Effect of Sodium Phenobarbital on Bilirubin Metabolism in an Infant with Congenital, Nonhemolytic, Unconjugated Hyperbilirubinemia, and Kernicterus

JOHN F. CRIGLER, JR. and NORMAN I. GOLD

From the Department of Medicine (Endocrine Division), The Children's Hospital Medical Center, and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Sodium phenobarbital and various hormones, compounds capable of hepatic enzyme induction, were given to an infant boy with congenital, nonhemolytic, unconjugated, hyperbilirubinemia and severe kernicterus for prolonged periods between the ages of 2 and 25 months to determine their effect on serum bilirubin concentrations. Phenobarbital, 5 mg/day orally, on two occasions decreased serum bilirubin concentrations approximately threefold over a period of 30 days. Withdrawal of phenobarbital after the first study resulted in a gradual (30 days) return of serum bilirubin to pretreatment levels. The lower serum bilirubin concentrations observed when phenobarbital therapy was reinstituted were maintained for 61 days on 2.5 mg/kg per day of the drug. Orally administered L-triiodothyronine, 0.05–0.1 mg/day for 71 days, intramuscular human growth hormone, 1 mg/day for 21 days, and testosterone propionate, 0.1 mg/day for 9 days, did not decrease serum bilirubin levels below lowest control values of 18 mg/100 ml.

Bilirubin-^{3}H was administered twice before and once with bilirubin-^{14}C during phenobarbital therapy to study the kinetics of bilirubin metabolism. Results of the first and second control studies and of the bilirubin-^{3}H and bilirubin-^{14}C phenobarbital studies, respectively, were as follows: total body bilirubin pools, 200, 184, 73, and 72 mg; half-lives, 111, 84, 37, and 39 hr; and turnover, 30, 37, 33, and 31 mg/day. The data show that the approximate threefold decrease in serum bilirubin concentration and total body pool resulted from a comparable decrease in bilirubin half-life without a significant change in turnover.

In vitro histological (electron microscopy) and enzymological studies of liver obtained by surgical biopsies before and during phenobaribtal administration showed that both the hepatocyte content of agranular endoplasmic reticulum (AER) and the ability of liver homogenate to conjugate *p*-nitrophenol were significantly increased during phenobarbital treatment.

The observations suggest that phenobarbital affects bilirubin metabolism by the induction of an enzyme(s) with a slow rate(s) of degradation (or rapid rate of degradation with limited capacity).

INTRODUCTION

In recent years, investigations in many animal species, including man, have shown that a variety of polycyclic hydrocarbons, insecticides, drugs, and hormones enhance the activities of microsomal and soluble enzymes and increase hepatic agranular endoplasmic reticulum (AER). Results of these investigations have been summarized by early workers in this field (1–6). The frequency of this pharmacological response is emphasized in a compilation by Conney (5) in 1965 which showed that stimulation of drug-metabolizing enzymes had been observed with over 100 drugs and other compounds.

Catz and Yaffe (7) and Arias, Gartner, Furman, and Wolfson (8) first demonstrated that the in vivo administration of various drugs and chemicals increased in vitro activity of the uridine diphosphate glucuronic acid transglucuronylase (UDPGA transferase) system for bilirubin in rats, rabbits, and mice. However, Arias

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et al. (8), in exploring the potential use of these drugs to accelerate the formation of bilirubin glucosiduronate in man, showed that the administration of chloroquine to pregnant women, for 1–8 wk before delivery, was unable to alter the hyperbilirubinemia present in their newborn infants.

The ability of phenobarbital to stimulate the transformations of drugs (9) and steroid hormones in various animals (10) and man (11) and our current use of this drug in studies of steroid hormone metabolism led us to investigate the effect of phenobarbital on the bilirubin metabolism of an infant with marked congenital, nonhemolytic, unconjugated hyperbilirubinemia, who had suffered severe, irreversible neurological damage during the 1st month of life. This paper reports changes observed in serum bilirubin concentrations and in the kinetics of bilirubin turnover before and during phenobarbital administration to this infant (12, 13). Coincident with these studies, Yaffe, Levy, Matsuzawa, and Baliah (14) described a similar effect of phenobarbital on serum bilirubin concentrations in an infant with congenital nonhemolytic jaundice and reported a drug-induced increase in the capacity to conjugate salicylamide as glucosiduronate.

METHODS

Clinical course and study periods. T. U. was hospitalized from 2 to 23 months of age and for 2 wk at 25 months of age. He was the seventh child of a 42 yr old gravida 7, para 7 woman. One day of bleeding occurred in the 2nd month of pregnancy. At 7 months, premature rupture of membranes was accompanied by a febrile illness of unknown cause which responded to antibiotics. Birth weight was 2.06 kg. Jaundice during the 1st wk of life was thought to be physiological. However, when he was seen at 2 months of age because of an umbilical hernia, marked jaundice was present.

The patient's maternal grandmother had intermittent, indirect hyperbilirubinemia (serum bilirubin, 1.7 mg/100 ml). She had been studied at another hospital, and no evidence of hemolysis or liver disease had been found. The patient's oldest brother had been jaundiced in the newborn period.

At 2 months of age, T. U. was 135 cm in length and weighed 4.38 kg. His skin was orange-yellow in color, and he had marked clinical evidence of kernicterus. There was a small umbilical hernia. The liver and spleen were not enlarged.

Initial laboratory studies were as follows: hemoglobin 12.7 g/100 ml, hematocrit 39.5%, reticulocytes 1.0%, and WBC 7000/mm^3 with a normal differential. Urinalysis was normal and was negative for bilirubin (Ictotest, Ames Co., Inc., Elkhart, Ind.) and urobilinogen (15). Serum bilirubin (16) was usually between 17 and 25 mg/100 ml, virtually all indirect reacting. Values from 35 to 39 mg/100 ml were observed after an exchange transfusion 7 days before his first admission and after surgery for liver biopsies (Table I footnotes). Other liver function tests [1] including total serum protein and serum protein electrophoresis, cephalin-cholesterol flocculation (19), thymol flocculation and turbidity (19), prothrombin time, and serum alkaline phosphatase (20) were within normal limits. Serum glutamic oxalacetic and glutamic pyruvic transaminase activities were increased and ranged from 105 to 180 and 75 to 95 U/ml, respectively. Lactic dehydrogenase activity, measured only on one occasion, was 265 U/ml, an elevated value. The explanation for these elevated serum enzyme activities in this patient with marked neuromuscular as well as hepatic dysfunction is not known.

