Adrenergic Regulation of Glycogenolysis in Rat Liver after Cholestasis

MODULATION OF THE BALANCE BETWEEN ALPHA$_1$ AND BETA$_2$ RECEPTORS

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ABSTRACT The effects of extrahepatic cholestasis upon adrenergic regulation of glycogenolysis and upon the numbers of adrenoceptors in rat liver were studied using isolated hepatocytes and plasma membranes, respectively. A 60% decrease in the number of alpha$_1$ adrenoceptors (285 *vs.* 680 fmol/mg protein) and a simultaneous 2.7-fold increase in the number of beta adrenergic sites (67 *vs.* 25 fmol/mg protein) were observed beginning 36 h after bile flow obstruction and persisted for at least 68 h. The reciprocal modification of the numbers of alpha$_1$ and beta adrenoceptors was accompanied by a change in the manner of stimulation of glycogen phosphorylase by catecholamines in hepatocytes; originally alpha$_1$ adrenergic in normal rats (phenylephrine K_a = 0.9 μM, isoproterenol K_a = 7.1 μM), the stimulation became predominantly beta adrenergic in cholestatic animals (phenylephrine K_a = 3.7 μM, isoproterenol K_a = 0.06 μM). In normal rats, activation of the enzyme by epinephrine was inhibited by the alpha blocker phentolamine, without inhibition by the beta blocker propranolol. In contrast, propranolol was more effective than phentolamine in cholestatic rat hepatocytes. Modification of the regulation of glycogenolysis after cholestasis did not seem to be secondary to an alteration in the metabolism of thyroid hormones or in the action of glucocorticoids. However, cholestasis provoked a 10-fold increase in the number of hepatic mitoses and in the incorporation of thymidine into liver DNA of cholestatic animals. Similar changes were observed in regenerating livers, following two-thirds hepatectomy. We propose that the changes following extrahepatic cholestasis might, as well, be explained by a regenerative process.

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INTRODUCTION

Modulation of the relative numbers of alpha and beta adrenoceptors in several tissues occurs in various physiologic or pathologic conditions (for a general review, see 1). For example, the regulation of carbohydrate metabolism by catecholamines in the liver undergoes a shift from mediation via beta adrenoceptors in the fetal rat liver (2, 3) to that by alpha adrenergic receptors in the normal adult rat liver (4–7). After adrenalectomy (8) or in the hypothyroid state (9, 10), glycogenolysis in the rat is again under beta adrenergic control. The beta adrenergic-mediated regulation of glycogen metabolism in these two pathologic states has been correlated with an increase in the number of beta receptors (11–13), without modification in the number of alpha adrenergic sites (8, 12–14). Recently, we found that extrahepatic cholestasis in rats leads to an increase in the response of hepatic adenylate cyclase to isoproterenol and to an augmentation of the number of beta adrenergic binding sites (15).

We therefore investigated (*a*) whether the number of alpha$_1$ receptors identified by [^{3}H]prazosin changes in cholestatic rats; (*b*) whether bile duct ligation has an influence on the adrenergic stimulation of glycogen phosphorylase in hepatocytes isolated from such cholestatic animals; and (*c*) what possible mechanism(s)

might be involved in the appearance of the observed changes. This study demonstrates that after bile duct ligation there is an opposite and simultaneous alteration in the numbers of $alpha_1$ and beta receptors, giving rise to a modification in the adrenergic stimulation of glycogen phosphorylase. Furthermore, there is an increase in the number of hepatic mitoses and in the incorporation of [^{3}H]thymidine into liver DNA in cholestatic rats. Similar alterations in the numbers of adrenergic receptors and in the regulation of glycogenolysis were demonstrated in rat livers 2 d after two-thirds hepatectomy. We suggest, on the basis of these results, that the modifications following extrahepatic cholestasis are the result of a regenerative process.

METHODS

Materials. (+)Isoproterenol (Sterling Winthrop Group Ltd, Surrey, England), protokylol (Lakeside Laboratories, Inc., Milwaukee, WI), (−)propranolol (Imperial Chemical Industries Ltd., London), phentolamine, (±)alprenolol (Ciba Pharmaceutical Company, Ciba-Geigy Corporation, Basel, Switzerland) were obtained as gifts. (−)Isoproterenol, (−)epinephrine, (−)norepinephrine, (−)phenylephrine, DNA from calf thymus, glycogen type II from oyster, alpha-D-glucose-1-phosphate grade 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and glycylglycine were from Sigma Chemical Co., St. Louis, MO. Collagenase (crude type CLS and type IV, 1190-6870) was from Worthington Biochemical Corp., Freehold, NJ. [^{3}H]Dihydroalprenolol (31–41 Ci/mmol) was supplied by New England Nuclear, Boston, MA. [*Alpha*-D-^{14}C]glucose-1-phosphate (0.3 Ci/mmol), [*methyl*-^{3}H]thymidine (54 Ci/mmol), and [^{3}H]prazosin (33 Ci/mmol) were supplied by the Radiochemical Centre (Amersham Corp., Arlington Heights, IL). All other chemicals were from Merck Sharp & Dohme International (Darmstadt).

Animals. Male, albino, Wistar rats (250 g body wt) were used for all experiments and served as the source of liver plasma membranes and hepatocytes.

Extrahepatic cholestasis. After anesthesia with ether, a short midline incision was made through which the bile duct was ligated just below the hilum. Hepatocytes were isolated 48 h after surgery. Plasma membranes were prepared from three or four livers from 3 to 68 h after surgery.

