive binding protein method [25] in the laboratory of Dr. Susan Henning. Only tissue from completely adrenalectomized rats was used for immunoblotting.

Adult rats were hypophysectomized at Harlan Sprague-Dawley. These animals were maintained on standard chow and were sacrificed approximately 7 weeks post-surgery and liver tissue removed for analysis.

RESULTS

Antibody against urate oxidase was affinity purified (AbUOx) and the specificity of the antibody was examined by immunoblot and immunolocalization studies. Immunoblot analysis of rat liver homogenates demonstrated reactivity of AbUOx with a single antigen, a protein with an apparent molecular weight of 35 kDa. AbUOx immunoblots of homogenates from sixteen different rat tissues determined that the expression of the immunoreactive 35 kDa protein was liver-specific. This tissue distribution of urate oxidase has been reported by other investigators, using both Western and Northern blot results; expression was found to be restricted to the liver [1,26]. Authentic anti-urate oxidase antibody obtained from J.K. Reddy (Northwestern University Medical School; see reference 1) was used to verify the identity of the 35 kDa protein. Identical nitrocellulose blots with two samples, the original immunogen mixture and purified rat liver peroxisomal crystalline cores, were developed with the affinity-purified AbUOx and the polyclonal antibody provided by Dr. J.K. Reddy, respectively. Both antibodies reacted specifically with the 35 kDa protein in the immunogen lane and with the crystalline core protein, urate oxidase (data not shown). The reactivity of AbUOx with the 35 kDa protein in both samples represents immunologic identity of the two antigens. As a final demonstration of the specificity of AbUOx, immunolocalization studies were performed on normal rat liver (Fig. 1). Using a colloidal gold-conjugated second antibody, AbUOx binding was localized to the crystalline core of hepatocyte peroxisomes (Fig. 1b–d). In control sections stained with preimmune antibody (Fig. 1a), peroxisomes and peroxisomal cores were devoid of gold particles. The crystalline core of rat liver peroxisomes has been well-defined biochemically; it is composed of a single protein, urate oxidase (see reference 2 for discussion). The collective results from both immunoblot and immunocytochemical analyses demonstrate that AbUOx is monospecific for urate oxidase. AbOUx was subsequently utilized for studies of urate oxidase expression in the developing rat liver.

To determine the expression of immunoreactive urate oxidase during the development of the rat, liver homogenates from animals of different developmental stages were analyzed on immunoblots. As shown in Figure 2, urate oxidase appears to be present at low levels soon after birth, with a slight decrease between days 7 and 10. A significant increase in urate oxidase level occurs between postnatal days 15 and 20, based on the width and intensity of the chloronaphthol signal. Figure 2 also demonstrates the consistency of the urate oxidase level in individual animals of each age group. A broad range of developmental ages, beginning with fetal tissue and continuing to sixty-day-old adult animals, is examined in Figure 3. Immunoreactive urate oxidase is not present in fetal liver; however, on the first day after birth detectable levels of protein appear. The increased expression seen at day 20 is maintained throughout subsequent development to adulthood.

In order to quantitate these results, immunoblots were analyzed by scanning densitometry and the densitometric signals expressed as antigen levels relative to controls run on the same blot. The immunoblot data presented in Figure 3 was scanned and urate oxidase levels expressed as a percent of the adult level (Fig. 4).