Liver biopsies for histological and enzymatic studies were obtained at surgery before (age 83 and 551 days) and during L-triiodothyronine (age 132 days) and phenobarbital (age 677 days) administration. Nitrous oxide–oxygen and halothane (2-bromo-2-chloro-1, 1, 1-trifluoroethane) anesthesia was administered after a 9 hr fast.

The various study periods during the patient's two hospitalizations are outlined in Table I, and the mean and standard deviation of serum bilirubin concentrations are given for each period. Serum bilirubin concentrations were measured once or twice daily on capillary blood (16) throughout the course of observation.

Kinetic studies of bilirubin metabolism were done at 496, 522, and 608 days of age (Table I, periods 15 and 16). During the first two studies, control 1 and control 2, the patient had intermittent fever with otitis media and left mastoiditis. Nasogastric tube feedings, required because of defective swallowing associated with kernicterus, led to reoccurring upper respiratory obstruction and infections. He received ampicillin from days 3 to 15 of the first bilirubin-^{3}H study (control 1) and nafcillin 4 days before and during the first 12 days of the second study (control 2). In spite of nafcillin therapy, he remained intermittently febrile with increased serum bilirubin concentrations during the second bilirubin-^{3}H study (control 2). On the 16th day of the latter study, 3 days after nafcillin was discontinued, his fever increased to 105°F, and a left mastoiditis was radiologically evident. This infection responded promptly to intravenous oxacillin and kanamycin. 12 days later (age 551 days), a gastrostomy was done to facilitate feedings. The third liver biopsy was obtained at this time. No further respiratory illnesses occurred during the period of study.

Kinetic studies. The general plan of these studies was based on observations of bilirubin metabolism in a similar patient originally reported by Schmid and Hammaker (21).

Preparation of bilirubin-^{3}H and bilirubin-^{14}C. Crystalline bilirubin was treated with tritium gas according to the procedure of Wilzbach (22). The crude product was washed with methanol and the methanol removed by evaporation. The residue was crystallized several times from hot pyridine (23). After addition of unlabled bilirubin, the prepara-

[1] Liver function tests were performed in the Clinical Laboratories of The Children's Hospital Medical Center. Total serum protein was measured using an ultra micro adaptation of the methods of Kingsley (17) and Gornall, Bardawill, and David (18) and protein electrophoresis carried out on a Beckman Model R paper electrophoresis system by procedure B described in the Instruction Manual (November, 1957). The Quick prothrombin time was done using the test procedure described by Hyland Division Travenol Laboratories, Inc., Los Angeles, Calif., who prepared the thromboplastin. Serum glutamic oxalacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), and lactic dehydrogenase (LDH) were determined using reagent tubes (Determatubes) prepared by the Worthington Biochemical Corp., Freehold, N. J., with slight modifications. Normal values in units per milliliter for infants are 10–40 for SGOT, 10–60 for SGPT, and 40–100 for LDH.

tion was dissolved in isopropanol: 0.5 N NaOH, 7:3 v/v, and allowed to sit 10 min at room temperature in the dark. Bilirubin, precipitated by lowering the pH to 2 with 6 N HCl. was extracted with chloroform. The chloroform was washed with water and removed by evaporation. From the residue, bilirubin-^{3}H, 1.75×10^6 dpm/mg, was obtained by two crystallizations from hot pyridine. Its specific activity was not altered by further treatment with alkali and acid. The molar extinction coefficient of the bilirubin-^{3}H preparation was 5960 in chloroform (23). Bilirubin-^{14}C, 12.2×10^6 dpm/mg, prepared from δ-aminolevulinic acid-^{14}C according to Ostrow, Hammaker, and Schmid (24), was supplied by Dr. Stephen Robinson. Dried, labeled bilirubin preparations, stored in the dark at room temperature until ready for use, retained their original specific activities.

Intravenous administration of bilirubin solutions. All intravenous solutions were prepared immediately before administration and injected in 1 min into the right external jugular vein. *Control 1:* 3.5 mg of bilirubin-^{3}H was placed in a sterile vial and dissolved in 3 ml of a pH 11 solvent (0.067 M phosphate buffer adjusted to pH with N NaOH). 20 ml of sterile 0.067 M phosphate buffer, pH 7.4, was added, and 21.9 ml, equivalent to 3.33 mg of bilirubin, and 5.8×10^6 dpm of ^{3}H were administered. *Control 2:* 3.3 mg of bilirubin-^{3}H was dissolved in 3 ml of the pH 11 solvent. In quick succession, 13.0 ml of the phosphate buffer and 4.0 ml of sterile 25% human serum albumin were added. 19 ml of this clear solution, equivalent to 3.15 mg of bilirubin and 5.5×10^6 dpm of ^{3}H was administered. *Phenobarbital study:* 6.6 mg of bilirubin-^{3}H and 0.158 mg of bilirubin-^{14}C were dissolved in 4 ml of pH 11 solvent. 18 ml of sterile pH 7.4 phosphate buffer was then added, and 20.5 ml of this solution, equivalent to 6.4 mg of bilirubin, 11.0×10^6 dpm of ^{3}H, and 1.83×10^6 dpm of ^{14}C were administered.

Blood, stool, and urine collections. Approximately 2 ml of blood was drawn for serum at 2, 10, 20, 30, 45, and 60 min and at 2, 3, 4, 6, 8, and 12 hr after the administration of labeled bilirubin. Thereafter, samples were taken twice daily, at 8:00 a.m. and 4:00 p.m., until serum radioactivity was too low for quantitative determination. Stools were collected as 3-day pools in *control 1,* and individually in *control 2* and *phenobarbital* studies with carmine markers used to estimate transit time. Continuous 24-hr urine collections were made. Serum, stools, and urines were kept frozen until analyzed.