Partial hepatectomy. A two-thirds hepatectomy was performed according to Higgins and Anderson (16). For the first 24 h after surgery, the rats were given a 20% aqueous solution of glucose ad lib. Plasma membranes hepatocytes were prepared 48 h after surgery.

Plasma membrane preparation. Liver plasma membranes from normal and cholestatic rats were prepared according to the procedure of Neville (17) up to step 11 and stored in liquid nitrogen until use. No loss of binding properties were detected even after storage of the membranes for 4 mo.

Hepatocyte isolation. Hepatocytes were isolated according to the procedure of Seglen (18) with the modifications previously described (19). Viability of hepatocytes was checked by staining with 0.45% trypan blue. Only preparations in which viability exceeded 85 and 75% for normal and cholestatic hepatocytes, respectively, were used. Cholestasis did not impair the yield of hepatocytes.

Hepatocyte incubation. The incubation was performed as previously described (19). Briefly, hepatocytes were preincubated at 37°C for 30 min with glucose (4 g/liter). The incubation with agonists was carried out for 1 or 2 min. For antagonists, the incubation was carried out for 6 min; then the agonist (−)epinephrine was added at a final concentration of 0.1 μM and the incubation stopped 1 min later. All incubations were stopped by quick freezing of the tubes in liquid nitrogen. The frozen tubes were kept at −80°C until the phosphorylase *a* was assayed.

Phosphorylase a assay. The frozen hepatocytes were homogenized according to Hue et al. (20). Glycogen phosphorylase was routinely assayed using [^{14}C]glucose-1-phosphate according to Gilboe et al. (21) as previously described (19). In a few cases, the enzyme was assayed according to Hue et al. (20). Phosphorous content was determined according to the procedure of Kallner (22). The phosphorylase activity is expressed in milliunits per milligram of protein; 1 U of enzyme activity corresponds to 1 μmol of product formed per minute.

Assay of [^{3}H]prazosin binding to liver plasma membranes. The assay was carried out as previously described (23) with the following modifications: the incubation at 37°C was started by the addition of 0.15 mg of membrane protein (0.1 ml) to various concentrations of tritiated prazosin in 50 mM Tris-HCl buffer, 10 mM $MgCl_2$, pH 7.6 containing 1 mM ascorbate, 1 mM catechol, 1 mM tropolone, and 120 mM NaCl. The final volume was 0.4 ml. After 10 min of incubation, the reaction was stopped by the dilution of 0.1 ml of the incubation mixture in 4 ml of ice-cold 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.6, followed by immediate filtration through a glass fiber filter Whatman GF/C (Whatman Inc., Clifton, NJ) (2.5-cm diam) under 0.7 atm vacuum. The filter was rapidly rinsed with 15 ml of ice-cold 50 mM Tris-HCl, 10 mM $MgCl_2$, and the retained radioactivity determined by liquid scintillation counting using 10 ml of Ready Solve EP (Beckman Instruments, Inc., Fullerton, CA) scintillation mixture. The counting efficiency of ^{3}H was 33%. Nonspecific binding, assessed in the presence of 10 μM phentolamine, represented 5–30% of the total radioactivity retained on the filters.

Assay of [^{3}H]dihydroalprenolol binding to liver plasma membranes. The assay was carried out according to Munnich et al. (24).

Protein determination. Protein was measured according to the procedure of Lowry et al. (25) using bovine serum albumin as standard.

Determination of K_a and EC_{50} values of adrenergic ligands for phosphorylase a. The K_a value for an agonist was taken as the concentration of the agent causing half the maximal phosphorylase *a* stimulation observed with that agent. EC_{50} is the concentration of antagonist causing a 50% inhibition of the activation obtained in the presence of 100 nM (−)epinephrine in the absence of any antagonist.

Thymidine incorporation into DNA. Rats, either normal or 48 h postoperative, were injected intraperitoneally with 20 μCi/100 g body wt of [^{3}H]thymidine in 0.5 ml of saline buffer. After 1 h the rats were killed by decapitation, and thymidine incorporation into DNA measured as described by Macmanus et al. (26) with the following modifications. Livers were removed and 1 g was homogenized in 3 ml of 7% ice-cold perchloric acid, using a high speed homogenizer (Ultraturrax, Janke & Kunfel, Staufen, Germany). The homogenate was centrifuged for 15 min at 25,000 *g*. The supernatant was kept and the pellet was washed once with 6 ml of 7% ice-cold perchloric acid and centrifuged as above. The supernatants were combined and designated "acid-soluble cell extract." The pellet was resuspended in 2 ml of 7% perchloric acid and heated at 80°C for 30 min. This material

was centrifuged for 10 min at 25,000 *g* and the supernatant containing DNA was kept. Radioactivity was measured by liquid scintillation counting. DNA was determined using the technique described by Croft and Lubran (27) with the following modifications: the standard DNA was heated at 80°C for 30 min in perchloric acid 7%; 1-ml aliquots of the standard DNA, the blank (perchloric acid 7%), or of the supernatants obtained above were added to 1 ml of the 2% diphenylamine reagent and the mixtures were kept at 30°C for 18 h. Absorption at 600 nm was measured against the blank.

Quantification of mitotic figures. Liver specimens obtained from two lobes from normal, cholestatic, and hepatectomized rats were immediately fixed in Bouin's fixative for 2 h, embedded in paraffin and cut into 3-μm thick sections, which were stained with hematoxylin and eosin. The numbers of mitotic figures and of hepatocytes were counted in 60 to 180 light microscopic fields at a magnification of ×250.

