JCI The Journal of Clinical Investigation

Studies of Y and Z, two hepatic cytoplasmic organic anion-binding proteins: effect of drugs, chemicals, hormones, and cholestasis

Humberto Reyes, … , Zenaida Gatmaitan, Irwin M. Arias

J Clin Invest. 1971;50(11):2242-2252. https://doi.org/10.1172/JCI106721.

Research Article

The process by which various anions, including bilirubin and several dyes, drugs, hormones and their metabolites, are transferred from plasma into the liver cell is poorly understood. Two hepatic cytoplasmic proteins, Y and Z, that bind various organic anions in vivo and in vitro have been postulated to be involved in this process.

The concentration of Y, the major organic anion-binding protein, increases in rat liver after administration of phenobarbital in association with enhanced organic anion transfer from plasma into liver as determined by initial plasma disappearance rate (K_1) and hepatic dye content for sulfobromophthalein (BSP) and indocyanine green (ICG), as well as increased relative hepatic storage of BSP. Acute bile duct ligation failed to alter plasma disappearance or hepatic content of BSP in normal or phenobarbital-treated rats.

Other drugs and chemicals which cause proliferation of hepatic smooth endoplasmic reticulum and enhancement of drug metabolism, such as allylisopropylacetamide, dieldrin, DDT, 3-methylcholanthrene, and benzpyrene increased Y and BSP K_1 and, where studied, hepatic BSP content. Alcohol feeding had no effect on Y, Z, or K_1 for BSP.

Hypophysectomy and thyroidectomy increased Y but decreased K_1 and, where studied, hepatic content of BSP. Of several hormones studied, only thyroxine restored Y and K_1 to normal in hypophysectomized or thyroidectomized rats. Mice with congenital pituitary insufficiency also manifested increased Y which returned to […]

Studies of Y and Z, Two Hepatic Cytoplasmic Organic Anion–Binding Proteins: Effect of Drugs, Chemicals, Hormones, and Cholestasis

HUMBERTO REYES, A. JONATHAN LEVI, ZENAIDA GATMAITAN, and IRWIN M. ARIAS

From the Division of Gastroenterology, Department of Medicine of the Albert Einstein College of Medicine, and Bronx Municipal Hospital Center, New York 10461

ABSTRACT The process by which various anions, including bilirubin and several dyes, drugs, hormones and their metabolites, are transferred from plasma into the liver cell is poorly understood. Two hepatic cytoplasmic proteins, Y and Z, that bind various organic anions in vivo and in vitro have been postulated to be involved in this process.

The concentration of Y, the major organic anion–binding protein, increases in rat liver after administration of phenobarbital in association with enhanced organic anion transfer from plasma into liver as determined by initial plasma disappearance rate (K_1) and hepatic dye content for sulfobromophthalein (BSP) and indocyanine green (ICG), as well as increased relative hepatic storage of BSP. Acute bile duct ligation failed to alter plasma disappearance or hepatic content of BSP in normal or phenobarbital-treated rats.

Other drugs and chemicals which cause proliferation of hepatic smooth endoplasmic reticulum and enhancement of drug metabolism, such as allylisopropylacetamide, dieldrin, DDT, 3-methylcholanthrene, and benzpyrene increased Y and BSP K_1 and, where studied, hepatic BSP content. Alcohol feeding had no effect on Y, Z, or K_1 for BSP.

Hypophysectomy and thyroidectomy increased Y but decreased K_1 and, where studied, hepatic content of BSP. Of several hormones studied, only thyroxine restored Y and K_1 to normal in hypophysectomized or thyroidectomized rats. Mice with congenital pituitary insufficiency also manifested increased Y which returned to normal after thyroxine administration. In hormone-deficient rats and mice, phenobarbital administration produced a further increase in Y suggesting that different mechanisms may be responsible for the change in Y resulting from drug administration and hormonal deprivation. Thyroxine, testosterone, or hydrocortisone did not alter BSP K_1 or Y in normal rats.

Cholestasis produced by ethinyl estradiol administration or biliary obstruction reduced Y, Z, BSP K_1, and hepatic BSP content.

These results support the hypothesis that Y and Z are involved in the transfer of BSP, ICG, and possibly other organic anions from plasma into the liver. The concentration of Y increased after administration of various drugs and chemicals as well as in thyroid deficiency. Thyroid hormone appears to be important in regulation of the intracellular concentration of Y. Because thyroid deficiency increased Y but decreased BSP K_1 and hepatic BSP content, other factors beside Y and Z influence hepatic organic anion uptake.

INTRODUCTION

Two cytoplasmic protein fractions, Y and Z, that bind bilirubin, sulfobromsulphalein (BSP),[1] and other organic anions in vivo and in vitro have been identified in vertebrate liver (1, 2). Y was measured in various rat tissues using a dye-binding technique, and was present exclusively in liver, whereas Z is also present in small in-

This work was performed during the tenure of Dr. Reyes's receipt of an International Fellowship from the U. S. Public Health Service (5 F05 TW 01319-03). The work was presented, in part, at the Annual Meeting of the American Society for the Study of Liver Disease, November 1969, Chicago, Ill.

Received for publication 20 October 1970.

[1] *Abbreviations used in this paper:* AIA, allylisopropylacetamide; BSP, sulfobromophthalein; ICG, indocyanine green; K_1, plasma disappearance rate; S, relative storage capacity; Tm, transport maximum.

testinal mucosa (1). A specific Y protein and a specific Z protein have been purified and characterized and are responsible for the major portion of organic anion binding by each fraction[2] (3). We have proposed that Y and Z proteins may be involved in the selectivity with which various organic anions are transferred from plasma into the liver cell. This hypothesis is based on (*a*) the tissue distribution of Y and Z (1); (*b*) competition between several organic anions for uptake in vivo and binding to Y and Z in vitro (1); (*c*) ontogenetic development of Y, the major organic anion-binding protein (1) in guinea pig (4) and in monkey (5); (*d*) apparent absence of Y and Z in fish and gill-breathing amphibia which lack selective hepatic BSP uptake (2), and (*e*) presence of Y and Z in liver from lung-breathing amphibia, reptiles, birds, and mammals, including man, which manifest selective hepatic organic anion uptake in vivo (2). In addition, (*f*) the concentration of Y in rat liver increases after administration of phenobarbital simultaneously with increased initial plasma disappearance rate (K_1) of BSP which was used as an indirect measure of hepatic BSP uptake (6).

In the present experiments, we have sought to confirm that hepatic organic anion uptake is increased after phenobarbital administration by estimating Y and Z as well as hepatic dye content and K_1 in rats utilizing BSP and indocyanine green (ICG), a nonmetabolized organic anion. Various drugs and chemicals, previously shown to result in hepatic smooth endoplasmic reticulum proliferation, increased the hepatic concentration of Y and enhanced hepatic uptake of BSP as determined by K_1 and, when studied, hepatic dye content. In order to investigate hormonal control of Y and Z, similar studies were performed in hypophysectomized and thyroidectomized rats and mice with an inheritable pituitary deficiency before and after treatment with various hormones and drugs.