Specific activity of serum bilirubin. 25–250 μl of serum was extracted according to the method of Weber and Schalm (25). The concentration of bilirubin in this extract was determined from its absorption at 450 mμ corrected for any contribution from free hemoglobin by measuring the absorption at 385 mμ and applying the following empirically derived equation: micrograms of bilirubin per milliliter of extract $= 9.3\ A_{450m\mu} - 0.79\ A_{385m\mu}$. The bilirubin content of several serum samples determined by this procedure agreed with values obtained with a diazotization procedure (16). To determine radioactivity, 0.5 ml of the Weber and Schalm extract and 2 ml of chloroform were placed in open 22-ml scintillation vials. To minimize quenching, the latter were placed, first beneath the short wave length (2537 A) lamp of a "Chromato-Vue" cabinet [2] for 10 min to bleach the bilirubin and then in a warm vacuum chamber for 20 min to remove quenching components volatile at 1 mm Hg. 15 ml of scintillation fluid was added to each treated vial. No loss of ^{3}H from such procedures was demonstrated by obtaining identical radioactivity from vials containing bilirubin-^{3}H standards given such treatment and from vials to which the bilirubin-^{3}H was added *after* treatment. The scintillation fluid consisted of 60 mg of 2,5-diphenyloxazole (PPO), 0.75 mg of 1,4-bis[2-(5-phenyloxazolyl)] benzene (POPOP), 0.294 ml of methanol, and 15 ml of toluene. A Nuclear-Chicago liquid scintillation system, Model 720, was used, with an efficiency of 20% for tritium alone, and 10% and 15% for ^{3}H and ^{14}C, respectively, when both isotopes were counted simultaneously (26). Backgrounds were 32 cpm for ^{3}H alone, and 8 and 13 cpm in scalers A (^{14}C) and C (^{3}H), respectively, when both isotopes were counted simultaneously. Sample vials, backgrounds, and standards were prepared in duplicate and counted 10 times for 10 min each. Toluene-^{3}H and toluene-^{14}C were used for internal quench corrections.

Crystallization of serum bilirubin. Unlabeled bilirubin was added to a pool of sera obtained from the bilirubin-^{3}H and bilirubin-^{14}C (phenobarbital) study and the bilirubin extracted from the pooled sera with the Weber and Schalm (25) reagent. A portion of extract was analyzed for radioactivity as previously described for measurement of serum specific activity. The extract which remained was washed with water, 10% NaCl, and water again, and the bilirubin which precipitated was crystallized once from methanol and once from chloroform-methanol. The ^{3}H/^{14}C values of the extract and crystallized bilirubin were 6.3 and 6.0 respectively. ^{3}H/^{14}C in the administered bilirubin was 6.0.

Radioactivity in stools. Stools were weighed wet and homogenized with 2 volumes of distilled water. Aliquots of the homogenates were dried in vacuo, with warming, transferred to tared vials for dry weight determination, and ground to a powder. 20–200 mg portions of dried stool were combusted in oxygen, according to Oliverio, Denham, and Davidson (27) using Nichrome baskets (28). The scintillation fluid used for ^{3}H alone and for ^{3}H plus ^{14}C counting was 270 ml of phenethylamine, 270 ml of methanol, 5 g of PPO, and 100 mg of POPOP made to 1 liter with toluene. Internal standards of toluene-^{3}H and toluene-^{14}C were used to correct for quenching. Accumulation of counts was the same as described for serum. Background values were 9 cpm, scaler A (^{14}C), and 24 cpm, scaler C (^{3}H), with unquenched efficiencies of 5% for ^{3}H and 20% for ^{14}C. Recoveries of ^{3}H and^{14} C, determined by combusting trace quantities of ^{3}H- and ^{14}C-labeled steroids in the presence of varying amounts of dried stool, were 100% and 85%, respectively, and independent of the stool sample weights used in this study. Similar values of disintegrations per milligram of stool over fourfold ranges of dry weight indicated completeness of combustion.

Radioactivity in urines. 1 ml of urine was added to each of six vials containing 15 ml of scintillation fluid (84 ml of Liquifluor, 416 ml of toluene, and 500 ml of methyl Cellosolve). Internal standards of ^3H_2O were added to two vials and toluene-^{14}C to another two to correct for quenching. 1 ml of H_2O served as background. For ^{3}H alone, background was 33 cpm, and the unquenched efficiency was 5%. For ^{14}C and ^{3}H, measured simultaneously, backgrounds were 12 cpm, scaler A (^{14}C), and 25 cpm, scaler C (^{3}H), with unquenched efficiencies of 25% and 4.5%, respectively. Accumulation of counts was the same as described for serum.

Mathematical analyses of kinetic data. The logarithm of the specific activity of serum bilirubin has been plotted as a function of time for each study. Exponentials 1 represent the best straight lines drawn by the method of least squares through the final, first-order portion of each curve. Successive subtractions of first-order plots (29) of the serum specific

[2] Ultra-Violet Products, Inc., San Gabriel, Calif.

activity curves of both control and phenobarbital studies resulted in three first-order slopes, exponentials 1, 2, and 3. The subtractions for exponentials 2 and 3 were performed graphically. Each curve, therefore, is described by the general equation, $y = a_1e^{-\lambda_1 t} + a_2e^{-\lambda_2 t} + a_3e^{-\lambda_3 t}$ (29), where y is serum bilirubin specific activity at time, t, and a_1, a_2, and a_3 the bilirubin specific activities at $t = 0$ of the respective slopes λ_1, λ_2, and λ_3.

The slope, λ_1, and its intercept a_1, which are considered to represent steady-state values after complete mixing, were used to calculate total body pool and turnover of bilirubin (29). The additional exponentials of the general equation describe, at least, two other biological compartments of bilirubin which are distinguished readily by the marked differences in their rates of mixing (λ_2 and λ_3) with the plasma pool. A compartmental analysis of the data, however, is not attempted in this paper.