RESULTS

Effect of cholestasis on the numbers of alpha$_1$ and beta$_2$ adrenoceptors. After bile duct ligation, the variation as a function of time of the numbers of alpha$_1$ and beta$_2$ adrenergic receptors in purified plasma membranes was assessed by the specific binding of [^{3}H]prazosin and [^{3}H]dihydroalprenolol, respectively (Fig. 1). No modification of the numbers of alpha$_1$ and beta$_2$ adrenoceptors was detected up to 6 h after surgery. A change in the number of both receptors was first observed 16 h after the operation. Maximum changes were present 36 h after surgery and remained constant at least up to 68 h. A 2.7-fold increase in the number of beta$_2$ sites occurred simultaneously with a decrease by three-fifths in the number of alpha$_1$ sites. It is striking that both phenomena took place at the same time. To determine whether the observed changes were due to modifications of the maximum numbers of alpha$_1$ and beta receptors in the membranes or to variations in the equilibrium dissociation constants of the radioligands for their respective binding sites, we studied, as a function of increasing concentrations of the radioligands, the binding of [^{3}H]dihydroalprenolol and of [^{3}H]prazosin to hepatic plasma membranes from normal rats or from rats 2 d after bile duct ligation.

Typical Scatchard plots (28) are presented in Fig. 2, and the results of several Scatchard analyses are summarized in Table I. Extrahepatic cholestasis resulted in a decrease of the maximal number of alpha adrenergic sites from 680 fmol/mg of protein for normal rats to 285 fmol/mg of protein for cholestatic rats, with a slight decrease in the affinity of the ligand for its sites (K_D = 0.11 nM and 0.25 nM, respectively, for normal and cholestatic plasma membranes). Simultaneously, the maximal number of beta adrenoceptors increased from 25 fmol/mg of protein for normal to 67 fmol/mg of protein for cholestatic rats, without any

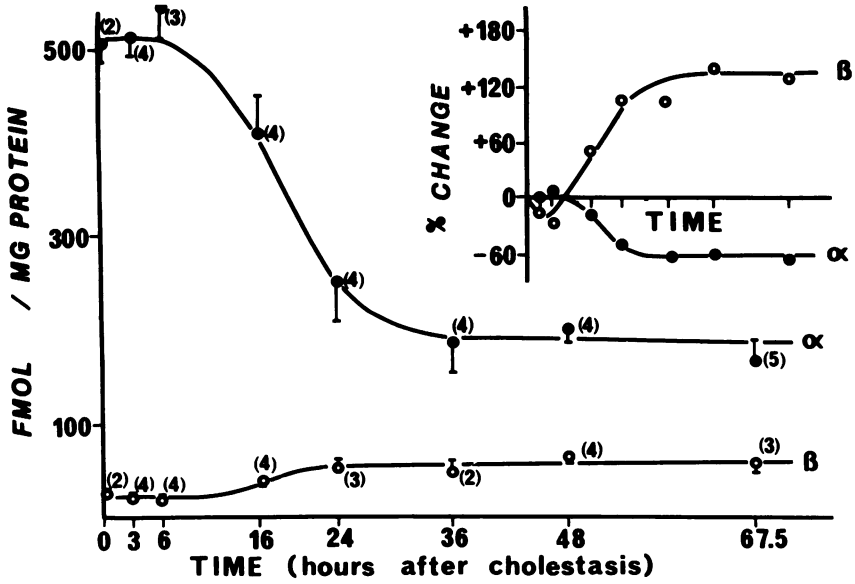

FIGURE 1 Time course of the variation in the numbers of prazosin and dihydroalprenolol binding sites after bile duct ligation. Plasma membranes were prepared at various times after bile duct ligation (Methods). Binding of [^{3}H]prazosin (●) or [^{3}H]dihydroalprenolol (○) was performed in the presence of, respectively, 0.15 and 0.74 mg of liver plasma membrane protein/ml. Each point represents the mean±SD of two to five experiments (exact number in parentheses), each performed in triplicate. Inset: The percent change of the number of binding sites relative to control values at time 0 is represented as a function of time after surgery.