Because ethinyl estradiol, which produces bile secretory failure (cholestasis) in rats (7), reduced Y and Z, the effect of mechanical cholestasis on the concentration of Y and Z was also investigated.

METHODS

Animals. Male Sprague-Dawley rats (250–350 g) (Marland Farms, Peekskill, N. Y.) were used in most of the experiments. Female Sprague-Dawley and male rats of Long Evans and Gunn (glucuronyl transferase-deficient Wistar) strains were used when indicated.

Male Sprague-Dawley rats were hypophysectomized or thyroidectomized (Charles River Breeding Laboratories, Wilmington, Mass.) when weighing 90–120 g and studied 40 days later except when other periods are specified. Unoperated male Sprague-Dawley rats with similar dates of birth served as controls.

Female mice of strains dw/dw (Snell's homozygous congenital hypopituitary dwarf mice) and DW/J (heterozygous) were obtained from the Jackson Laboratory, Bar Harbor, Maine (8, 9).

Animals were fed Purina rat or mouse chow and water *ad libitum*. Drugs were injected subcutaneously daily to groups of 3–10 animals for 6–14 days.

Drugs. Sodium phenobarbital was dissolved in 0.9% NaCl. Adult rats received 8 mg/100 g body weight daily except in the dose-response study when daily doses ranged from 0.2 to 15 mg/100 g. To permit survival, the highest dose group received increasing amounts of drug for 3 wk and then 15 mg/100 g daily for 10 days. Hypophysectomized and thyroidectomized rats were given 4 mg/100 g body weight daily. Mice received 7 mg/100 g body weight intraperitoneally.

Allylisopropylacetamide (AIA), dissolved in 0.9% NaCl, was injected daily into normal (30 mg/100 g) and hypophysectomized (10 mg/100 g) rats for 6 days.

Dieldrin (hexachloro-octahydro-dimethanonaphthalene) and DDT (trichloro-*p*-chlorophenylethane) were dissolved in corn oil and 2 mg/100 g body weight was injected for 6 days to normal rats. The same dose of DDT was used in hypophysectomized rats.

3-Methylcholanthrene and 3,4-benzpyrene were dissolved in corn oil and 1 mg/100 g was injected for 6 and 10 days, respectively, to normal rats. The same dose of 3-methylcholanthrene was injected into hypophysectomized rats.

Five adult female Sprague-Dawley rats received a diet for 5½ wk in which 36% of calories were derived from ethyl alcohol. Five pair-fed rats received an alcohol-free, complete diet.

Hormones. L-Thyroxine dissolved in 0.9% NaCl, was administered daily as follows: normal rats, 5 μg/100 g for 6 days; hypophysectomized rats, 14 μg/100 g for 6 days, and thyroidectomized rats, 0.9–5.5 μg/100 g for 6 days.

Hydrocortisone sodium succinate, dissolved in 0.9% NaCl, was administered daily to normal rats (96 or 250 μg/100 g for 8 days) and hypophysectomized (100 μg/100 g for 8 or 13 days).

Testosterone propionate, dissolved in corn oil, was injected into normal male rats (0.7 mg/100 g for 6 days).

Ethinyl estradiol was dissolved in corn oil. Female normal rats received 0.75 mg/100 g daily for 11 days.

Control groups consisted of three or four normal, hypophysectomized, or thyroidectomized rats, and DW/J or dw/dw mice which received similar volumes of saline or oil injections for 6–10 days. The results in these animals were similar to those observed in untreated animals. Data obtained from adult male rats injected as controls were pooled with results from 11 untreated animals and used as "normals" for statistical comparison with results from drug or hormone-injected male rats.

Methods. All experiments were performed 15–20 hr after the last injection. Y and Z protein fractions were quantitated using a standard procedure which has been demonstrated to be reproducible (1). Portions of 100,000 *g* supernate, representing 1 g of liver from each animal, were mixed with 3.75 mg of BSP and placed on a Sephadex G-75 column (2.5 × 43 cm). Elution was performed with 0.01 M phosphate buffer, pH 7.4, using pump-driven upward flow. Protein concentration was estimated by absorbance at 280 mμ, and protein-bound BSP by absorbance after alkalinization with NaOH at 580 mμ using a Beckman-Gilford Spectrophotometer (Beckman Instruments, Fullerton, Calif.). Y

[2] Levi, A. J., G. Fleischner, J. Robbins, Z. Gatmaitan, and I. M. Arias. 1971. Purification and characterization of an organic anion-binding protein from liver cytoplasm. In preparation for publication.

and Z fractions were measured by triangulation of the area under each peak of 580 mμ absorbance and expressed as units/gram of liver or units/100 g body weight. 1 unit is arbitrarily equivalent to 1 mg of BSP bound per fraction. Liver weight was determined before perfusion but after the organ was washed in cold saline and subsequently blotted. Y and Z were quantitated in livers of animals which received BSP injections for plasma disappearance studies. The background of BSP in these livers did not affect quantitation of Y and Z as excess BSP was added before fractionation on Sephadex G-75.

In separate experiments, Y and Z proteins were quantitated by acrylamide gel electrophoresis and densitometry. Portions of 100,000 *g* supernate, equivalent to 4 g of rat liver, were mixed with 7.5 mg of BSP and chromatographed on a Sephadex G-75 column (3 × 90 cm). Similar volumes of eluate from the peak tubes in each fraction were concentrated 5 times (Y fraction) or 15 times (Z fraction) by ultrafiltration. Portions of eluate representing similar volumes of each original fraction were submitted to vertical electrophoresis (E-C Apparatus Corp., Philadelphia, Penn.) in 5% acrylamide gel in 0.1 M barbital HCl, pH 9.6. The protein bands were stained with Amido-Schwarz and quantitated using a recording densitomer. Any BSP which was present in Y or Z fraction was removed during electrophoresis and did not interfere with the study of proteins. Y and Z proteins were identified and quantitated densitometrically by comparison with known amounts of standard purified protein which was electrophoresed simultaneously in each gel. Standard optical density–concentration curves using purified rat albumin and Y protein were obtained under the same conditions. Y concentrations from 5 to 175 μg per gel slot gave straight lines after densitometry. Each sample contained less than 150 μg of Y or Z proteins.

Because there is no completely satisfactory method for quantitating plasma-liver cell bidirectional organic anion–transfer rates, the following indirect measures were used:

1. The initial disappearance rates from plasma of single intravenous doses of BSP (5 mg/100 g) or ICG (1 mg/100 g), expressed as the first order rate–constant, K_1, were determined from four to eight carefully timed plasma samples obtained from 2 to 7 min after dye administration (10, 11).