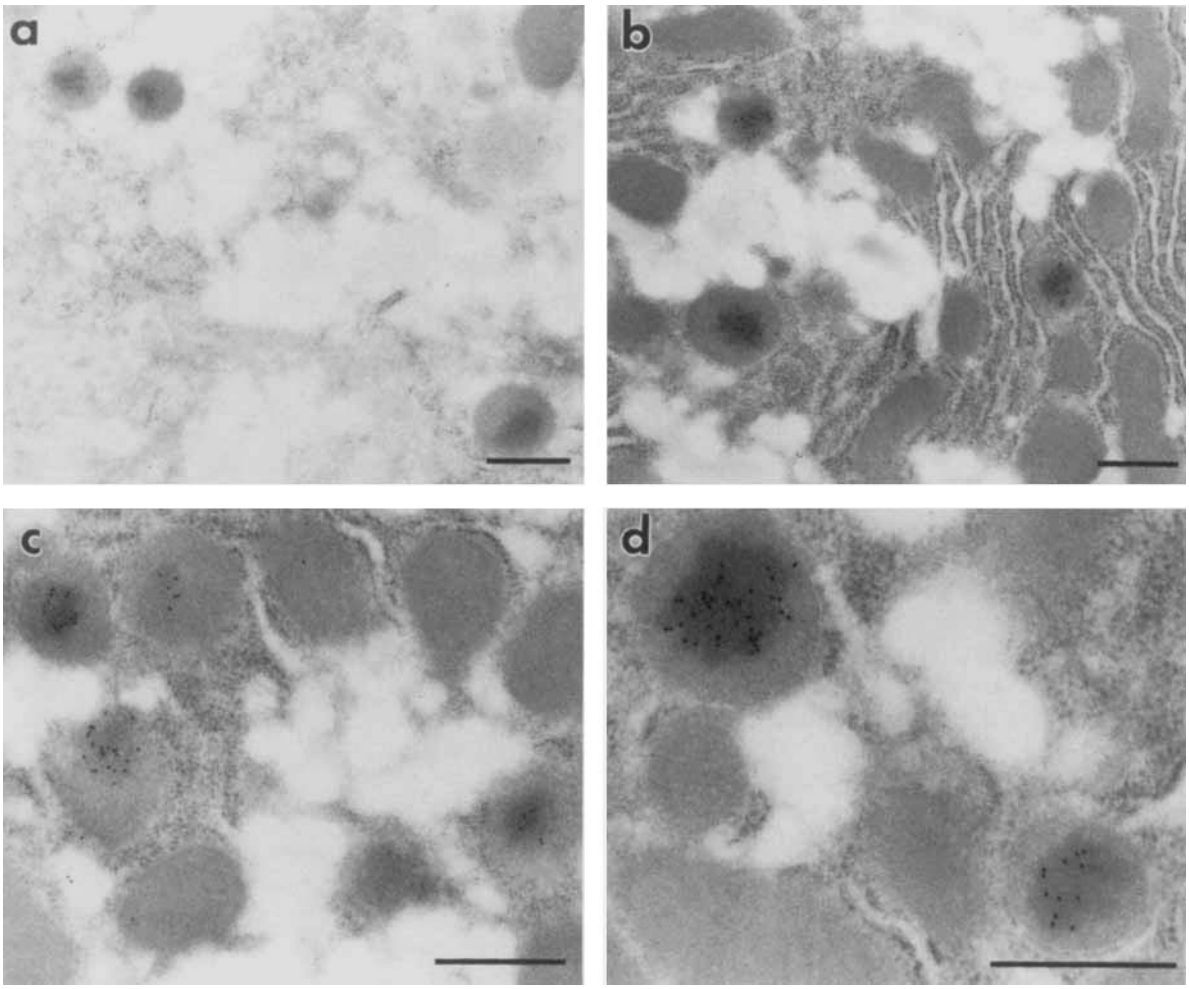

Fig. 1. Immunoelectron microscopy of rat liver sections with AbUOx. Rat liver specimens in Lowicryl were sectioned and the sections incubated with affinity-purified AbUOx. The primary antibody was followed by incubation with colloidal gold-conjugated second antibody. **a:** Preimmune IgG. **b–d:** Urate oxidase affinity-purified AbUOx. Bar, 0.5 μm.

This analysis corroborates the major changes that can be discerned from the immunoblot; urate oxidase levels increase to a plateau at day 20.

An identical immunoblot was developed with an anti-actin antibody to serve as a marker for constitutive cellular proteins. Actin is maintained at a relatively static level over the developmental timecourse used in this experiment (Fig. 4). Therefore, the differential developmental expression of urate oxidase cannot be explained by a general increase in all cellular proteins. Actin served as a control in subsequent experiments to demonstrate that the different homogenate preparations contain similar amounts of total cellular protein. The developmental expression of another peroxisomal enzyme, catalase, was also determined by immunoblot analysis of these liver homogenates. As shown in Figure 4, catalase expression, in general, reflects the developmental differences observed for urate oxidase; catalase undergoes the abrupt increase in antigen level between days 15 and 20.

During the third week of life, circulating levels of two hormones, corticosterone and thyroxine, increase dramatically in the rat [27]. The relationship between thyroid hormone levels and peroxisomal proteins has previously been explored [13,14,15,16]. This study was designed to determine other possible factors responsible for the differential expression of urate oxidase in development. One class of molecules which regulate developmental changes, the sex steroids, was found not to be involved in influencing urate oxidase levels. In studies of both immature and adult animals, no difference in urate