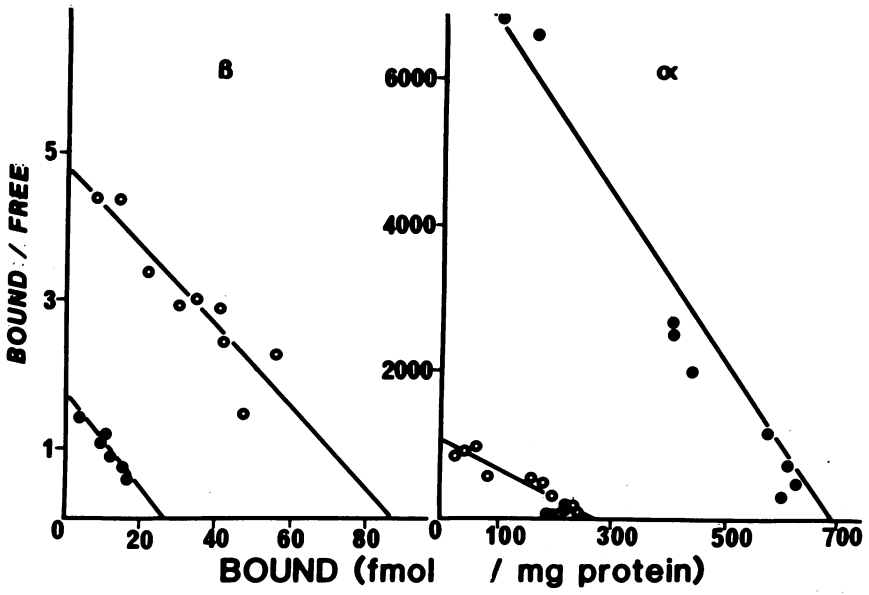

FIGURE 2 Scatchard plots of the binding of [^{3}H]prazosin and [^{3}H]dihydroalprenolol binding to rat liver plasma membranes of normal and cholestatic rats. Right panel: plasma membranes from normal (●), (0.058 mg/ml) or cholestatic (○), (0.117 mg/ml) rats were incubated at 37°C for 10 min with increasing concentrations of [^{3}H]prazosin, in the presence or in the absence of 0.01 mM phentolamine. The ratio of bound prazosin (femtomoles per milligram protein) to free prazosin (nanomolar), B/F, is plotted as a function of bound prazosin (femtomoles per milligram protein). The correlation coefficients are $r = 0.97$, $P < 0.001$ and $r = 0.91$, $P < 0.001$, respectively, for normal and cholestatic rats. Left panel: plasma membranes from normal (●) (1.75 mg/ml) or cholestatic (○) (1.2 mg/ml) rats were incubated at 30°C for 10 min with increasing concentrations of [^{3}H]dihydroalprenolol in the presence or in the absence of 5 μM (±)alprenolol. The ratio of bound dihydroalprenolol (femtomoles per milligram protein) to free dihydroalprenolol (nanomolar), B/F, is plotted as a function of bound dihydroalprenolol (femtomoles per milligram protein). The correlation coefficients are $r = 0.95$, $P < 0.001$ and $r = 0.91$, $P < 0.001$, respectively, for normal and cholestatic rats. The equations of the lines were calculated by linear regression.

TABLE I
Number and Affinity of Alpha and Beta Adrenoceptors in Normal, Cholestatic, and Hepatectomized Rat Liver Plasma Membranes

	Alpha adrenoceptors	Beta adrenoceptors
Normal rats		
Bmax (*fmol/mg protein*)	680 (2)	25 (2)
K_D (*nM*)	0.11	17
Cholestatic rats		
Bmax (*fmol/mg protein*)	285 (2)	67 (3)
K_D (*nM*)	0.25	13
Hepatectomized rats		
Bmax (*fmol/mg protein*)	490 (2)	73 (2)
K_D (*nM*)	0.38	8

Numbers in parentheses represent the number of separate experiments performed. K_D values were obtained from Scatchard analysis (28).

change in the affinity of the ligand for its sites (K_D = 17 nM and 13 nM, respectively, for normal and cholestatic plasma membranes).[1]

The decreased number of prazosin binding sites observed was not related to a slower association of the ligand to its site since equilibrium was reached 8 min after the beginning of the association experiment for membranes prepared from normal or cholestatic rats (data not shown). We also verified that the decrease of the specific binding of [^{3}H]prazosin to plasma membranes after bile duct ligation was not due to an in-

[1] In this report, the number of beta adrenoceptors in normal rats was 25 fmol/mg protein whereas in previous reports (12, 15) it was 60 fmol/mg protein. This difference is due to the fact that we used male Wistar rats weighing 250 g in the present study instead of the female Wistar rats weighing 100 g used in the previous studies. Furthermore, these changes are consistent with findings of Blair et al. (30) who showed that beta-mediated regulations decrease in the aging rat.

terference of bile salts, the concentration of which is enhanced after surgery. This concentration was measured according to Mahu et al. (29) and found to be <15 nmol/mg of protein in purified plasma membranes derived from the livers of cholestatic rats, which corresponds at most to a concentration of 5.7 μM of the salts in the binding assay of tritiated prazosin. This concentration of taurocholate, the major bile salt in the rat (29), does not alter the binding of [^{3}H]prazosin binding to plasma membranes since for both normal and cholestatic plasma membranes, the specific binding of the radioligand begins to decrease only at bile salt concentrations >30 μM (data not shown). Taken together, these data demonstrate that extrahepatic cholestasis is accompanied by an actual increase in the number of beta adrenoceptors simultaneous with a decrease in the number of alpha$_1$ adrenergic sites.

Effect of cholestasis on the adrenergic activation of glycogen phosphorylase. Since catecholamine-mediated glycogenolysis in rat liver appears to be controlled via beta adrenoceptors in several physiopathological states (2, 3, 8–10, 30), we investigated the possibility that the sensitivity of glycogen phosphorylase to adrenergic agonists could be modified by extrahepatic cholestasis. All the studies were performed on parenchymal cells prepared from rats either normal or 2 d after bile duct ligation.