2. 5½ min after intravenous administration of BSP to rats, which were lightly anesthetized with ether, laparotomy was performed and, in less than 1 min, the liver was removed, weighed, and rapidly perfused through the hepatic and/or portal vein with 10–20 ml 0.9% NaCl in order to remove blood. A 10% liver homogenate was prepared in distilled water and 1 ml of saturated toluenosulfonate was mixed with each 1 ml of homogenate. 1.0 ml of 50% trichloroacetic acid was added to precipitate proteins from which BSP had been displaced. After centrifugation for 20 min at 2000 rpm in an International centrifuge, the supernate was filtered through Whatman No. 2 filter paper. 5 ml of clear supernatant, 2 ml of concentrated NaOH, and 3.0 ml distilled water were mixed, and optical density of the solution was determined in a Beckman DU spectrophotometer at 580 mμ. BSP was quantitated from a standard curve prepared after similar analysis of known amounts of the dye. ICG was estimated in liver homogenates after perfusion in a similar manner. The toluenosulfonate-treated filtrate was neutralized, brought to pH 7.0–7.4 with NaOH, and 5 ml of filtrate was diluted with distilled water and read against blank at 800 mμ in a Beckman DU spectrophotometer. ICG was quantitated by comparison with a standard curve prepared with known amounts of the dye. Recovery of BSP or ICG added to rat liver homogenates in the range found in various experiments ranged from 78 to 96%. Benzpyrene and phenobarbital added in excess to liver homogenate or plasma did not interfere with the chemical estimation of BSP in plasma or liver. In several control and drug-treated rats, bile duct and duodenal contents were examined visually during laparotomy for ICG and for BSP after alkalinization with NaOH; no dye was apparent. A small terminal sample of the liver perfusion was tested and trace amounts of dye were present but were not quantitated.

3. BSP hepatic removal rate, relative storage capacity (S), and transport maximum (Tm) were determined in four rats which received 8 mg phenobarbital/100 g daily for 4 days; four rats which received a similar daily dose of phenobarbital for 15 days, and seven saline-injected controls, using the modifications proposed by Klaasen and Plaa (12) of the method of Wheeler, Meltzer, and Bradley (13). Under phenobarbital anesthesia, BSP (2.5 mg/min per kg) was infused intravenously for 90 min at a constant rate (0.034 ml/min) by a motor-driven pump (Harvard Apparatus Co., Inc., Millis, Mass.). Plasma volume was determined by plasma dilution of intravenously administered Evans blue (T-1824) (14).

4. In an effort to minimize the possibility that phenobarbital increased K_1 by increasing bile flow, K_1, Y, and Z, and hepatic BSP content were determined after bile duct ligation which was performed under light ether anesthesia An increase in K_1 under these circumstances probably reflects enhanced hepatic uptake rather than biliary excretion.

The effect of chronic biliary obstruction on K_1, Y, and Z, and hepatic BSP content was studied 3 days (three rats), 6 days (three rats), or 12 days (two rats) after bile duct ligation and section.

During experiments requiring anesthesia, rectal temperature was monitored and maintained at 37–38°C with a heating lamp.

Student's *t* test and Pearson's correlation coefficient were used for statistical analysis of results (15). *P* values equal to or less than 0.05 were considered significant.

RESULTS

The following results concern the relationship between changes in Y and various measures of hepatic organic anion uptake in normal and phenobarbital-treated rats:

1. Table I presents dose-response relationships between Y, Z, and K_1 in rats which received 0.2–15 mg of phenobarbital/100 g daily for 10 days. Hepatic BSP content was separately estimated in rats similarly treated with phenobarbital (Table I). Y, K_1, and hepatic BSP content significantly increased with administration of 1.0–4.0 mg phenobarbital/100 g daily for 10 days. Within the limits of the number of studies performed, the results are not significantly different at the 4.0, 8.0, and 15.0 mg dosage level. Increase in all three parameters occurred up to the 4.0 mg dosage range. Mean liver/body weight ratios increased with increasing doses of phenobarbital. Z was 0.39 ±0.06 units/100 g in control rats and was unaffected by phenobarbital administration.

2. In 13 normal adult, male Sprague-Dawley rats which received a single intravenous dose of ICG, K_1

TABLE I

Effect of Different Doses of Phenobarbital on Y, Liver Per Body Weight Ratio, K_1 BSP, and Hepatic BSP Content 5½ min after Intravenous Administration of 5 mg dye/100 g

Phenobarbital dosage for 10 days	No. rats	Y	BSP K_1	No. rats	Hepatic BSP content	Ratio, mean
mg/100 g per day		*U/100 g*			*mg/100 g*	*liver/body wt*
Control	10	0.62 ±0.04	0.198 ±0.006	4	1.60 ±0.09	3.6
0.2	4	0.67 ±0.10	0.228 ±0.007	2	1.61, 1.71	3.7
1.0	10	1.01 ±0.08	0.242 ±0.010	4	1.95 ±0.11	4.1
4.0	4	1.11 ±0.12	0.258 ±0.007	4	2.10 ±0.10	4.6
8.0	10	1.33 ±0.10	0.252 ±0.006	2	2.19, 1.98	4.9
15.0	4	1.38 ±0.09	0.255 ±0.004	2	1.96, 2.06	5.3

Studies of hepatic dye content were performed in different rats from those utilized in the other studies. Mean liver per body weight ratios include all rats. Results are expressed as mean ±SE. See text for further procedural details.

TABLE II

Effect of Phenobarbital on Hepatic Removal Rate, Transport Maximum (Tm), and Relative Storage Capacity (S) of BSP in Adult Rats

	Bile flow	Bile BSP concentration	BSP removal rate	Tm	S
	µl/min per kg	*mg/ml*	*mg/min*	*mg/min per kg*	*mg/100 kg*
Controls (7)	57.8 ±5.5	14.2 ±0.5	0.59 ±0.02	0.82 ±0.07	0.52 ±0.09
Phenobarbital treated					
14 days (4)	80.6* ±9.7	16.9 ±0.6	0.66* ±0.02	1.36* ±0.16	0.32 ±0.05
4 days (4)	66.4 ±6.3	14.9 ±0.5	0.63* ±0.02	0.88 ±0.09	0.61* ±0.06

Figures indicate mean ±SE.
* $P < 0.05$.

was 0.218 ±0.007; in 4 other normal rats, hepatic ICG content 5½ min after injection of 1 mg dye/100 g was 0.49 ±0.09 mg/100 g. In nine rats pretreated with 8 mg phenobarbital/100 g body weight for 10 days, K_1 for ICG was 0.269 ±0.016; in four rats similarly pretreated, hepatic ICG content was 0.61 ±0.11 mg/100 g. Differences in K_1 and ICG content of liver between control and treated rats were statistically significant ($P < 0.01$).