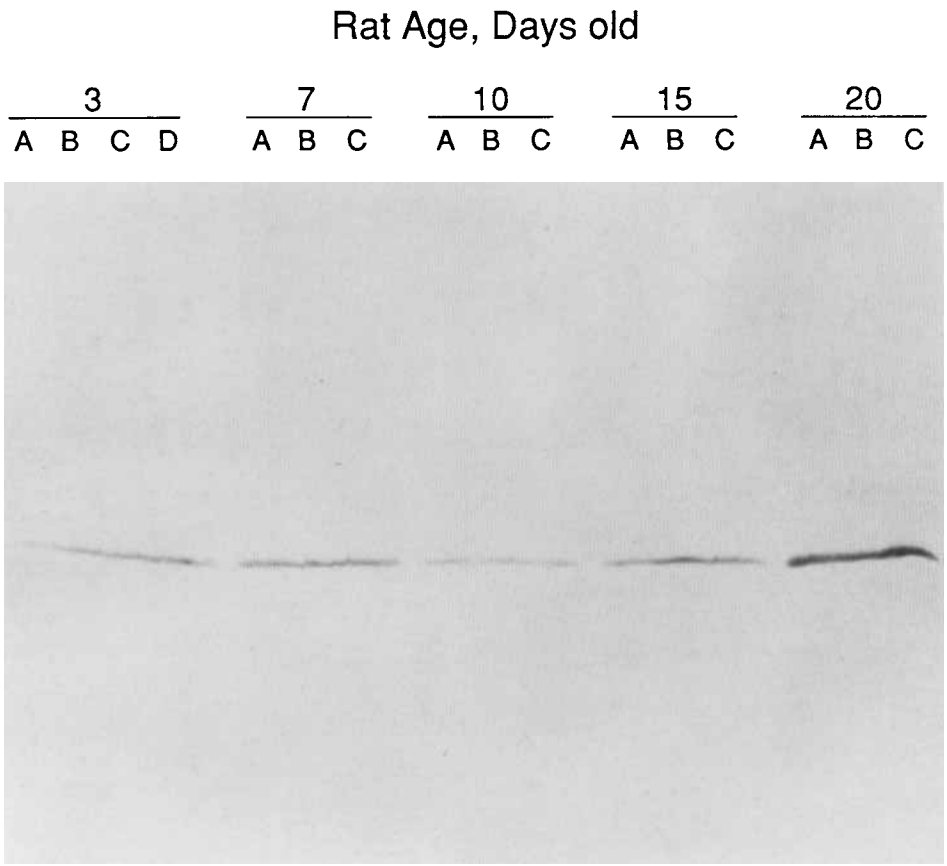

Fig. 2. Urate oxidase levels in rat liver homogenates during development. Equivalent amounts of rat liver tissue homogenates (25 μl/lane) were separated by SDS-gel electrophoresis and transferred to nitrocellulose. The nitrocellulose sheet was incubated with AbUOx, then developed with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The letters over the individual lanes represent individual animals used as source of tissue.

oxidase expression was detected between male and female rats by AbUOx immunoblot analysis. For all studies described there, the sex of the rats was randomized if the animals were sacrificed when less than 20 days old; female rats were utilized at postnatal day 20 and older. To investigate the putative role of another developmental modulator, corticosterone, glucocorticoid regulation of rat liver urate oxidase levels was examined in both immature and adult animals. The relationship between the increases in urate oxidase and in circulating corticosterone was studied by injecting 10-day-old rats with hydrocortisone or saline, then collecting liver samples at day 15 to be assayed by immunoblots. The results are presented in Figure 5. As demonstrated with the saline-injected animals, urate oxidase levels are normally "pre-peak" at day 15. In hydrocortisone-treated animals, however, the urate oxidase levels were increased approximately 2.5-fold in comparison to day 10 levels. The increase in the mean urate oxidase levels in hydrocortisone-treated animals compared to saline-treated animals was statistically significant (at $P = 0.01$). This elevation resembles the physiological increase seen in developing rats between postnatal days 15 and 20. Immature rats were also examined for the effect of adrenalectomy on urate oxidase levels. Animals were adrenalectomized at 10 days of age, before the normal surge in glucocorticoids occurs, and sacrificed 13 or 16 days later, after the surge of urate oxidase expression normally occurs. The mean (± standard deviation) circulating levels of corticosterone in serum was 2.87 ± 1.85 μg/dl for sham animals (n = 6), and 0.61 ± 0.18 μg/dl for adrenalectomized animals (n = 9) at the time of sacrifice. The results of quantitative AbUOx immunoblot analysis of these liver homogenates are presented in Figure 6. When the corticosterone is effectively removed, as the serum values indicate, urate oxidase is expressed at the level similar to those seen in sham-operated animals. These results suggest that in immature ani-