The basal level of the glycogen phosphorylase activity was slightly decreased in cells isolated from cholestatic animals. In the presence of 50 mM glucose-1-phosphate, the activities were 24.4±4.1 mU/mg of protein (n = 23) and 18.8±4.9 mU/mg of protein (n = 19) for normal and cholestatic rats, respectively. The abilities of an alpha agonist, (−)phenylephrine, and of a beta agonist, (−)isoproterenol, to activate glycogen phosphorylase in normal and cholestatic rat hepatocytes are depicted in Fig. 3. In normal rat liver, the activation of the enzyme was mediated by alpha adrenergic receptors since (−)phenylephrine (K_a = 0.9 μM) was eight times more potent than (−)isoproterenol (K_a = 7.1 μM). In contrast, (−)isoproterenol (K_a = 60 nM) appeared to be 62 times more efficient than (−)phenylephrine (K_a = 3.7 μM) in stimulating the enzyme after extrahepatic bile duct ligation. Protokylol, a potent beta$_2$ agonist (31), was unable to activate glycogen phosphorylase in normal hepatocytes. Strikingly, however, it was the most potent agonist (K_a = 25 nM) for stimulating the enzyme in cholestatic rat hepatocytes. The potency of various adrenergic agonists to activate the enzyme is indicated in Table II. From the results, it is evident that glycogen phosphorylase is preferentially stimulated by beta agonists in hepatocytes isolated from cholestatic rats. Also indicated in Table II is the fact that the levo-isomer of isoproterenol was 35-fold more potent than the dextro-isomer.

To confirm the results obtained with adrenergic agonists, we studied the effects of adrenergic blockers on the stimulation of glycogen phosphorylase by (−)epinephrine in normal and cholestatic animals (Table III). As expected from previous results (19), the

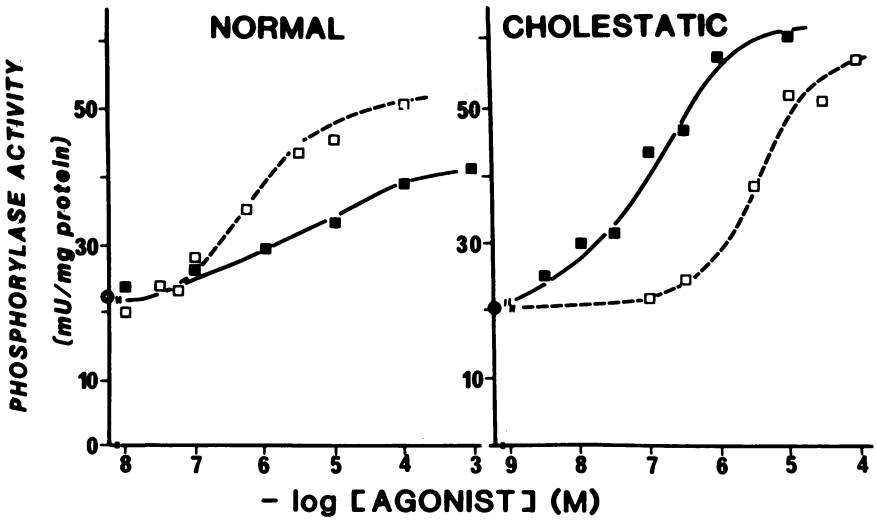

FIGURE 3 Activation of glycogen phosphorylase by adrenergic agonists in hepatocytes isolated from normal and cholestatic rats. Hepatocytes from normal (left panel), (52 × 10^6 cells in 5.8 ml) or cholestatic (right panel), (45 × 10^6 cells in 5.8 ml) rats were incubated with increasing concentrations of (−)phenylephrine (□), (−)isoproterenol (■), or buffer (Methods). Glycogen phosphorylase was assayed as described in Methods.

TABLE II

Activation of Glycogen Phosphorylase by Agonists in Normal, Cholestatic, and Hepatectomized Rat Hepatocytes

Agonists	K_a		
	Normal rats	Cholestatic rats	Hepatectomized rats
		μM	
Protokylol	NA (3)	0.025 (3)	NT
(−)Isoproterenol	7.1 (4)	0.06 (4)	0.014 (2)
(−)Epinephrine	0.52 (6)	1.02 (3)	NT
(−)Norepinephrine	0.27 (6)	1.35 (2)	NT
(+)Isoproterenol	NT	2.10 (2)	NT
(−)Phenylephrine	0.9 (7)	3.70 (3)	1.6 (2)

Numbers in parentheses represent the number of separate experiments performed for each compound.

K_a values were obtained as reported in Methods. NA, no activation up to 1 mM; NT, not tested.

alpha adrenoceptor blocker phentolamine (20 μM) prevented the activation of the enzyme by the catecholamine in normal rats, while the beta adrenoceptor blocker (−)propranolol was ineffective at the same concentration (Table III). In contrast, (−)propranolol significantly inhibited the stimulation of glycogen phosphorylase in cholestatic rats. Phentolamine also decreased the stimulation of the enzyme by epinephrine although the decrease was not statistically significant.

Since the inhibition of glycogenolysis appeared to be both beta and alpha adrenergically mediated after cholestasis, we performed dose-response curves for the antagonists (Fig. 4). In normal rats (left panel), phentolamine displayed a biphasic curve while inhibition by propranolol appeared as a monophasic one. The alpha adrenergic component (i.e., the "high-affinity" part of the phentolamine curve) represented 70–80% of the total inhibition in all the experiments. In cholestatic animals (right panel), both propranolol and phentolamine displayed biphasic curves. In the experiment depicted in Fig. 4, the beta adrenergic inhibition (i.e., the "high affinity" part of the propranolol curve and the "low affinity" part of the phentolamine curve) was 80% of the total inhibition. However, in different experiments, the beta adrenergic component varied from 60 to 80%, while the alpha component varied between 40 and 20% of the inhibition.