In vitro liver studies

Electron microscopy. 1-mm cubes of liver obtained by biopsy before (age 551 days) and during (age 677 days) phenobarbital therapy were fixed either in 5% glutaraldehyde-sodium cacodylate buffer, followed by washing and further fixation in Dalton's chrome-osmium solution, or directly, in Dalton's chrome-osmium solution. Tissues were processed for electron microscopy as described by Uzman, Foley, Farber, and Lazarus (30). 1-μ sections of several blocks of each specimen were examined by phase microscopy and appropriate areas selected for thin sectioning and electron microscopic examination. Two types of analyses were done to establish whether or not phenobarbital treatment significantly altered the amount of AER in the hepatocyte population. First, several blocks of each specimen were cut and random electron micrographs taken and blind-sorted on the basis of the amount of AER present. Second, the amount of AER in hepatocytes adjacent to portal areas of the tissue obtained during phenobarbital therapy was compared to that of hepatocytes near the central part of the lobule of the specimen obtained before phenobarbital administration. The latter comparison was made as, perhaps, the most demanding test of the effect of phenobarbital on AER proliferation since Burger and Herdson (31) and Becker and Lane (32)

TABLE I

Study Periods and Serum Bilirubin Concentrations in an Infant Boy with Congenital, Nonhemolytic, Unconjugated Hyperbilirubinemia and Kernicterus

Period	Age	Test substance	Total serum bilirubin *mg/100 ml*	
			No.	Mean ±SD
	days			
1	68–96	None	25	26.3 ±4.7*
2	97–114	L-Triiodothyronine, 0.05 *mg/day*, oral	16	18.4 ±1.7
3	115–140	L-Triiodothyronine, 0.075 *mg/day*, oral	17	24.9 ±3.0‡
4	141–158	L-Triiodothyronine, 0.1 *mg/day*, oral	16	24.4 ±1.5
5	159–167	L-Triiodothyronine, 0.1 *mg/day*, oral plus human growth hormone, 1 *mg/day*, i.m.	9	21.5 ±1.8
6	168–179	Human growth hormone, 1 *mg/day*, i.m.	11	27.2 ±2.8
7	180–185	None	6	30.0 ±1.5
8	186–191	Phenobarbital, 15 *mg* 2× *day*, oral	9	24.3 ±1.8
9	192–197	Phenobarbital, 15 *mg* 2× *day*, oral plus testosterone propionate, 0.1 *mg/day*, i.m.	9	18.2 ±3.0
10	198–209	Testosterone propionate, 0.1 *mg/day*, i.m.	21	13.5 ±1.5
11	210–227	None	32	17.7 ±1.5
12	228–236	Testosterone propionate, 0.1 *mg/day*, i.m.	14	18.3 ±1.2
13	237–266	Testosterone propionate, 0.1 *mg/day*, i.m. plus phenobarbital, 15 *mg* 2× *day*, oral	58	12.1 ±4.1
14	267–308	Phenobarbital, 15 *mg* 2× *day*, oral	77	8.9 ±2.0
15	309–567	None	298	22.7 ±4.0§
16	568–699	Phenobarbital, 20 *mg* 2× *day*, p.g.‖	104	6.7 ±1.2¶
	700	Discharged on phenobarbital, 10 *mg* 2× *day*, p.g.		
17	755–761	Phenobarbital, 10 *mg* 2× *day*, p.g.	11	7.1 ±0.4
18	762–768	Phenobarbital, 8 *mg/day*, p.g.	14	6.6 ±0.9

* Admitted to The Children's Hospital Medical Center on 68th day of age. Serum bilirubin increased to 34 mg/100 ml after 500 ml exchange transfusion on 61st day of age and to 38 mg/100 ml after liver biopsy on 83rd day of age. Serum bilirubin 17 mg/100 ml on the 96th day of age.

‡ Values obtained for 9 days after liver biopsy not included.

§ Values of first 30 days off phenobarbital (see Fig. 2) not included.

‖ Per gastrostomy.

¶ Values of first 30 days on phenobarbital (see Fig. 1 B) not included.

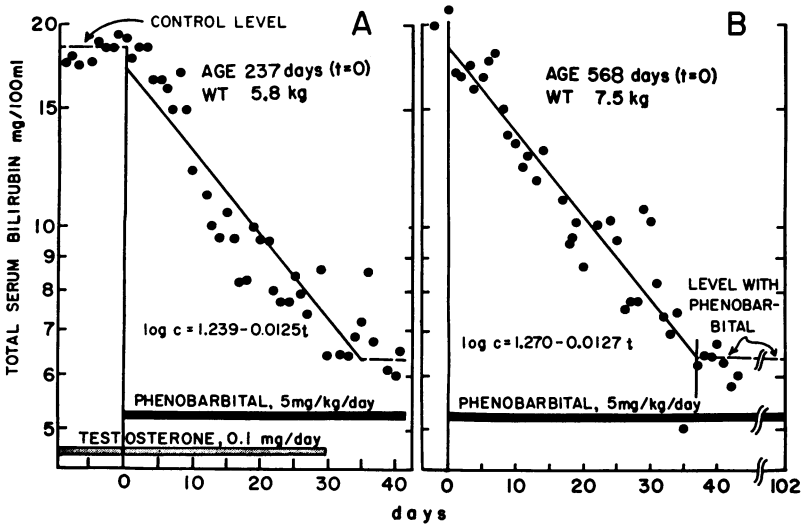

FIGURE 1 Decrease in serum bilirubin concentration with phenobarbital therapy in two study periods. Each point represents the mean of two analyses. Similar rate of decrease in concentration, c, in studies A and B are evident from the identical slopes of the first-order plots (see Appendix I). Testosterone, in study A, did not lower the control level or augment the phenobarbital effect.

have shown that in rats induction of AER occurs more slowly and to a lesser extent in the portal area of the liver lobule. Statistically valid lobular analyses of AER content of hepatocytes of the liver biopsies of this patient were not possible because of limitations of sample size.

Conjugation of p-nitrophenol (PNP). Liver obtained from the patient during L-triiodothyronine (age 132 days) and phenobarbital (age 677 days) therapy and from a 10 month old girl with idiopathic hypoglycemia who had received only human growth hormone and epinephrine during the previous 2 months was assayed for its ability to conjugate PNP in vitro.[3] The livers were homogenized at 0°C with two times their volume of 0.25 M sucrose in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The crude homogenate was centrifuged at 0°C and 9000 g for 15 min, and the supernatant was used as the homogenate for incubations similar to those described by Isselbacher, Chrabas, and Quinn (33). Each incubation, performed in air at 37°C, was 2 ml in volume and contained 5 μmoles of Tris buffer, pH 7.4, 1.5 μmoles of nicotinamide adenine dinucleotide phosphate (NADP), 0.5 U of glucose-6-phosphate dehydrogenase, 20 μmoles of glucose-6-phosphate, 5 μmoles of $MgCl_2$, 1 μmole of uridine disphosphoglucuronic acid, 0.6 μmole of uridine diphospho-*N*-acetyl glucosamine (34), 2 μmoles of PNP, and 0.2 ml of homogenate, the latter equivalent to 67 mg wet weight of liver. The reaction was stopped by saturation with $(NH_4)_2SO_4$ and by the addition of 2 ml of ethanol. After adjustment of the contents to 50 ml with 0.1 N NaOH, the absorbance of a filtered portion was determined at 400 mμ (33, 34), and the quantity of unconjugated PNP remaining was calculated from its molar extinction coefficient of 18.