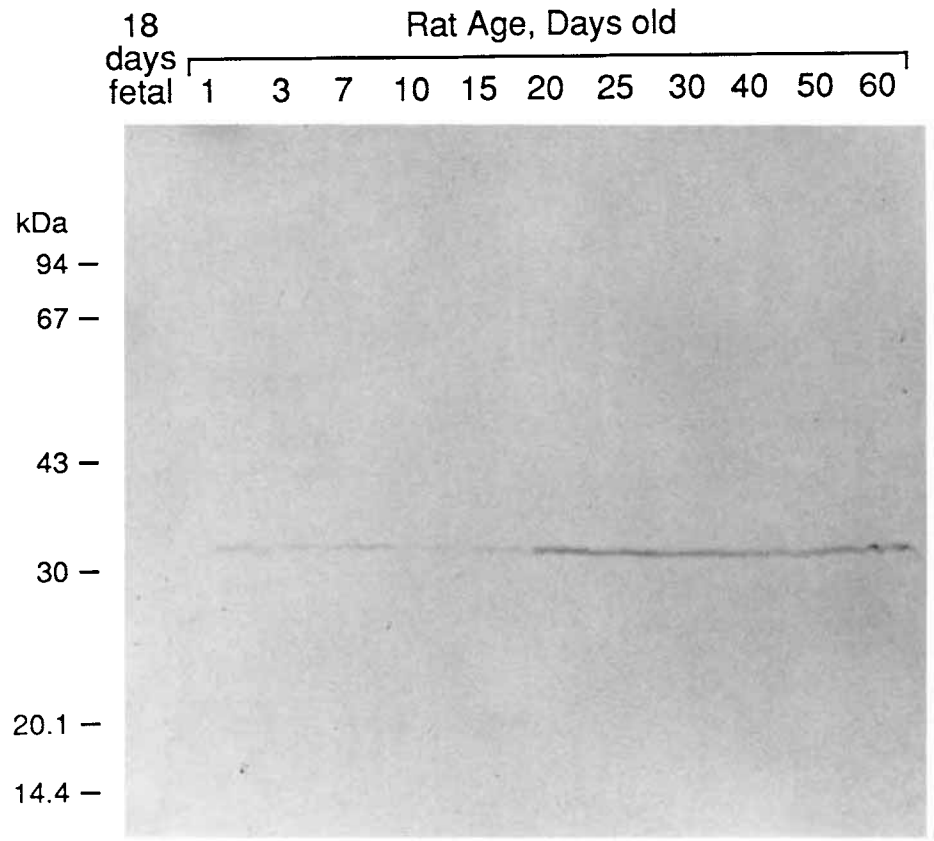

Fig. 3. Urate oxidase levels in rat liver homogenates during development. Equivalent amounts of rat liver tissue homogenates (25 μl/lane) were separated by SDS-gel electrophoresis and transferred to nitrocellulose. The nitrocellulose sheet was incubated with AbUOx, then developed with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The positions of molecular weight marker proteins are indicated to the left.

mals, although exogenous glucocorticoid can directly elevate urate oxidase levels, corticosterone does not solely regulate urate oxidase, as adrenalectomy has no effect.

Although immature rats were found to have significantly increased levels of urate oxidase after hydrocortisone administration (see above), hydrocortisone treatment of adult rats (Fig. 7) had only a slight effect which was not statistically significant (at $P = 0.01$). Adrenalectomy of adult animals resulted in a decrease in urate oxidase levels to about 40% of the sham level (Fig. 8); this reduction in the mean urate oxidase level was statistically significant (at $P = 0.01$) in comparison to the mean sham value. It has been reported in adult animals, a few days after adrenalectomy, that the gonads begin production of corticosterone to offset the loss of adrenal tissue as the hormone source [28]. In contrast to the protocol used for adrenalectomized immature animals, adult rats were sacrificed only 4 days post-adrenalectomy to avoid the interference of gonadally produced hormones in the study. The serum concentrations

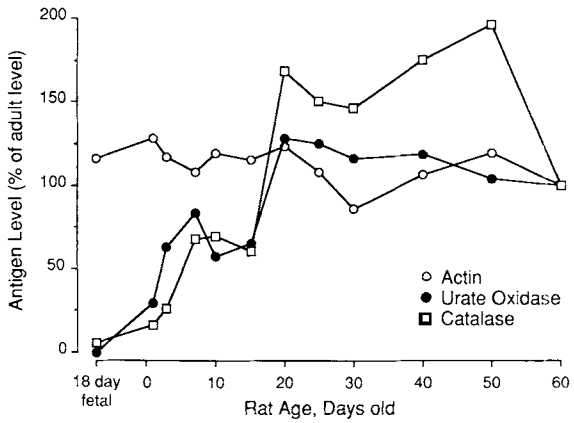

Fig. 4. Antigen levels in rat liver homogenates during development. Immunoblots of liver homogenates (prepared as in Methods) from rats of different developmental ages were developed using AbUOx, anti-catalase antibody, or anti-actin antibody as primary antibody, followed by peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane calculated. Antigen levels were expressed as the percent of the adult (60-day-old) level and plotted versus rat age. Each point represents the antigen level determined in one liver homogenate per age set.

of corticosterone in all adult adrenalectomized animals at the time of sacrifice were below the level of detection in the standard assay, indicating that 4 days post-surgery is a sufficient time period to achieve glucocorticoid deficiency.