Possible cellular changes during cholestasis. An augmentation in the number of beta adrenoceptors, which we report here after bile duct ligation, also occurs in adrenalectomized rats (11, 12). Since the structural formulas of bile salts and glucocorticoids are partly similar, the effect of cholestasis may actually be indirect; that is, the effects observed in cholestatic animals might be due to competition between increased concentrations of bile salts and glucocorticoids for the glucocorticoid receptors, leading to an "adrenalectomy-like" effect limited to the liver. We thus compared the potency of hydrocortisone in inducing the tyrosine aminotransferase activity, assayed according to Diamondstone (32), in livers from both normal and cholestatic rats. As shown in Table IV, the glucocorticoid produced a two- to threefold induction of the enzyme in both normal and cholestatic rats,[2]

[2] The induction of tyrosine aminotransferase reported in Table IV is smaller than the ones usually reported in the literature, since we used a dose of hydrocortisone five times smaller than the dose usually utilized, in order not to overcome a possible effect of bile salts.

TABLE III

Inhibition by Antagonists of Glycogen Phosphorylase Stimulated by Epinephrine

Additions	Percent stimulation over basal level		
	Normal rats	Cholestatic rats	Hepatectomized rats
(−)Epinephrine 100 nM	168±32 (7)	221±53 (5)	312 − 271 (2)
(−)Epinephrine 100 nM + phentolamine 20 μM	50±21* (4)	157±73 (4)	276 − 186 (2)
(−)Epinephrine 100 nM + (−)propranolol 20 μM	156±40 (3)	50±8‡ (3)	112 − 89 (2)

Basal levels of the enzyme were 24 and 19 mU/mg of protein, respectively, in control and cholestatic rats, and 14 mU/mg of protein in hepatectomized rats.

Experiments were performed as described in the Methods.

Number in parentheses represent the number of separate experiments.

* $P < 0.001$ compared with epinephrine 100 nM for control rats.

‡ $P < 0.01$ compared with epinephrine 100 nM for cholestatic rats.

TABLE IV

Influence of Cholestasis upon the Induction of Tyrosine Aminotransferase by Hydrocortisone

	Normal rats		Cholestatic rats	
	Control	+Hydrocortisone	Control	+Hydrocortisone
Activity±SD (*μmol/min/g of liver*)	1.12±0.32 (8)	2.2±0.6 (9)	1.12±0.98 (4)	3.35±0.81 (7)
Fold activation		1.96		2.99

Male Wistar rats (250 g body wt) had or had not undergone surgery. 2 d later, the rats in each group were injected intraperitoneally with a submaximal dose of hydrocortisone acetate[2] (0.5 mg/100 g body wt) or solvent 7 h before killing. Liver homogenates were prepared (1 g of liver/3 vol KCl 0.14 M, pH 7.3), filtered and centrifuged at 31,000 *g* for 30 min. Tyrosine aminotransferase activity was measured in the supernatant according to Diamondstone (32).

Numbers in parentheses represent the number of separate determinations.

and the induction was even higher after bile duct ligation. This indicates that there is no blockade of the effect of the steroid in cholestasis.

Alternatively, the effects of cholestasis on liver function might be mediated via altered thyroid hormone levels. Modification of the adrenergic regulation of glycogenolysis after hypothyroidism has been described (9, 10), and during the course of acute hepatic disorders, changes in the level of thyroid hormones may occur (33). We, therefore, measured the level of triiodothyronine and of thyroxine in the blood of cholestatic and normal rats. The level of thyroxine was 3.60 and 4.37 μg/100 ml and that of triiodothyronine was 57.33 and 66.67 ng/100 ml, respectively, for the normal and cholestatic rats. For both hormones, the differences in the levels between cholestatic and normal rodents was not significant ($P < 0.1$ and $P < 0.65$, respectively, for thyroxine and triiodothyronine by the nonparametric Mann-Whitney test). Thus, the effects of bile duct ligation do not seem to be due to a modification of the thyroid hormone level. However, it cannot be excluded that the sensitivity of liver to thyroid hormones was changed in the cholestatic rats.

A third explanation for the observed effects is that the trauma induced by extrahepatic cholestasis leads to a "regenerating-like" state of the hepatic cell. In

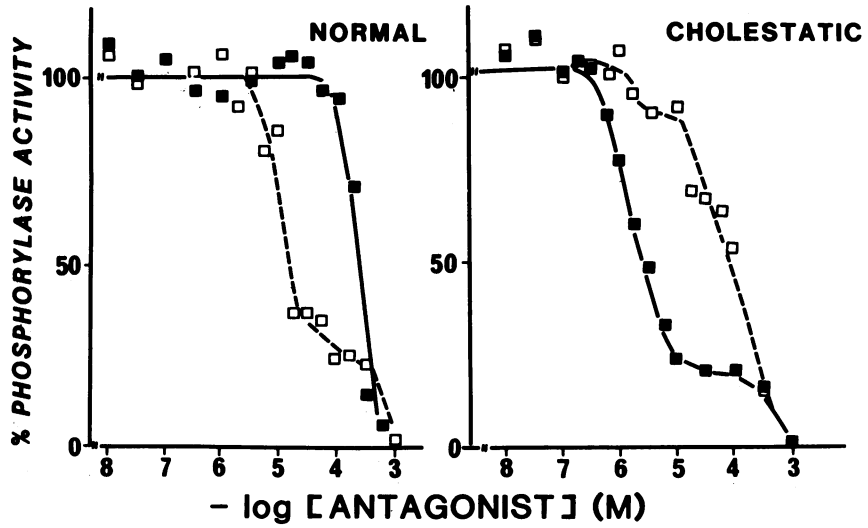

FIGURE 4 Inhibition of glycogen phosphorylase by adrenergic antagonists in hepatocytes isolated from normal and cholestatic rats. Hepatocytes from normal (left panel), (50×10^6 cells in 5.8 ml) or cholestatic (right panel) (40×10^6 cells in 5.8 ml) rats were incubated with increasing concentrations of phentolamine (□) or (−)propranolol (■) (Methods). Basal glycogen phosphorylase activities were 22.5 and 8.5 mU/mg protein and epinephrine-stimulated ones were 57.5 and 32.5 mU/mg protein, respectively, for normal and cholestatic rats.