3. Phenobarbital administration for 4 days significantly increased BSP removal rate, and S without increasing Tm, bile flow, or bile BSP concentration (Table II). Phenobarbital administration for 14 days increased bile flow, bile BSP concentration, and Tm; S was not significantly increased. K_1 and Y were increased by phenobarbital pretreatment ($P < 0.02$ and 0.001, respectively), even when the common bile duct was acutely ligated for 15 min before measurement of these parameters (Fig. 1). Hepatic BSP content was not measured in these animals. The results are similar to those observed in normal rats (Table I).

Because in another study (16), phenobarbital administration failed to increase BSP binding by rat liver cyto-

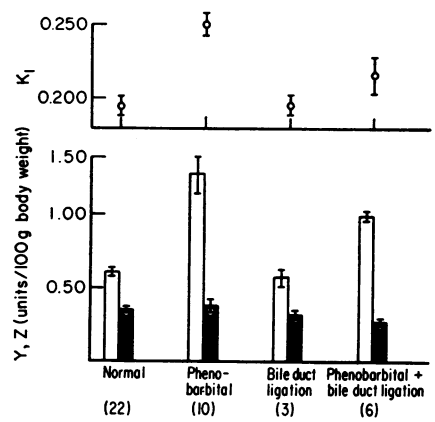

FIGURE 1 Effect of acute bile duct ligation on K_1, Y, and Z in rats and the effect of pretreatment with phenobarbital. Phenobarbital (8 mg/100 g) was administered daily for 6 days to adult, male rats. Common bile duct ligation was performed in control and phenobarbital-treated rats. 15 min later, BSP was injected intravenously and K_1 was estimated. Approximately 25 min after bile duct ligation, the animals were killed and Y and Z were quantitated.

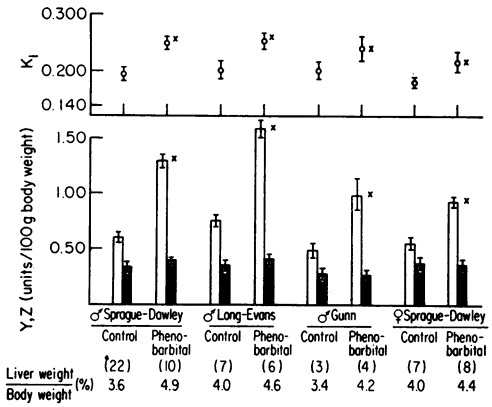

FIGURE 2 Effect of phenobarbital administration on K_1, Y, and Z in rats of both sexes and different strains. $P < 0.05$ as compared with the respective control groups. In this and all subsequent figures, Y is designated by open bars and Z by solid bars.

plasmic proteins, the possibility that strain or sex differences were involved in this effect was investigated. Phenobarbital administration increased Y and simultaneously increased K_1 in Sprague-Dawley rats of both sexes, and in male Long Evans and Gunn rats (Fig. 2). After phenobarbital administration, Y increased in each group of male animals from 164 to 225% of basal values. The increases in Y and K_1 were less in female Sprague-Dawley rats than in males of the same strain.

Administration of phenobarbital, AIA, dieldrin, DDT, benzpyrene, and 3-methylcholanthrene increased Y, K_1, and liver weight relative to body weight in each group (Fig. 3). DDT and dieldrin administration reduced Z by 20 to 60%. BSP content of liver was increased after benzpyrene administration by 18, 29% (two rats); after AIA by 21, 29% (two rats), and after DDT by 18, 24% (two rats).

In 32 normal and 39 drug-treated male rats in which K_1 and Y were determined, Pearson's correlation coefficient was 0.72 ($P < 0.02$). Pearson's correlation coefficient between hepatic BSP content, measured in 4 normal and 25 drug-treated rats, and K_1, measured in 32 normal and 39 drug-treated rats was 0.86 ($P < 0.02$). In the same rats, the correlation coefficient between hepatic BSP content and Y was 0.84 ($P < 0.05$).

In eight alcohol-fed rats, Y was 0.47 ±0.02 (SE) and Z was 0.11 ±0.03 (SE). BSP K_1 was measured in other similarly fed rats and did not differ from results obtained in pair-fed controls.

The effect of several hormones on K_1, Y, and Z was studied in rats of both sexes. Dose and length of treatment are indicated in Methods. Thyroxine or testosterone did not alter K_1, Y, Z, or liver/body weight ratios. Hydrocortisone (96 μg/100 g) did not increase K_1 or Y in five rats; however, in five rats receiving 250 μg/100 g, K increased by 25% and Y increased by 10% when compared to saline-injected control rats. Ethinylestradiol administration to four female rats resulted in 55% reduction in Y, ($P < 0.05$), 49% reduction in K_1 ($P < 0.05$); 42% reduction in Z ($P < 0.001$), and liver/body weight ratios were unchanged.

Hypophysectomy in male rats resulted in rapid increase in Y and a slow decrease in Z (Fig. 4). 7 days after hypophysectomy, Y increased 80% over control values ($P < 0.05$) and did not change 40 or 280 days after operation. Z was unchanged 7 days after hypophysectomy, but was reduced to 40% of normal at 40 and

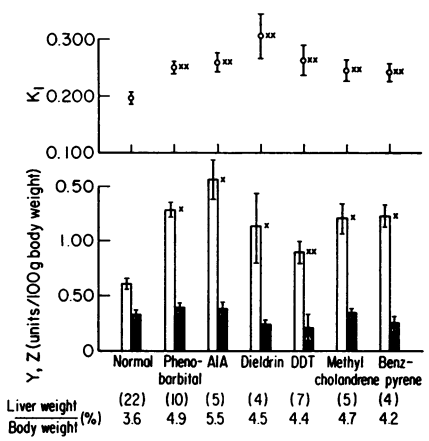

FIGURE 3 Effect of drugs and chemicals on K_1, Y, and Z. $^{x}P < 0.001$ as compared with normals; $^{xx}P < 0.005$ as compared with normals.

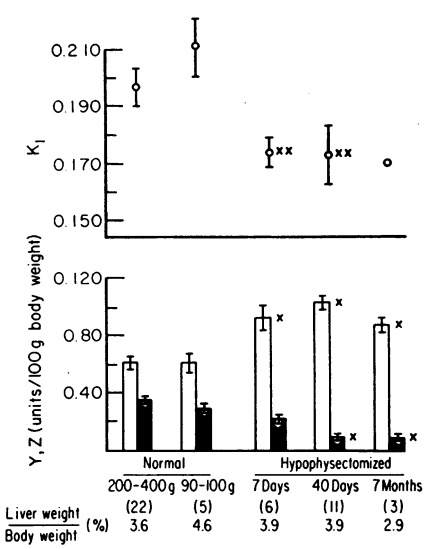

FIGURE 4 Effect of hypophysectomy on K_1, Y, and Z. $^{x}P < 0.001$ as compared with normals; $^{xx}P < 0.02$ as compared with normals.