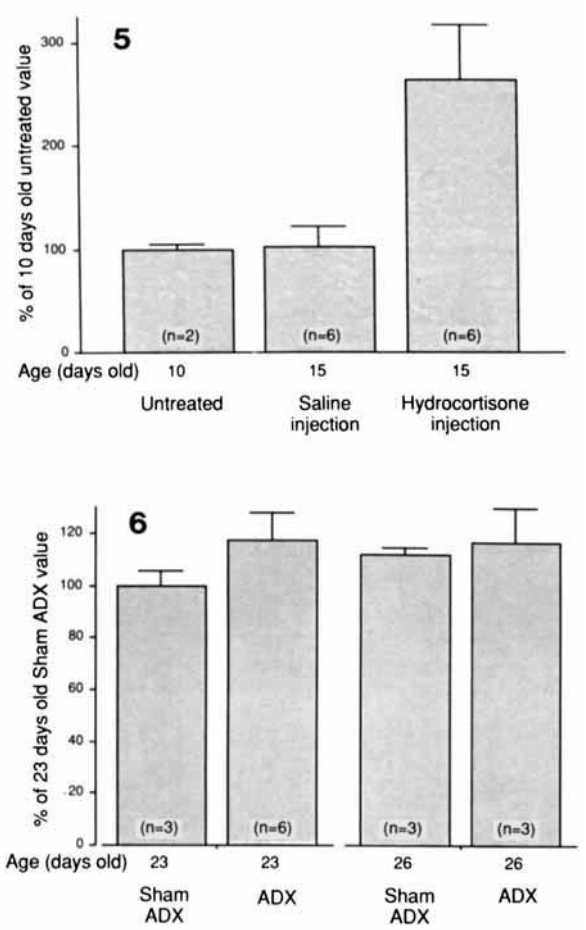

Fig. 5. Effect of hydrocortisone acetate administration on urate oxidase levels in immature rats. Suckling rats from two litters were injected at 10 days of age with saline or hydrocortisone acetate (50 μg/gram body weight). Five days after treatment, these rats were sacrificed and liver homogenates were prepared. Liver samples were also collected from untreated rats at 10 days of age. Tissue homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with AbUOx before development with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane calculated. Urate oxidase levels were expressed as percent of antigen level in 10-day-old untreated littermate rats. Each value is the mean antigen level as determined from homogenates of two untreated rats or six injected rats; error bars represent the standard deviation. An identical immunoblot was developed with an anti-actin antibody and the 4-chloro-1-naphthol signals quantitated. Actin levels in 15-day-old animals, expressed as the percent of the level in the liver of 10-day-old untreated littermate rats, were 128.5 ± 34.7% (n = 6) for saline-injected rats, and 94.8 ± 26.8% (n = 6) for hydrocortisone-injected rats.

Just et al. [13] demonstrated that increases in catalase activity correlate with peroxisomal proliferation in rat liver. To determine if the types of changes described for urate oxidase levels in hydrocortisone-injected immature rats and adrenalectomized adult rats were similar to another peroxisomal protein, the experimental samples were analyzed by quantitative immunoblotting with an anti-catalase antibody (Fig. 9). Catalase levels remain relatively constant despite variation of the glucocorticoid status of the animal.

To further define the role of glucocorticoids and other modulators in the regulation of urate oxidase, additional in vivo studies were designed. The ability of exogenous hormone administration to alter decreased urate oxidase levels in adrenalectomized adult rats is examined in Figure 10. As in Figure 8, adult rats that have been adrenalectomized have decreased urate oxidase levels as assayed by AbUOx immunoblot. Hypophysectomized adult rats, which have decreased adrenocorticotropin function, also demonstrated a marked decrease in liver urate oxidase expression.

To assess the role of two major adrenocortical hormones, aldosterone and corticosterone, in influencing liver urate oxidase levels, sham-adrenalectomized and adrenalectomized adult rats were treated with either aldosterone or hydrocortisone acetate four days post-surgery. Rats were sacrificed five days after initiation of hormone administration and liver homogenates analyzed on AbUOx immunoblot (Fig. 10). When compared to uninjected sham-adrenalectomized animals, neither hormone appeared to affect

Fig. 6. Effect of adrenalectomy at postnatal day 10 on urate oxidase levels in immature rats. Two litters of rats were subjected to adrenalectomy or sham adrenalectomy at 10 days of age. Rats were sacrificed at 23 or 26 days of age and liver homogenates prepared. Tissue homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with AbUOx before development with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane were calculated. Urate oxidase levels were expressed as the percent of antigen level in sham-operated 23-day-old littermate rats. Each value is the mean antigen level as determined from three or six rat liver homogenates; error bars represent the standard deviation. An identical immunoblot was developed with an anti-actin antibody and the 4-chloro-1-naphthol signals quantitated. Actin levels, expressed as the percent of the level in the liver of sham-operated 23-day-old littermate rats, were 76.3 ± 16.8% (n = 6) for adrenalectomized 23-day-old rats, 114.7 ± 3.1% (n = 3) for sham-operated 26-day-old rats, and 81.3 ± 6.9% (n = 3) for adrenalectomized 26-day-old rats.

liver urate oxidase levels in sham-operated animals. However, in the adrenalectomized animals, where the liver urate oxidase levels are decreased, hydrocortisone but not aldosterone treatment restored normal levels of urate oxidase. This glucocorticoid stimulation of urate oxidase expression in adrenalectomized rats demonstrates that glucocorticoid administration alone is sufficient to compensate for the loss of adrenal tissue in these adult animals.