RESULTS

Effect of various hormones on serum bilirubin concentration. Before the demonstration of the ability of phenobarbital to lower the concentration of serum bilirubin, the patient was given L-triiodothyronine (T_3) for 71 days and growth hormone and testosterone propionate alone and with T_3 and phenobarbital, respectively, for shorter periods (Table I, periods 2–6, 9–10, and 12). These hormones failed to decrease serum bilirubin levels.

Effect of phenobarbital on serum bilirubin concentration. The ability of sodium phenobarbital, 5 mg/kg per day, to reduce serum bilirubin concentration from approximately 20 mg/100 ml to levels of about 6 mg/100 ml at two ages, is shown in Fig. 1. On both occasions, approximately 30 days of treatment were required to reach the new steady-state level. Maintenance of the decreased serum concentration of bilirubin was interrupted in the first study by withdrawal of therapy, but was maintained for the duration of our observations, a period of 200 days after reintroducing phenobarbital therapy (Table I, periods 16–18). Reducing the dose of phenobarbital to 2.5 mg and to 1 mg/kg per day for the last 68 days of observation did not change serum bili-

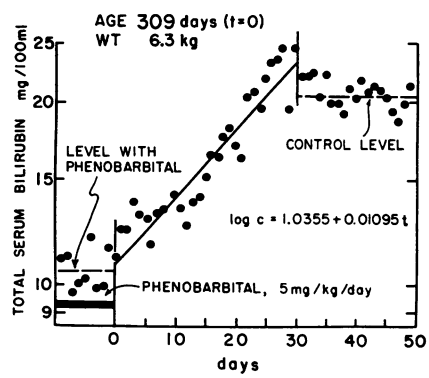

FIGURE 2 Increase in serum bilirubin concentration on discontinuing phenobarbital. Each point represents the mean of two analyses. The absolute rate of *increase* in concentration, c, is similar to its rate of *decrease* upon initiation of phenobarbital therapy (Fig. 1 and Appendix I).

[3] A similar in vitro assay of the conjugation of PNP, carried out on liver obtained from the patient before any drug or hormonal therapy (age 83 days), was technically unsatisfactory.

rubin concentrations (Table I, periods 17 and 18). Withdrawal of phenobarbital (Fig. 2), however, led to a gradual increase in serum bilirubin concentration until the pretreatment level was reestablished.[4]

Serum bilirubin concentrations in Figs. 1 and 2 were plotted logarithmically as a function of time with best straight lines drawn by the method of least squares to obtain slopes characterizing the processes of change during phenobarbital administration and withdrawal. These plots serve only as a convenient means of mathematical description and are not meant to imply that the kinetics of this change are known to be first order. In

[4] In Fig. 2, the bilirubin concentrations of 10.5 mg/100 ml, observed for approximately 30 days before discontinuing phenobarbital administration, and of 22–24 mg/100 ml, noted after phenobarbital withdrawal, are somewhat higher than those observed during the periods illustrated in Fig. 1. The difference in bilirubin concentrations is assumed to be due to a change in the "steady-state" conditions of the patient resulting from a persistent, low-grade respiratory infection during the interval shown in Fig. 2 (see Methods).