280 days after operation. K_1 was 87% of normal values 7 days after hypophysectomy and did not change thereafter. Hepatic BSP content in five rats 40 days after hypophysectomy was 71 ±7.1% of control values.

Phenobarbital and AIA increased Y and liver/body weight in hypophysectomized rats without changing Z (Fig. 5). In the doses used, DDT and 3-methylcholanthrene did not alter Y, Z, K_1, or liver/body weight ratio. It was difficult to obtain adequately timed blood samples in hypophysectomized rats, which explains the different number of K_1 determinations compared with Y and Z measurements. In four phenobarbital injected hypophysectomized rats, K_1 increased but was not significantly different from K_1 in normal rats. In two similarly treated hypophysectomized rats, hepatic BSP content was within the normal range.

Hydrocortisone, testosterone, or progesterone, injected daily for 8 days in hypophysectomized rats, did not change the concentration of Y or Z ($P > 0.05$), (Fig. 6). Thyroxine reduced Y to normal levels ($P < 0.01$ when compared with results in hypophysectomized rats) but did not change Z. K_1 was not significantly modified in hypophysectomized rats treated with hydrocortisone or testosterone ($P > 0.05$); however, thyroxine (14 μg/100 g) restored K_1 to normal in hypophysectomized rats ($P < 0.01$ when compared with results in hypophysectomized rats).

Because thyroxine was the only hormone tested which returned Y and K_1 to normal in hypophysectomized rats, studies were performed in thyroidectomized rats (Fig. 7). Thyroidectomy increased Y to 130%, reduced Z to 48%, reduced K_1 to 66%, and reduced BSP content in three animals to 70% of normal. Pheno-

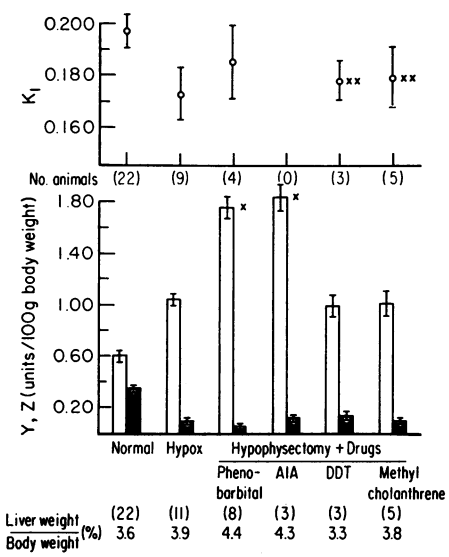

FIGURE 5 Effect of drugs and chemicals on K_1, Y, and Z in hypophysectomized rats. 40 days after hypophysectomy, male Sprague-Dawley rats received phenobarbital, AIA, DDT, or 3-methylcholanthrene. Results are compared with saline-injected hypophysectomized and normal rats. $^{x}P < 0.001$ as compared with untreated hypophysectomized rats; $^{xx}P < 0.02$ as compared with normal rats, and 0.05 as compared with untreated hypophysectomized rats.

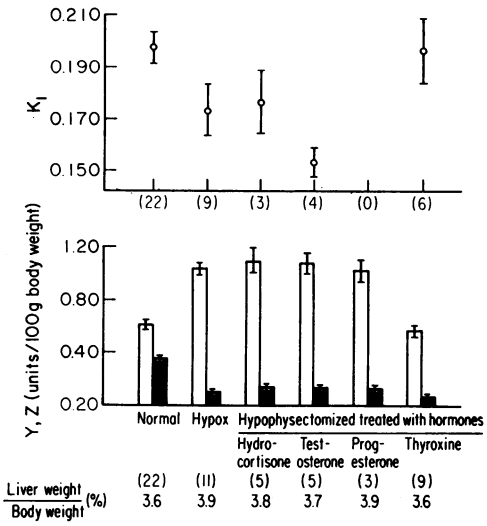

FIGURE 6 Effect of hormones on K_1, Y, and Z in hypophysectomized rats. 40 days after hypophysectomy, male Sprague-Dawley rats received hydrocortisone, testosterone, methoxyethynylestradiol, progesterone, or thyroxine daily, for 10 days. K_1 for BSP was measured when possible. Results are compared with saline-injected hypophysectomized and normal rats.

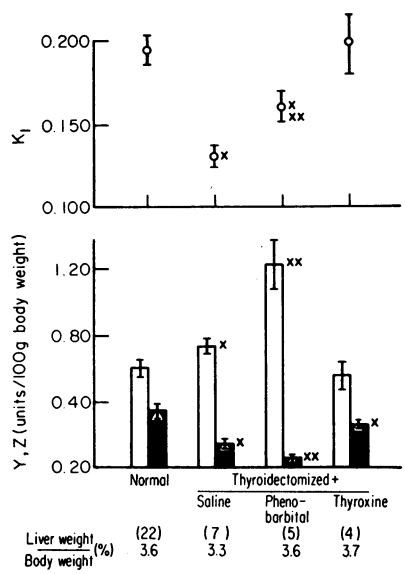

FIGURE 7 Effect of thyroidectomy and administration of phenobarbital or thyroxine to thyroidectomized rats, on K_1, Y, and Z. 40 days after thyroidectomy, male Sprague-Dawley rats (100–120 g) received phenobarbital, thyroxine, or saline, daily for 7 days. Results are compared with normal adult male rats. $^{x}P < 0.05$ as compared with normal rats; $^{xx}P < 0.05$ as compared with saline-injected thyroidectomized rats.

TABLE III

Comparison between Y and Z Estimated by Densitometry Scanning of Stained Polyacrylamide-Gel after Electrophoresis, and by BSP Binding Using Sephadex-G75

	Normal	Hypox.	Hypox. and phenob.	Hypox. and T_4	Thyrox.	Thyrox. and T_4
Studies with *Y* Densitometry						
Y fraction, *μg protein/50 μl*	185	208	218	128	160	100
Y protein, *μg/50 μl*	47	64	92	37	48	42
Y protein in *Y* fraction, %	26	31	42	29	30	42
Y, *% of normal*	100	136	196	79	102	92–89
BSP binding						
Y, *units/g liver*	0.19	0.29	0.42	0.17	0.25	0.18
% of normal	100	155	221	89	128	95
Studies with *Z* Densitometry						
Z protein, *μg/50 μl*	147	129	44	51	91	138
% of normal	100	88	30	35	62	94
BSP binding						
Z units/g liver	0.11	0.04	0.03	0.03	0.06	0.08
% of normal	100	44	33	33	66	88

Data were obtained in two separate experiments.

barbital administration further increased Y and reduced Z; K_1 increased when compared with results in untreated thyroidectomized rats but did not reach normal values. Administration of thyroxine (5.5 μg/100 g) reduced Y or normal, increased Z, and restored K_1, liver/body weight ratio, and, in three rats, BSP content to normal. A presumed physiologic dose of thyroxine (0.9 μg/100 g) restored K_1 to normal, increased Z, but did not reduce Y/100 g probably because of an increase in liver/body weight ratio. When Y was expressed per gram of liver, normal values were obtained.