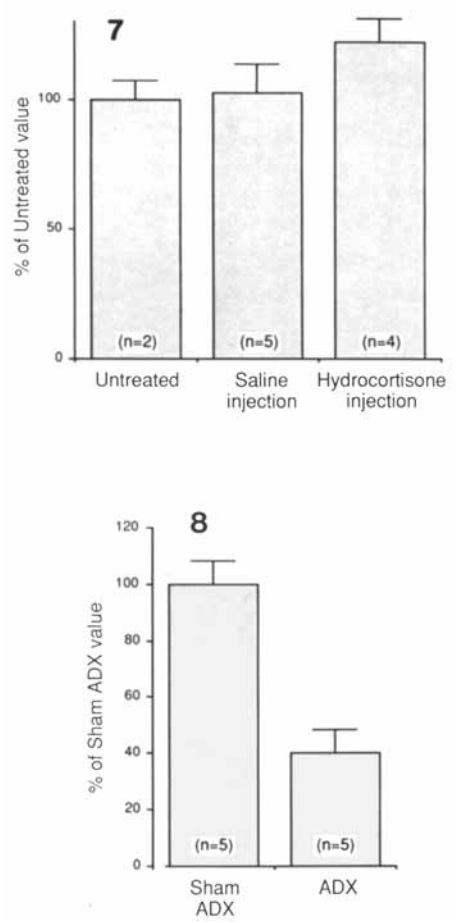

Fig. 7. Effects of hydrocortisone acetate administration on urate oxidase levels in adult rats. Sixty-day-old adult rats were injected with saline or hydrocortisone acetate (50 μg/gram body weight). Five days after treatment, these rats were sacrificed and liver homogenates were prepared. Liver samples were also collected from two sixty-day-old untreated rats. Tissue homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with AbUOx before development with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane calculated. Urate oxidase levels were expressed as the percent of antigen level in 60-day-old untreated rats. Each value is the mean antigen level as determined from homogenates of five saline-injected or four hydrocortisone-injected rats; error bars represent the standard deviation. An identical immunoblot was developed with an anti-actin antibody and the 4-chloro-1-naphthol signals quantitated. Actin levels, expressed as the percent of the level in 60-day-old untreated rat liver, were 87.4 ± 2.5% (n = 5) for saline-injected animals and 92.8 ± 6.2% (n = 4) for hydrocortisone-injected animals.

DISCUSSION

Differential Expression of Liver Proteins

Investigations of the differential expression of liver proteins have focussed on two aspects: developmental differentiation and the influence of endogenous or exogenous factors such as hormones or pharmacologic agents. Greengard [29] classified liver-specific proteins into three categories based on the developmental age at which they were induced: 1) late fetal, around the 17th day of gestation; 2) early neonatal, occurring at or immediately after birth; and 3) late suckling, during the third postnatal week, just prior to weaning. If urate oxidase were to be assigned to one of these categories of developmental expression, the results of the present study (Fig. 4) suggest that urate oxidase belongs in the third group, being elevated just prior to weaning. It is of interest to note that the enzymes activated in the late suckling period, perhaps representing the final differentiation of hepatocytes in the formation of adult liver, are diverse in their functions and appear to be unrelated [29].

Having established the pattern of expression for urate oxidase, possible mechanisms responsible for the induction of urate oxidase in the third week of development were explored. Three physiologic changes which occur at or just prior to the third week of life are weaning, increased levels of corticosterone, and increased levels of

Fig. 8. Effect of adrenalectomy on urate oxidase levels in adult rats. Sixty-day-old adult rats were subjected to adrenalectomy or sham adrenalectomy. Rats were sacrificed four days after surgery and liver homogenates prepared. Tissue homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with AbUOx before development with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane calculated. Urate oxidase levels were expressed as the percent of antigen level in sham-operated animals. Each value is the mean antigen level as determined from five rat liver homogenates; error bars represent the standard deviation. An identical immunoblot was developed with an anti-actin antibody and the 4-chloro-1-naphthol signals quantitated. Actin levels, expressed as the percent of the level in liver homogenates of sham-operated animals, were 65.0 ± 22.4% (n = 5) for adrenalectomized adult rats.