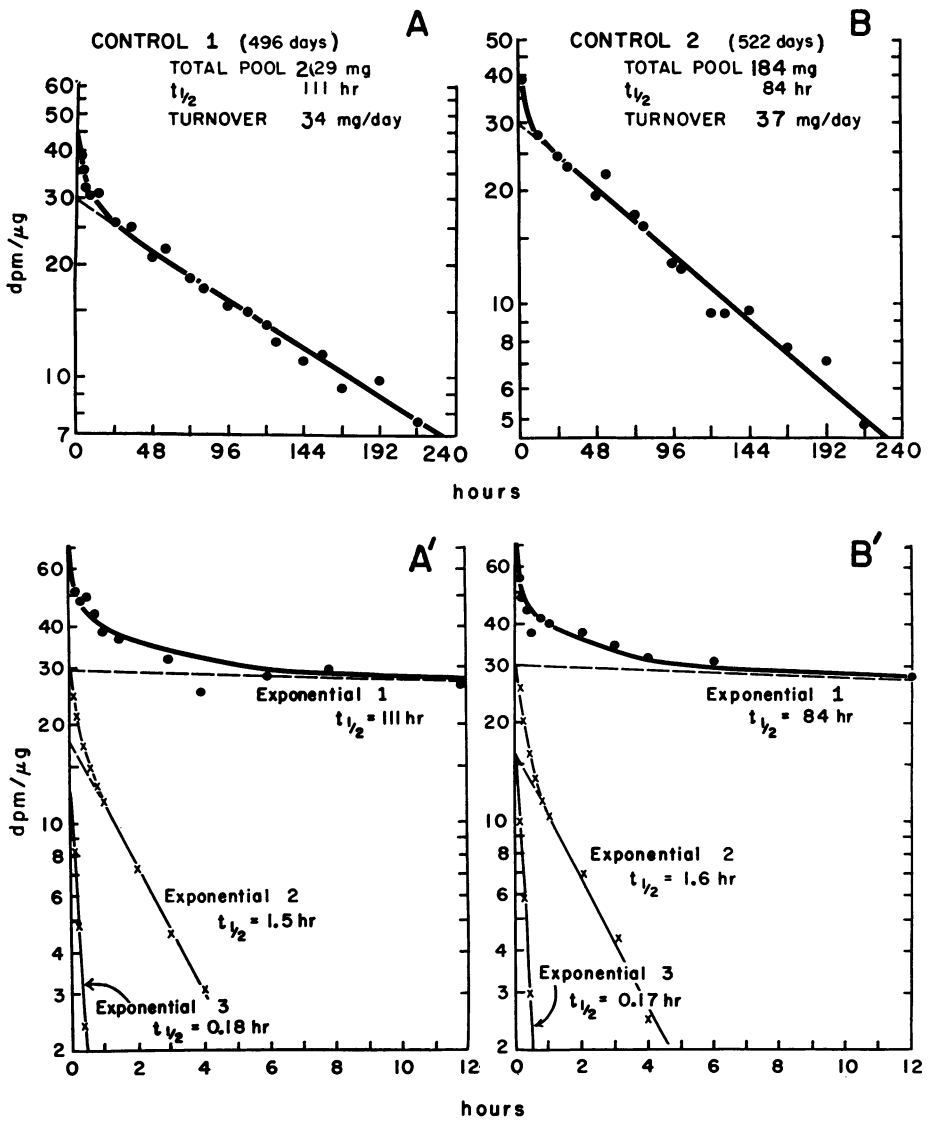

FIGURE 3 Turnover of bilirubin-^{3}H during two control periods. A′ and B′ represent expansions of the early portions of A and B. Values: measured ●——●; extrapolated from first-order lines ———; by difference ×——×. Equations (see Methods): Control 1, $y = 29e^{-0.0062t} + 18e^{-0.46t} + 12e^{-3.8t}$; Control 2, $y = 30e^{-0.0083t} + 16e^{-0.43t} + 16e^{-4.1t}$, where y = disintegrations per minute per microgram, and t = hour.

TABLE II

Effect of Phenobarbital on Bilirubin Metabolism in an Infant Boy with Congenital, Nonhemolytic, Unconjugated Hyperbilirubinemia and Kernicterus

	Control 1	Control 2	Phenobarbital*	
	496 days 7.3 kg	522 days 7.3 kg	608 days 7.4 kg	
Distribution factors	^3H	^3H	^3H	^{14}C
Serum bilirubin, *mg/100 ml*	20	23	6.7	
dpm of bilirubin administered $\times 10^{-6}$	5.8	5.5	11	1.83
Bilirubin turnover, *mg/day*‡	30	37	33	31
Bilirubin half-life, *hr*				
Exponentials 1	111	84	37	39
2	1.5	1.6	4.2	4.8
3	0.18	0.17	0.40	0.48
Bilirubin pools				
Total, *mg*§	200	184	73	72
Vascular, *mg*‖	83	96	28	28
Extravascular, *mg*¶	117	88	45	44
Extravascular/vascular	1.4	0.9	1.6	1.6
Turnover constant, k_t, per *day***	0.15	0.20	0.45	0.43

* 5 mg/kg per day, per gastrostomy.

‡ Total pool $\times$ 0.693/$t_{\frac{1}{2}} \times$ 24.

§ Disintegrations per minute administered/a_1; a_1, bilirubin specific activity at t = 0, was obtained by extrapolation of exponential 1 to t = 0.

‖ Serum concentration $\times$ plasma volume, the latter estimated as 5.7% of body weight.

¶ Total pool minus vascular pool.

** k_t = 0.693/$t_{\frac{1}{2}} \times$ 24 for exponential 1; $k_t = \lambda_1$, see text.

spite of considerable variation in the approximation of the data to first-order kinetics, the absolute values of the slopes describing the over-all rates of decrease of serum bilirubin on *introduction* of phenobarbital and of increase on *withdrawal* were identical (Figs. 1, 2). The equations for these lines are given in the figures, and the highly significant statistical similarity of their slopes is shown in Appendix I.

Effect of phenobarbital on bilirubin metabolism as determined from studies using bilirubin-^3H and bilirubin-^{14}C. Serum. Bilirubin specific activities as functions of time for both control studies (Fig. 3) and for the phenobarbital study (Fig. 4) adhered to first-order kinetics after 24 hr.

Controls 1 and 2. The following equations describe the serum bilirubin specific activity curves shown in Fig. 3: control 1, $y = 29e^{-0.0092t} + 18e^{-0.46t} + 12e^{-3.8t}$; control 2, $y = 30e^{-0.0083t} + 16e^{-0.43t} + 16e^{-4.1t}$, where y = disintegrations per minute per microgram and t = hours. The slopes of the initial portions of the control curves (Figs. 3A′ and B′) are similar. The early distribution and metabolism of bilirubin-^3H therefore, appear to be the same whether the labeled bilirubin is injected in a buffered aqueous solution (control 1) or in 5% human serum albumin (control 2).

The turnover data for the control studies (Table II) showed, unexpectedly, that the higher serum concentration of unconjugated bilirubin in control 2, 23 mg/100 ml vs. 20 mg/100 ml, was associated with a significant decrease in bilirubin half-life, 84 hr vs. 111 hr, a slight decrease in total body bilirubin pool, 184 mg vs. 200 mg, and a moderate increase in bilirubin turnover, 37 mg/day vs. 30 mg/day. It is not possible to assign any significance to the apparent differences in the vascular and extravascular pools, expressed in Table II by the extravascular pool : vascular pool ratios. Variations between the two control studies are thought to be due to biological changes in the patient which resulted from infection that began during the first study and persisted, in spite of antibiotic therapy, throughout the second study (see Methods).

Phenobarbital therapy. The specific activity curves for bilirubin-^3H and bilirubin-^{14}C plotted in Fig. 4 are described by the following equations: ^3H, $y = 151e^{-0.019t} + 85e^{-0.165t} + 151e^{-1.73t}$; ^{14}C, $y = 25e^{-0.018t} + 4e^{-0.144t} + 25e^{-1.44t}$, where y = disintegrations per minute per microgram and t = hours. A difference in the early distribution and metabolism of bilirubin-^3H- and bilirubin-^{14}C, suggested by differences in the half-life values calculated from the slopes of exponentials 2 and 3 and detectable in the $^3H/^{14}C$ of sequential serum bilirubin samples and stools, was not sufficient to influence significantly the turnover and body distributions of bilirubin calculated from the kinetic data for each isotope (Fig. 4, Table II).