fetal (23) and suckling (34) rat livers, the regulation of glycogenolysis is, indeed, beta and not alpha adrenoceptor dependent. Also, Wright (35) showed that the beta adrenergic activation of adenylate cyclase is increased in regenerating liver, being maximal 60 h after partial hepatectomy. Similarly, Hornbrook (36) has reported that glycogen phosphorylase becomes more responsive to beta adrenergic agonists 1 d after partial hepatectomy. To assess the state of regeneration, we measured the incorporation of tritiated thymidine into DNA in livers from cholestatic as compared with normal rats. We observed an 11-fold increase in the incorporation of tritiated thymidine into DNA in cholestatic as compared with normal rats (Table V). No modification of the entry of the radiolabeled nucleoside into the cell or of the pool size of intermediates between [^{3}H]thymidine and DNA was evident as seen from the measurements of incorporation of radioactivity into the acid-soluble cell extract (data not shown). The DNA content per gram of liver did not vary significantly in normal and operated rats (Table V). Further, there was a 10-fold augmentation of the number of mitotic figures in the livers of rats 2 d post bile duct ligation as compared with normal rat livers (154 vs. 15 mitoses/10,000 cells, Table V). After a two-thirds hepatectomy, we found 87 mitoses/10,000 cells (Table V), a result close to those obtained by Bucher (37) and by Morley and Royse (38) (1.2% mitoses 48 h after partial hepatectomy.

To confirm that regeneration of the liver was responsible for the changes in the adrenergic system previously described, we assessed the numbers of alpha$_1$ and beta adrenoceptors as well as the catecholamine regulation of glycogenolysis 48 h after partial hepatectomy. We found, as compared with normal rats, a threefold augmentation in the number of beta adrenergic sites and a 28% reduction of the number of alpha$_1$ adrenoceptors (Table I). Glycogen phosphorylase was stimulted more efficiently by (−)isoproterenol (K_a = 14 nM) than by (−)phenylephrine (K_a = 1.6 μM) (Table II). Moreover, the epinephrine-stimulated enzyme was blocked preferentially by propranolol as compared with phentolamine (Table III). These results are in agreement with those reported by Hornbrook (36).

DISCUSSION

Two major findings emerge from the experiments reported here. Firstly, surgical extrahepatic cholestasis in the rat results in a modification of the balance between alpha$_1$ and beta$_2$ adrenergic receptors in the liver. This change leads to a shift from alpha$_1$ to beta$_2$ adrenergic control of the stimulation of glycogen phosphorylase by catecholamines in isolated hepatocytes. Secondly, the modifications induced by bile duct ligation appear to be linked to a regenerative process.

With respect to the first point, the most striking result of the binding studies is the simultaneity of the appearance of beta adrenergic sites and of the disappearance of alpha$_1$ adrenoceptors. The 2.7-fold increase of the beta adrenergic sites was concomitant with a decrease by 58% of alpha$_1$ receptors, without significant modification of the affinities of the ligands for their binding sites (Table I). The synchronism of the changes undergone by both types of receptors might be considered to be the result of an interconversion of alpha and beta adrenergic binding sites as it has been postulated by Nickerson and Kunos (39) for heart receptors under different circumstances. However, studies from our laboratory on rat liver (40) are in favor of marked structural differences between alpha and beta adrenoceptors. Furthermore, there is not a one-to-one relationship between the increase of beta receptors and the decrease of alpha$_1$ adrenergic sites (appearance of 42 fmol of [^{3}H]dihydroalprenolol binding sites/mg of protein and disappearance of 395 fmol of [^{3}H]prazosin binding sites/mg of protein, 2 d

TABLE V

[^{3}H]Thymidine Incorporation into DNA of Livers from Normal, Cholestatic, or Hepatectomized Rats

	Normal rats	Cholestatic rats	Hepatectomized rats
[^{3}H]Thymidine (*cpm/mg* DNA±SD)	6,468±2,963 (6)	73,525±15,236 (6)	ND
DNA (*mg/g* of liver±SD)	0.93±0.25	1.08±0.32	ND
Number of mitoses per 10,000 cells	15 (3)	154 (3)*	87 (4)*
Number of cells per ×250 field	141±11	132±14	143±6

Tritiated thymidine incorporation into DNA and DNA concentration were measured as described in Methods.

The number of mitoses was evaluated in 63–178 fields under a light microscope at the magnification of ×250.

Numbers in parentheses represent the number of separate determinations. ND, not determined.

* Significantly different from the control value ($P < 0.001$).

after bile duct ligation). Thus, it seems unlikely that interconversion of receptors occurs during cholestasis.