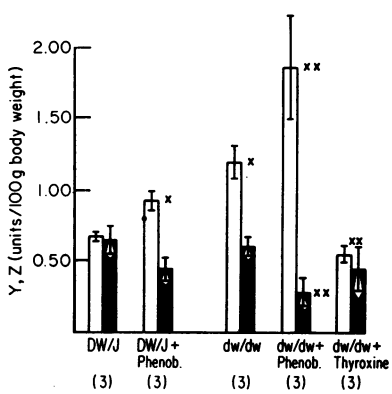

FIGURE 8 Y and Z protein fractions in hypopituitary mutant (dw/dw and normal mice (DW/J). Effect of phenobarbital and thyroxine. Female hypopituitary dwarf mutant mice (dw/dw) and female normal mice of the same strain (DW/J) received phenobarbital (7 mg/100 g body weight per day) or thyroxine (8 μg/100 body weight per day) intraperitoneally for 6 days. Controls received saline intraperitoneally for the same period. Each study was done using livers pooled from three to four animals. $^{x}P < 0.025$ as compared to DW/J; $^{xx}P < 0.05$ as compared to dw/dw.

Acrylamide gel electrophoresis and densitometry were used to quantitate Y and Z proteins in hypophysectomized and thyroidectomized rats before and after treatment with phenobarbital or thyroxine. Densitometric quantitation of Y and Z in various experiments were compared with estimates of Y and Z by BSP binding using Sephadex G75 (Table III). The correlation coefficients between Y and Z measured by both methods were 0.99 and 0.83, respectively. Increase in Y concentration was observed in hypophysectomized or thyroidectomized rats, and was restored to normal after thyroxine administration. Phenobarbital administered to hypophysectomized rats gave the greatest increase in Y. Z decreased in all experiments.

Y and Z in Snell's hypopituitary mutant (dw/dw) and heterozygous mice (DW/J), and the effect of phenobarbital and thyroxine are shown in Fig. 8. Mutants (dw/dw) with hypopituitary dwarfism (8, 9) have almost double the concentration of Y per gram of liver as compared to heterozygous, phenotypically normal mice of the same strain (DW/J). Phenobarbital administration increased Y in mutants and normal mice to 186%. Z decreased in the mutants ($P < 0.05$). Administration of thyroxine to dw/dw mice for 6 days reduced

Y to normal and slightly reduced Z. No studies of hepatic dye uptake were performed, as mutants weigh only 5–6 g.

The effect of chronic mechanical cholestasis produced by common bile duct ligation is shown in Fig. 9, and Table IV. Y and Z for BSP and hepatic BSP content progressively decreased. The results are comparable with the effects of prolonged administration of ethinyl estradiol in doses which result in bile secretory failure (7). Y and Z were measured densitometrically as well as by BSP binding using Sephadex G75 (Table IV) and the results with each method showed correlation coefficients of 0.95 for Y, and 0.98 for Z.

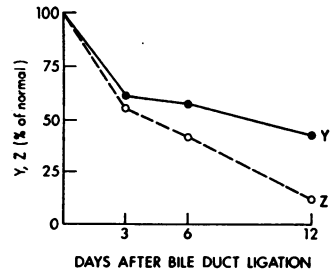

FIGURE 9 Effect of prolonged bile obstruction on Y and Z. Livers were processed 3, 6, or 12 days after bile duct obstruction. Results are expressed as per cent of mean values in 22 normal male Sprague-Dawley rats.

DISCUSSION

We have previously reported that after phenobarbital administration to rats, Y, the major hepatic cytoplasmic organic anion–binding protein, increases progressively to attain maximal values by 6 days and returns to normal approximately 9 days after drug withdrawal (6). The full time course of response coincided with an increase in the initial plasma disappearance rate (K_1) of intravenously administered BSP used as an indirect measure of hepatic uptake of an organic anion (7). Using a different method of preparation, Grodsky, Kolb, Fanska, and Nemechek (16) described a bilirubin and BSP-binding protein fraction in rat liver; however, dye binding by this fraction was not increased after phenobarbital administration. In the present experiments, correlation was shown between the dose of phenobarbital and increase in Y, K_1, and hepatic content of BSP. 8 mg of phenobarbital/100 g maximally increased Y and, therefore, was used in subsequent experiments in adult rats.

Strain and sex differences in hepatic drug metabolism and biliary excretory capacity for organic anions, including bilirubin and BSP, have been reported in rats (17–21). In the present study, the response of Y and K_1 after phenobarbital administration was not unique to either sex or to a specific strain of rat. In Sprague-Dawley rats of both sexes, and male rats of three different strains, Y increased significantly after administration of a fixed dose of phenobarbital for 6 days and simultaneously, K_1 for BSP increased. Although sex and strain differences were found in the amounts of Y and Z in untreated rats, the magnitude of the response

TABLE IV

The Effect of Cholestasis on Y and Z as Determined Densitometrically and by BSP Binding

	Normal	Bile duct ligation	Ethinyl estradiol
Studies with *Y*			
Densitometry			
Y fraction, *μg protein/50 μl*	149	119	112
Y protein, *μg/50 μl*	37	30	22
Y protein in *Y* fraction, %	25	25	20
Y, *% of normal*	100	64	34
BSP binding			
Y, *units/g liver*	0.18	0.12	0.07
% of normal	100	67	39
Studies with *Z*			
Densitometry			
Z protein, *μg/50 μl*	114	68	33
% of normal	100	60	29
BSP binding			
Z, *units/g liver*	0.11	0.07	0.03
% of normal	100	66	33

Data were obtained in two separate experiments.

of Y to phenobarbital administration was similar in each group. The relative Y reduction in Gunn as compared with Sprague-Dawley rats (Fig. 2) was not further studied by gel electrophoresis and densitometry, and may result from competition for binding between BSP and bilirubin endogenously bound to Y.

Because measurement of Y and Z by gel filtration and dye binding is influenced by the size of the Sephadex column, standardization of chromatography is essential (1). The comparative quantitative results are significant and are retained if a different size column is used; however, the absolute number of units of Y and Z vary (1). In the present study, a standardized gel filtration system was utilized and its reproducibility has been previously established. In some experiments, Y and Z were separately quantitated by densitometry after gel electrophoresis. The results correlated well with results obtained by dye binding on Sephadex (Tables III, IV).