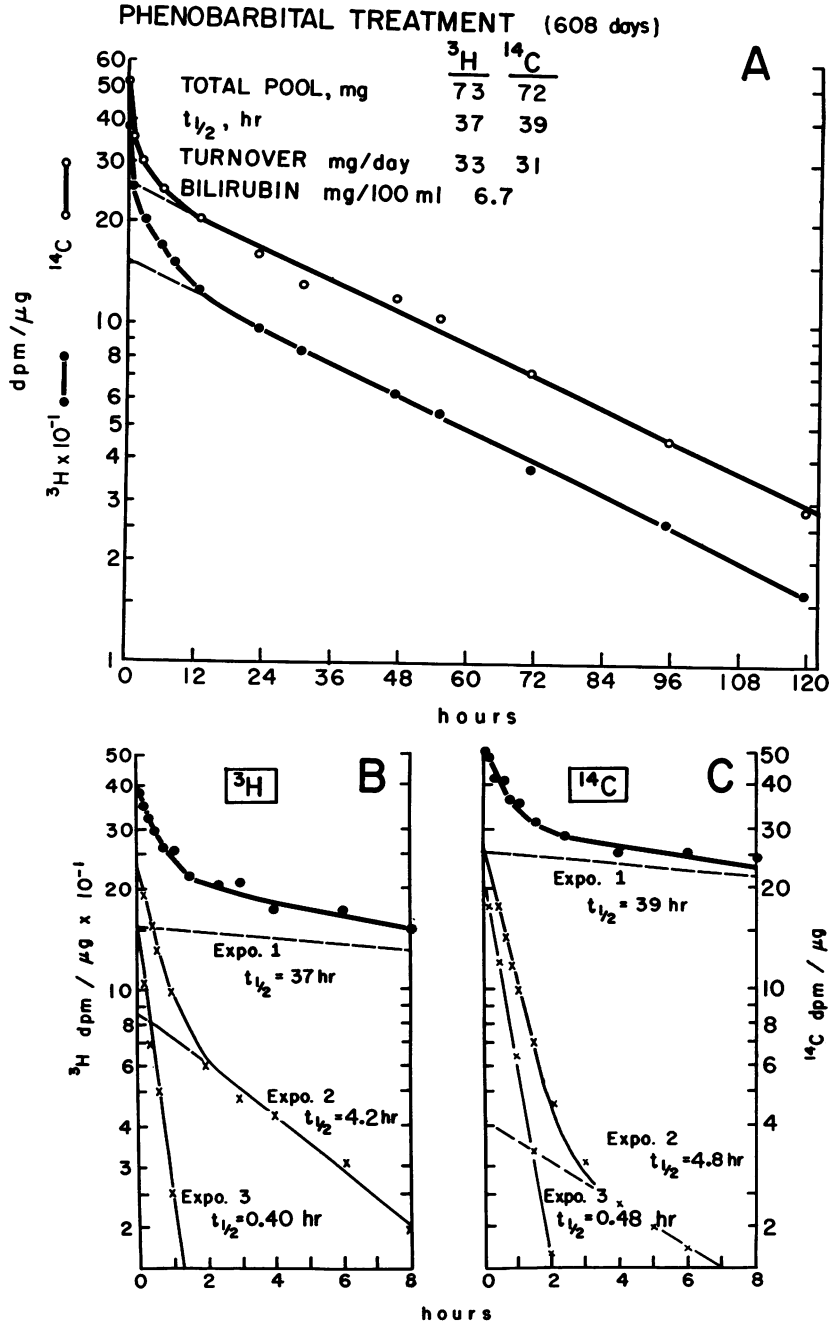

FIGURE 4 Turnover of bilirubin-^{3}H and bilirubin-^{14}C during phenobarbital therapy. B and C are expansions of the early portions of the ^{3}H and ^{14}C curves, respectively, of A. Curve construction is identical with Fig. 3. Equations (see Methods) : y (^{3}H) $= 151e^{-0.019t} + 85e^{-0.165t} + 151e^{-1.73t}$; y (^{14}C) $= 25e^{-0.018t} + 4e^{-0.144t} + 25e^{-1.41t}$.

In addition, the ^{3}H/^{14}C value of bilirubin crystallized from a pool of the sera was the same as that of the administered labeled bilirubins (see Methods).

The decreased mean serum bilirubin concentration, 6.7 mg/100 ml, during phenobarbital administration was accompanied by a comparable reduction in total bilirubin pool without a significant change in extravascular pool: vscular pool ratio or in bilirubin turnover (Table II). The change in the total body bilirubin pool, therefore, resulted from a two- to threefold decrease in bilirubin half-life.

Stool and urine. The rate of excretion of ^{3}H or ^{14}C

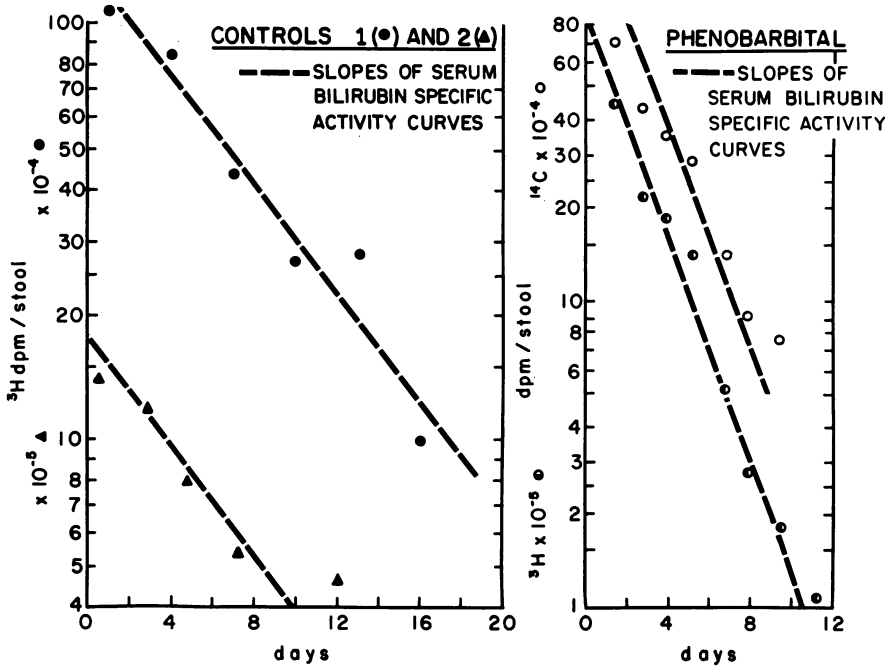

FIGURE 5 Rate of excretion of radioactivity in stool compared with that in serum. Each point represents either total radioactivity per 3-day collection (control 1, ●) or per single stool (control 2, ▲; phenobarbital: ^{14}C, ○; ^{3}H, ◐) and are plotted vs. time allowing 24 hr for transit through the intestines (measured repeatedly using carmine markers). Slopes of serum bilirubin specific activity curves are plotted to demonstrate how closely data approach those expected under steady-state conditions.