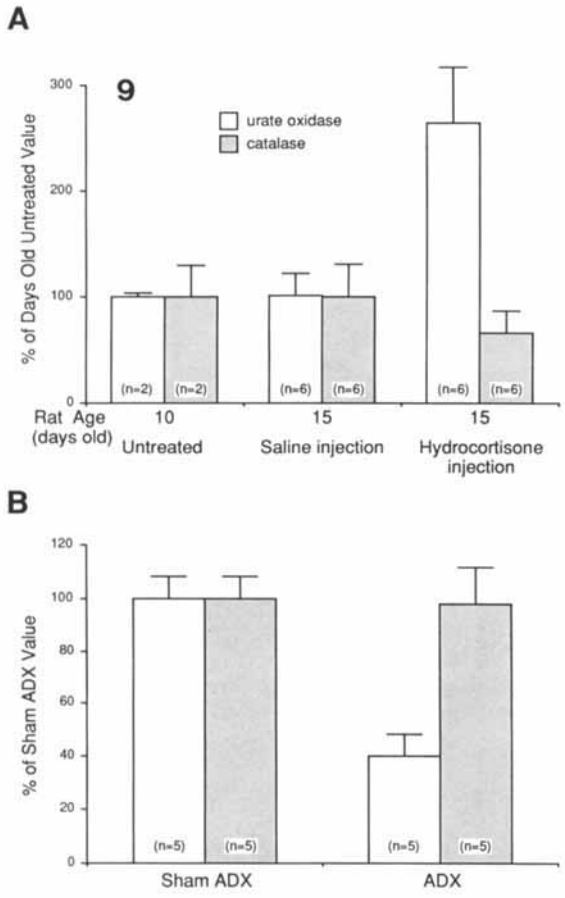

Fig. 9. A: Response of peroxisomal proteins to hydrocortisone acetate administration at 10 days of age. Suckling rats from two litters were injected at 10 days of age with saline or hydrocortisone acetate (50 μg/gram body weight). Five days after treatment, these rats were sacrificed and liver homogenates were prepared. Liver samples were also collected from two untreated rats at 10 days of age. Tissue homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with either AbUOx or an anti-catalase antibody before development with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane calculated. Urate oxidase and catalase levels were expressed as percent of antigen levels in 10-day-old untreated littermate rats. Each value is the mean antigen level as determined from homogenates of two untreated rats or six injected rats; error bars represent the standard deviation. **B:** Response of peroxisomal proteins to adrenalectomy at 60 days of age. Sixty-day-old rats were subjected to adrenalectomy or sham adrenalectomy. Rats were sacrificed four days after surgery and liver homogenates prepared. Tissue homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis, transferred to nitrocellulose, and incubated with either AbUOx or an anti-catalase antibody before development with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. The immunoblots were scanned with a densitometer and the resulting peak sizes for each lane calculated. Urate oxidase levels were expressed as the percent of antigen level in sham-operated animals. Each value is the mean antigen level as determined from five rat liver homogenates; error bars represent the standard deviation.

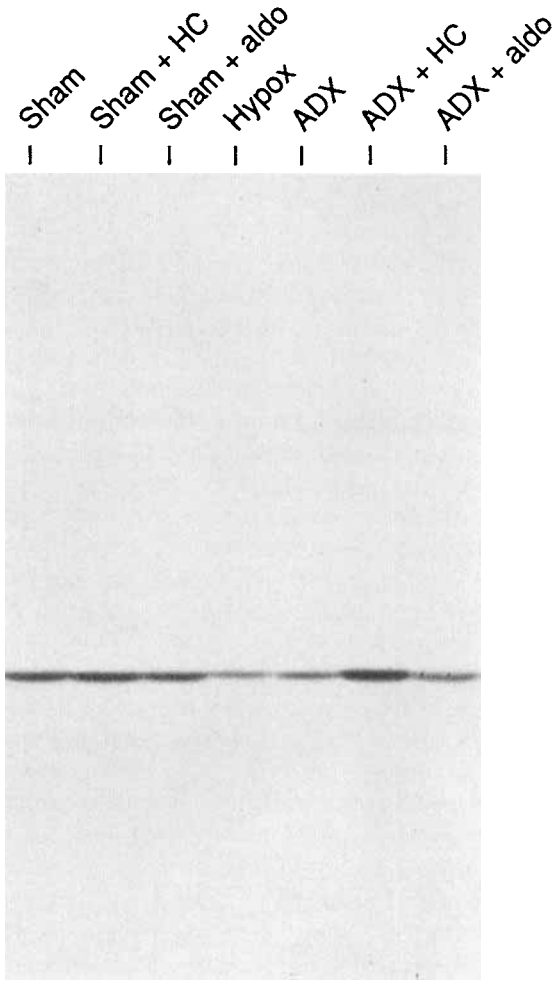

Fig. 10. In vivo regulation of urate oxidase in adult rat liver. Rat liver homogenates (prepared as in Methods) were separated by SDS-gel electrophoresis and transferred to nitrocellulose. The nitrocellulose sheet was incubated with AbUOx, then developed with peroxidase-conjugated second antibody and 4-chloro-1-naphthol. Each individual lane represents an individual animal used as source of tissue. Rats which were not hormone treated were sacrificed four days post-surgery, with the exception of hypophysectomized rats, which were sacrificed 7 weeks post-surgery. Rats received hydrocortisone acetate (50 μg/gram body weight; single dose) or aldosterone (50 μg/kilogram body weight; twice a day for 5 days) injections beginning on the fourth post-surgical day; rats were then sacrificed 5 days after initiation of hormone treatment. Abbreviations: ADX, adrenalectomized; +Aldo, administered aldosterone; +HC, administered hydrocortisone; Hypox, hypophysectomized.

thyroxine [27]. The relationship between weaning and levels of urate oxidase in immature rats was examined through the use of the "prevention of weaning" protocol (see Methods). It was determined that rats that were weaning-delayed still demonstrated the typical increase in urate oxidase expression at 20 days of age. In fact,

most enzymes which are induced in the third week of life have been found to be weaning-independent in the onset of their increased expression [27]. Jejunal sucrase, an enzyme required upon weaning in order to digest the high-carbohydrate solid diet, is not inhibited in its typical onset after postnatal day 17 by the prevention of weaning [30].

The circulating levels of corticosterone, the major glucocorticoid in the rat [31], and thyroxine begin to increase around postnatal day 12 [27]. Plasma levels of these two hormones increase suddenly in the 17-day fetal rat, then decline from postnatal days 2 through 10 before the surge seen after postnatal day 12 [29]. These hormonal inflections approximate the changes in urate oxidase levels in immature rat liver, demonstrated in Figure 4. The effect of thyroid hormones on expression of peroxisomal proteins, including urate oxidase, has been previously investigated [13,14,15,16]. However, no definitive study of the influence of glucocorticoid hormones on peroxisomal proteins has been reported. The possible role of glucocorticoid hormones in modulating urate oxidase was examined in this study.