The transfer of BSP from plasma to bile includes uptake into the liver cell, storage, partial conjugation with glutathione, and subsequent excretion into the bile canaliculus. Each of these parameters may be increased after phenobarbital administration (20–23) and increased BSP plasma disappearance could theoretically result from any or several of these effects. In the present studies, enhanced hepatic dye uptake probably occurs after drug administration for the following reasons: (*a*) initial plasma BSP disappearance rate was increased when determined 2–7 min after a single intravenous injection when exponential disappearance of dye is significantly influenced by hepatic uptake (10); (*b*) suppression of bile flow by acute bile duct ligation did not eliminate the increase in K_1 for BSP in rats treated with phenobarbital; (*c*) K_1 for ICG, an organic anion which is also selectively and rapidly transferred from plasma to bile but without biotransformation in the liver (24), also increased after phenobarbital administration; (*d*) hepatic content of BSP and ICG were increased after phenobarbital administration, and (*e*) relative hepatic storage of BSP was increased in rats treated with 8 mg phenobarbital/day for 4 days at a time when Tm, but not bile flow was unaffected. Relative hepatic storage of BSP was not significantly different from normal in rats treated with phenobarbital for 14 days, at which time bile flow and Tm were increased (Table III).

Hepatic uptake in terms of bidirectional molecular transfer across the plasma membrane cannot be measured directly. BSP is partially bound to serum albumin (25) and a nonenergy-dependent mechanism for its rapid and selective transfer into the liver has been proposed (26). Direct measurement of hepatic BSP uptake requires simultaneous kinetic estimates of bidirectional flux across the hepatic cell plasma membrane. Neither plasma disappearance data nor measurements of hepatic dye content are entirely satisfactory as measures of hepatic organic anion uptake. Each method has several assumptions which may be questioned. However, in the present study in normal rats, each of five different estimates of hepatic uptake increased in association with an increase in Y.

Administration of AIA, dieldrin, DDT, benzpyrene, and 3-methylcholanthrene results in proliferation of hepatic smooth endoplasmic reticulum, and increase in various drug metabolizing enzymes and other hepatic microsomal proteins (27).[3] When administered to normal adult rats, these drugs produced effects similar to phenobarbital; Y increased whereas Z was normal or decreased. When measured, hepatic BSP content increased. The relationship between interaction of the "inducer," hepatic endoplasmic reticulum, and increased Y concentration requires further study.

Female rats receiving ethinyl estradiol in a dose shown to produce cholestasis (7) had reduced Y, Z, and K_1 for BSP. Direct measurement of Y and Z by gel electrophoresis and densitometry confirmed the reduction noted by dye binding (Table IV). None of the other hormones studied significantly influenced Y, Z, or K_1 in normal rats. Prolonger biliary obstruction rapidly and progressively decreased Z; Y was reduced more slowly and to a lesser extent (Fig. 9, Table IV). The mechanisms by which biliary obstruction and ethinyl estradiol administration reduce the concentration of Y and Z and the relationship to cholestasis require further study.

Hypophysectomy in rats and hypopituitarism in dw/dw mice doubled the concentration of Y; Z was slowly reduced to less than 50%. Similar changes were observed in thyroidectomized rats, although Y increased by only 30%. In hypophysectomized or thyroidectomized rats and dw/dw mice, phenobarbital administration produced a further increase in Y and, in some experiments, Z was reduced (Figs. 5, 7, and 8). The additive effect on Y of hormonal deprivation and drug administration suggests that each factor may increase the concentration of Y by a different mechanism.

The apparent increase in Y after drug administration or hormonal deprivation can theoretically result from increased synthesis (induction), decreased degradation (stabilization), or altered affinity of the protein for the dye utilized in quantitation. Phenobarbital, insecticides, and carcinogens increase microsomal protein synthesis (28, 29). Because Y increases in response to drugs which increase microsomal proteins and proliferate he-

[3] Wolff, J. A., A. D. Munro Faure, A. W. Peck, A. Bye, R. Chang, and M. Jacobson. Effects of environmental chemicals on the metabolism of drugs, carcinogens, and normal body constituents in man. In press.

patic smooth endoplasmic reticulum, Y may be synthesized in the endoplasmic reticulum and subsequently released in the cytoplasm. Y and Z are soluble proteins and account for approximately 5 and 2% of total cytoplasmic proteins, respectively.[3] Their site of origin and other possible subcellular localizations cannot be ascertained by available methodology and require immunologic study.

Y and Z are probably not the sole determinants of hepatic organic anion uptake particularly as estimated by plasma disappearance rates or hepatic content of BSP. For example, K_1 and hepatic dye content are reduced in hypophysectomized and thyroidectomized rats although Y is increased. In addition, mutant Southdown sheep with impaired organic anion uptake in vivo have Y and Z fractions in their liver although precise quantitation has not been performed (1). Changes in body temperature, hepatic blood and lymph flow, plasma membrane of the hepatocyte, and availability of competing exogenous organic anions, probably influence hepatic organic anion uptake. Body temperature was maintained in the present experiments. Hepatic blood flow was not measured. Hepatic plasma membranes bind BSP and other organic anions in vitro (30) and competition for binding by other organic anions occurs in vivo (31).

Thyroxine was the only hormone tested which, in physiologic doses, restored the concentration of Y to normal in hypophysectomized and thyroidectomized rats. Z increased after thyroxine administration only in thyroidectomized rats. In these animals, weight gain and general improvement were obvious; K_1 and where studied, BSP content became normal. In hypophysectomized animals, changes in weight and body temperature were minimal during thyroxine administration, and K_1 returned to normal only in animals treated with large doses of the hormone. In dw/dw mice, thyroxine restored Y to normal. Thyroxine administration did not increase K_1 beyond normal values whether the hormone was administered to normal, hypophysectomized, or thyroidectomized rats. These observations suggest that thyroid hormone is important in the control of Y and perhaps Z.

Increase in plasma disappearance of drugs has been observed in animals pretreated with several drugs and chemicals, including phenobarbital, insecticides, and carcinogens,[3] and in man after administration of phenobarbital and other drugs or exposure to insecticides (29). This change has usually been attributed to induction of microsomal drug-metabolizing enzymes. Our experiments suggest an alternate possibility. The increase in plasma disappearance of various compounds after drug administration may be related to induction of cytoplasmic binding proteins which facilitate hepatic uptake of the respective organic anions.

The mechanisms whereby drugs, chemicals, and thyroid regulate the intracellular concentration of Y are currently under investigation. Despite the indirect measures utilized to estimate hepatic organic anion uptake, the present results support the hypothesis that Y and Z are important in transfer of organic anions from plasma into the liver.

ACKNOWLEDGMENTS

We gratefully appreciate the assistance of Doctors E. S. Russell and Seldon Bernstein of the Jackson Laboratory, Bar Harbor, Maine in providing dwarf mice; Dr. Charles Lieber of the Bronx Veterans Administration Hospital for providing liver from ethanol-fed rats and their controls, and to Dr. Donald P. Tschudy of the National Institutes of Health for a gift of allylisopropylacetamide.