The change in the balance of catecholamine receptors resulting from extrahepatic cholestasis is accompanied by a change in the adrenergic control of glycogenolysis. In normal male rats, this study and that by Studer and Borle (41) demonstrated a preferential alpha adrenergic stimulation of glycogen phosphorylase. Isoproterenol was unable to stimulate the enzyme to the same extent as phenylephrine (Fig. 3). Phentolamine, but not propranolol, inhibited efficiently the epinephrine-stimulated enzyme (Table III, Fig. 4). Following bile duct ligation, there is a preferential beta adrenergic stimulation of glycogen phosphorylase. Protokylol, a $beta_2$ adrenergic agonist (31) was the most efficient agonist tested in rats after bile duct ligation (Table II). This drug, which possesses a good affinity for the alpha adrenergic receptor in rat liver (42), was unable to stimulate the enzyme in hepatocytes isolated from normal rats (Table II) and acted as an alpha antagonist (data not shown). The finding that propranolol antagonized the action of catecholamines on glycogen breakdown more efficiently than phentolamine in parenchymal cells isolated from cholestatic animals is also consistent with a preferential beta adrenergic control. However, phenylephrine was still potent in stimulating the phosphorylase (Table II) and the beta adrenergic inhibition of the enzyme represented 60 to 80% of the total inhibition (Fig. 4). These data imply that, in cholestasis, the control of glycogenolysis is mainly mediated via beta adrenoceptors, although a limited $alpha_1$-adrenergic regulation is still present.

Reciprocal changes of adrenergic receptors in many tissues in other pathological states have been reported (for a general review, see 1). Studies from this and other laboratories have shown that in the livers of adrenalectomized rats there is an increase in the number of beta adrenergic receptors (11, 12) with no change in the number of alpha adrenergic sites (8, 12); in addition, Chan et al. (8) observed a reduction of the alpha adrenergic and an enhancement of the beta adrenergic-mediated glycogenolysis in isolated hepatocytes. In hypothyroid rats, Malbon et al. (9) and Preiksaitis and Kunos (10) described a beta adrenergic-mediated hepatic glycogen breakdown. Malbon (13) linked this modification of the catecholamine regulation of carbohydrate metabolism with a two- to threefold increase to the maximum number of beta adrenoceptors, whereas the number of alpha adrenoceptors remained constant (14) or was slightly diminished (43). Since we observed a somewhat similar modification of the adrenergic regulation of glycogenolysis, we carefully examined whether bile flow obstruction was accompanied by hypothyroidism or by an "adrenalectomy-like" state. The changes that we observed are not due to alterations in glucocorticoid action or thyroid hormone levels but rather to a more direct hepatic effect of cholestasis. Okajima and Ui (44) have demonstrated the conversion of the stimulation of glycogenolysis from an alpha adrenergic type in freshly isolated hepatocytes to a beta adrenergic one in a primary culture of the same cells. This phenomenon is very rapid since a modification is already observed 4 h after the start of the culture. Maximal changes, obtained at 8 h of culture, remained constant up to 24 h. This time course is not very different from the one observed here for adrenoceptor changes due to cholestasis since a substantial modification of the numbers of $alpha_1$ and beta adrenoceptors is detected 16 h after bile duct ligation (Fig. 1). Extrahepatic cholestasis in the rat, therefore, appears to be one among several processes which can bring about the same overall change, namely, a new balance between alpha and beta adrenoceptors, and, as a result, a modification of the pathway by which catecholamines induce glycogenolysis.

The second major finding of this paper was that the observed changes after cholestasis appeared due to a regenerative process. An increased incorporation of tritiated thymidine into DNA is known to occur in regenerating liver after partial hepatectomy (26, 45, 46). In this experimental model, adenylate cyclase is more sensitive to beta adrenergic agonists (35), and glycogenolysis is stimulated through beta adrenoceptors (36). Since an increased sensitivity of adenylate cyclase to isoproterenol also results from cholestasis (15), it was crucial to gather information of the state of DNA synthesis in this system. Twenty years ago, Mac Donald and Pechet (47) reported an augmentation in the incorporation of tritiated thymidine into the nuclei of hepatocytes and in the number of mitotic figures in livers from cholestatic rats. More recently, Bagheri et al. (48) demonstrated a rise in the incorporation of [^{3}H]thymidine in mouse liver 36 h after lithocholate (a bile salt) ingestion. It was preceded by a similar effect in the gallbladder and could be mimicked by deoxycholate or cholate. In our system, we found a 10-fold augmentation of the number of mitoses and of the incorporation of the radiolabeled nucleoside in rat liver DNA 2 d after bile duct ligation compared with normal animals (Table V).

The mechanism by which bile flow obstruction leads to a regenerative state of the liver is unknown. Okazaki et al. (49) hypothesized recently that biliverdin, the precursor of bilirubin, could initiate in vivo rat liver regeneration. Lafarge-Frayssinet et al. (50) reported that biliverdin could increase by two- to threefold the incorporation of tritiated thymidine into DNA in cultured cells originating from liver; however, other au-

thors were unable to stimulate the proliferation of hepatocytes in primary culture by the same compound (51). Clearly, more studies are needed to elucidate this point. However, the simplest hypothesis may be that bile stasis in the liver provokes a hepatic damage similar to carbon tetrachloride poisoning, in which regeneration is promoted (52). In this case, the level of alpha fetoprotein was shown to increase 2 d after poisoning. Alpha fetoprotein is also present in cultured hepatocytes (53), the system in which Okajima and Ui (44) demonstrated a change in the adrenergic regulation of glycogenolysis.

In conclusion, it would appear that a liver undergoing a regenerative process (extrahepatic cholestasis, partial hepatectomy) can revert to a dedifferentiated "fetal state" and thus reacquire characteristics of such a state, for example beta adrenergic control of carbohydrate metabolism (2, 3). The adrenergic system of liver can, therefore, be considered as a good marker of the state of differentiation of this organ.

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