The identification of glucocorticoid receptors in the liver has implicated glucocorticoids as regulators of liver protein expression [32]. Studies focusing on the role of glucocorticoids as developmental modulators have described several enzymes which are precociously induced by the administration of exogenous hormone. Some of these proteins have also been examined after the removal of endogenous glucocorticoids by adrenalectomy, and were found to be delayed in the naturally occurring rise of enzyme activity [27]. Among liver functions studied in this manner, tryptophan oxygenase and ornithine aminotransferase have been determined to be induced by glucocorticoid and developmentally delayed by adrenalectomy [33,34].

Development of the Hepatic Peroxisomal System

Ultrastructural and morphometric analyses of rat hepatocytes during prenatal and postnatal development have allowed quantification of the alterations in liver peroxisomes which occur during this time period. In the fetal rat, few peroxisomes were detected at the 15th day of gestation in electron micrographs of hepatocytes; those peroxisomes which were present lacked the typical core structure [35]. By the 18th–19th day of gestation, many more peroxisomes per hepatocyte were noted, the enzyme activity of the organelles has increased, and peroxisomal crystalline cores are visible for the first time [35,36]. Quantitative data have also been obtained for postnatal peroxisomal development. Morphometric data demonstrate that the number of peroxisomes per hepatocyte relative to the number at birth are two- to threefold higher between postnatal days 5 and 14, and was almost sevenfold greater in the adult animal [37].

The relationship of the differential expression of urate oxidase in rat liver to the postnatal development of peroxisomes was examined in the present study. Catalase was used as a marker enzyme representative of the number of peroxisomes present, as increased catalase activity had previously been correlated with peroxisomal proliferation [13]. Accordingly, liver homogenates from rats of different developmental ages were assayed for the amount of catalase present (Fig. 4). The developmental expression pattern for catalase reflects the pattern obtained for urate oxidase. Therefore, it appears that the increasing levels of urate oxidase previously described do not represent a unique event for urate oxidase, but rather a trend for peroxisomal proteins. With respect to the morphometric data concerning the number of peroxisomes per hepatocyte during development [37], it appears that the changing levels of urate oxidase and catalase represent a peroxisomal proliferation in immature liver. Using these two enzymes as markers now, peroxisomal proliferation seems to level off until a steady state is achieved near adulthood (Fig. 4). This rapid proliferation of peroxisomes in immature rat liver may represent a maturational process required for the functions associated with adult liver tissue.

Effect of Glucocorticoids on Urate Oxidase Expression

Urate oxidase was examined for the effects of glucocorticoids on the developmental rise in enzyme levels for immature animals and on the maintenance of adult levels of enzyme. Administration of glucocorticoid (hydrocortisone acetate) at 10 days of age, prior to the age at which a developmental rise in urate oxidase is typically seen, elicited a premature increase in urate oxidase level (Fig. 5). This data suggested that glucocorticoid action may modulate the developmental expression of urate oxidase in immature rats. Urate oxidase levels were also quantitated in immature rats after adrenalectomy to further

assess the contribution of glucocorticoids to urate oxidase expression. Animals were adrenalectomized before the naturally occurring surge in corticosterone previously described; liver tissue was examined from rats sacrificed at an age when urate oxidase levels would have already peaked near adult levels. However, adrenalectomy at 10 days of age did not produce decreased levels of urate oxidase in the fourth week of life, as compared to animals subjected to sham adrenalectomy (Fig. 6). Although hydrocortisone can induce a precocious increase in urate oxidase levels, this hormone is not the only developmental regulator of urate oxidase expression. Administration of glucocorticoids can alter the level of many other hormones [27], the effects of which could be the modulation of urate oxidase. Developmental increases in some proteins, such as liver tryptophan oxygenase, are abolished by adrenalectomy [33], while other enzymes are delayed in their eventual developmental increase [27]. In the present study, adrenalectomy may temporarily delay increased expression of urate oxidase in the immature rat liver.

In adult animals, administration of glucocorticoids in the same dose (based on weight) as that given to immature rats did not produce a significant increase in the urate oxidase levels of rats examined after a similar time period (Fig. 7). However, rats adrenalectomized at sixty days of age which were sacrificed four days post-surgery had urate oxidase levels less than half that of sham-operated animals (Fig. 8). In adults, a maximal level of expression may exist for urate oxidase; the system responsible for producing urate oxidase may not be inducible much beyond the normal adult level. Therefore, additional stimulation by exogenous glucocorticoids would have no effect, although decreases in glucocorticoids may cause decreased expression of the enzyme.

Glucocorticoids can be regulators of liver development and are implicated as one constituent of a complex control mechanism governing the level of liver urate oxidase. Because catalase was so strikingly similar to urate oxidase in its developmental expression, it was interesting to note that glucocorticoids did not affect catalase levels (Fig. 9). Although the developmental expression of urate oxidase appears to reflect a general proliferation of peroxisomes, glucocorticoids regulate urate oxidase in a specific manner.

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