This work was supported by grants from the U. S. Public Health Service AM-02019 and AM 5384, the New York Heart Association and Heart Fund, Inc., and the G. D. Searle Company.

REFERENCES

1. Levi, A. J., Z. Gatmaitan, and I. M. Arias. 1969. Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein, and other anions. *J. Clin. Invest.* **48**: 2156.
2. Levine, R. I., H. Reyes, A. J. Levi, Z. Gatmaitan, and I. M. Arias. 1971. Phylogenetic study of organic anion transfer from plasma into the liver. *Nature* (*London*). **231**: 277.
3. Fleischner, G., J. Robbins, H. Reyes, A. J. Levi, and I. M. Arias. 1971. Immunologic studies of Y, the major organic anion-binding protein in rat liver cytosol. *Gastroenterology*. **60**: 185.
4. Levi, A. J., Z. Gatmaitan, and I. M. Arias. 1969. Deficiency of hepatic organic anion-binding protein as a possible cause of non-haemolytic unconjugated hyperbilirubinaemia in the newborn. *Lancet*. **2**: 139.
5. Levi, A. J., Z. Gatmaitan, and I. M. Arias. 1970. Deficiency of hepatic organic anion-binding protein, impaired organic anion uptake by liver and "physiologic" jaundice in newborn monkeys. *N. Engl. J. Med.* **283**: 1136.
6. Reyes, H., A. J. Levi, Z. Gatmaitan, and I. M. Arias. 1969. Organic anion-binding protein in rat liver: drug induction and its physiologic consequence. *Proc. Nat. Acad. Sci.* **64**: 168.
7. Karkavy, M., and N. B. Javitt. 1969. Effect of ethynyl estradiol on hepatic excretory function of the rat. *In* Metabolic Effects of Gonadal Hormones and Contraceptive Steroids. H. A. Salhanick, D. M. Kipnis, and R. L. Vande Wiele, editors. Plenum Publishing Corporation, New York. 11.
8. Snell, G. D. 1929. Dwarf, a new Mendelian recessive character of the house mouse. *Proc. Nat. Acad. Sci.* **15**: 733.
9. Smith, P. E., and E. C. Mac Dowell. 1930. An hereditary anterior-pituitary deficiency in the mouse. *Anat. Rec.* **46**: 249.
10. Berthelot, P., and B. H. Billing. 1966. Effect of bunami-

odyl on hepatic uptake of sulfobromophthalein in the rat. *Amer. J. Physiol.* **211**: 395.
11. Wheeler, H. O., W. I. Cranston, and J. I. Meltzer. 1958. Hepatic uptake and biliary excretion of indocyanine green in the dog. *Proc. Soc. Expt. Biol. Med.* **99**: 11.
12. Klaasen, C. D., and G. L. Plaa. 1967. Determination of sulfobromophthalein storage and excretory rate in small animals. *J. Appl. Physiol.* **22**: 1151.
13. Wheeler, H. O., J. I. Meltzer, and S. E. Bradley. 1960. Biliary transport and hepatic storage of sulfobromophthalein sodium in the unanesthetized dog, in normal man, and in patients with hepatic disease. *J. Clin. Invest.* **39**: 1131.
14. Metcoff, J., and C. B. Favour. 1944. Determination of blood and plasma volume partitions in the growing rat. *Amer. J. Physiol.* **141**: 695.
15. Goldstein, A. 1964. Biostatistics, An Introductory Text. The Macmillan Company, New York. 51.
16. Grodsky, G. M., H. J. Kolb, R. E. Fanska, and C. Nemechek. 1970. Effect of age of rat on development of hepatic carriers for bilirubin: a possible explanation for physiologic jaundice and hyperbilirubinemia in the newborn. *Metab. (Clin. Exp.).* **19**: 246.
17. Kato, R., and J. R. Gillette. 1965. Sex differences in the effects of abnormal physiological states on the metabolism of drugs by rat liver microsomes. *J. Pharmacol. Exp. Ther.* **150**: 285.
18. Furner, R. L., T. E. Gram, and R. E. Stitzel. 1969. The influence of age, sex and drug treatment on microsomal drug metabolism in four rat strains. *Biochem. Pharmacol.* **18**: 1635.
19. Page, J. G., and E. S. Vesell. 1969. Hepatic drug metabolism in ten strains of Norway rat before and after pretreatment with phenobarbital. *Proc. Soc. Exp. Biol. Med.* **131**: 256.
20. Hart, L. G., A. M. Guarino, and R. H. Adamson. 1969. Effects of phenobarbital on biliary excretion of organic acids in male and female rats. *Amer. J. Physiol.* **217**: 46.
21. Klaasen, C. D., R. J. Roberts, and G. L. Plaa. 1969. Maximal biliary excretion of bilirubin and sulfobromophthalein during various rates of infusion in rats of different weights and strains. *Toxicol. Appl. Pharmacol.* **15**: 143.
22. Roberts, R. J., and G. L. Plaa. 1967. Effect of phenobarbital on the excretion of an exogenous bilirubin load. *Biochem. Pharmacol.* **16**: 827.
23. Klaasen, C. D., and G. L. Plaa. 1968. Studies on the mechanism of phenobarbital-enhanced sulfobromophthalein disappearance. *J. Pharmacol. Exp. Ther.* **161**: 361.
24. Barbier, F., and G. A. De Weerdt. 1964. Chromatography and I. R. spectrography of indocyanine green. *Clin. Chim. Acta.* **10**: 549.
25. Baker, K. J., and S. E. Bradley. 1966. Binding of sulfobromophthalein (BSP) sodium by plasma albumin. Its role in hepatic BSP extraction. *J. Clin. Invest.* **45**: 281.
26. Goresky, C. A., and G. G. Bach. 1970. Membrane transport and the hepatic circulation. *Ann. N. Y. Acad. Sci.* **170**: 18.
27. Biempica, L., N. S. Kossower, and A. B. Novikoff. 1967. Cytochemical and ultrastructural changes in rat liver in experimental porphyria. *Lab. Invest.* **17**: 171.
28. Arias, I. M., D. Doyle, and R. T. Schimke. 1969. Studies on the synthesis and degradation of proteins of the endoplasmic reticulum of rat liver. *J. Biol. Chem.* **244**: 3303.
29. Conney, A. H. 1967. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**: 317.
30. Cornelius, C. E., J. Ben-Ezzer, and I. M. Arias. 1967. Binding of sulfobromophthalein sodium (BSP) and other organic anions by isolated hepatic cell plasma membranes *in vitro*. *Proc. Soc. Exp. Biol. Med.* **124**: 665.
31. Andrews, W. H. H., and T. G. Richards. 1960. The activity of bile salts and certain detergents on the hepatic storage and protein-binding of sulfobromophthalein. *Quart. J. Exp. Physiol. Cog. Med. Sci.* **45**: 275.