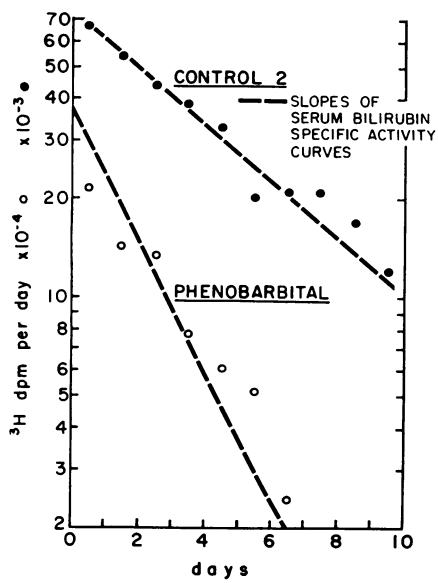

FIGURE 6 Rate of excretion of radioactivity in urine compared with that in serum. Each point represents total radioactivity from bilirubin-^{3}H excreted per 24 hr in urine during control 2 (●) and phenobarbital (○) studies. Data for control 1 were not obtained. Slopes of serum bilirubin specific activity curves are plotted to demonstrate how closely data approach those expected under steady-state conditions.

in stool (Fig. 5) and ^{3}H in urine (Fig. 6) during control and phenobarbital studies was similar to the rate of decrease of serum bilirubin specific activities in the respective studies, an observation consistent with steady-state conditions. Cumulative recovery of ^{3}H and ^{14}C from stool and urine for the three studies is shown in Table III. Most of the radioactivity, as expected, is found in stool with only 7% of the injected dose excreted in urine in times equivalent to 4 or more half-lives of bilirubin. No explanation for the 48% recovery of total radioactivity in control 1 is evident since early

TABLE III
Cumulative Recovery of ^{3}H and ^{14}C from Stools and Urines

Period of study	Stools			Urines	
	Cumulative period	^{3}H	^{14}C	Cumulative period	^{3}H
	days	*% of dose*		*days*	*% of dose*
Control 1	16	48		7	3
Control 2	14	73		14	7
Phenobarbital*	12	84	90	7	7

* Urinary content of ^{14}C was inadequate for quantitative determination.

values of serum specific activity exclude a significant error in measuring the injected dose, and no error in the analyses of the stool and urine radioactivity was detected.

Effect of phenobarbital on liver structure. Examination of the liver biopsies obtained before and during phenobarbital administration by light microscopy, carried out by Dr. Gorden Vawter, showed no significant differences and no pathological abnormalities. Electron microscopy (Fig. 7) performed by Dr. B. G. Uzman, however, revealed a marked increase in AER after phenobarbital administration, a drug-induced change in hepatic cells described in other animal species (6, 31, 32, 35, 36) but not previously documented in man. The changes in AER content of hepatocytes were readily distinguished by blind-sorting of electron micrographs from specimens before vs. those during phenobarbital therapy and were clearly evident when cells from the portal area of the tissue removed during phenobarbital therapy were compared to centrally located hepatocytes of the pretreatment specimen (Fig. 7 *a* and *b* vs. *c* and *d*). The latter analysis was based on studies in rats (31, 32) which showed that phenobarbital-induced increases of AER occurred more slowly and to a lesser extent in the portal area of the liver lobule.

Conjugation of PNP. The rate of conjugation of PNP, presumably as glucosiduronate (33), was calculated from the zero-order kinetics of the first 30 min of incubation with all three liver homogenates (Fig. 8). The number of millimicromoles of PNP conjugated per gram of wet weight of liver per minute was greatest, 232, during phenobarbital administration to T.U. and lowest, 130, for T.U. when he was receiving T_3 but not receiving the barbiturate. An intermediate rate of 175 was observed in P.M. who was not receiving phenobarbital.

DISCUSSION

Attempts to alter conjugation or to affect other metabolic transformations of bilirubin with T_3 did not result in lowered serum bilirubin levels or decreased clinical jaundice, although observations by others (37) and by us (38) suggest that excess thyroid hormone augments glucosiduronate conjugation of steroid hormones in human beings, and neonatal hypothyroidism is known to be associated with increased unconjugated hyperbilirubinemia (39, 40). Human growth hormone and testosterone propionate were given because testosterone and other anabolic steroids in some animals stimulate glucosiduronate formation (41) and augment the response of enzyme systems to drugs (41–43). Serum bilirubin concentrations of the patient, however, were not decreased by human growth hormone or testosterone (Table I, periods 5, 6, and 12).

The detailed data on bilirubin metabolism reported in this paper were obtained to elucidate independent observations originally described by us (11, 12) and by Yaffe et al. (14) of the ability of phenobarbital to markedly reduce serum bilirubin concentrations to two infants with congenital, nonhemolytic, unconjugated hyperbilirubinemia. Serum bilirubin concentrations in both infants decreased over a period of 3–4 wk to 3 and 7 mg/100 ml, values approximately one-third of those observed before phenobarbital was begun, and returned to pretreatment levels at a similar rate when the drug was discontinued. The fall in serum bilirubin concentration was associated with a significant decrease in clinical jaundice, suggesting either decreased bilirubin turnover, increased fractional turnover (decreased $t_{\frac{1}{2}}$), or sequestration of bilirubin in a nonvisible compartment. The reduction in serum bilirubin concentration during phenobarbital therapy was not due to a decrease in the rate of bilirubin formation since this was normal in control studies and was not significantly affected by phenobarbital (Table II).[5] Although there was a slight increase in the proportion of total body bilirubin in the extravascular pool during phenobarbital administration (Table II), a finding which might reflect some alteration in the binding of bilirubin to plasma proteins (45, 46), the major effect of phenobarbital was on bilirubin half-life which was decreased two- to threefold. Thus, phenobarbital increased the rate of removal of bilirubin from the body pool and, thereby, proportionally diminished the amounts of both *vascular* and *extravascular* bilirubin.

Several distinctive properties of the manner in which phenobarbital lowers the serum bilirubin concentration are evident. First, the approximately 30 days required to reach a new level of 5–7 mg of bilirubin per 100 ml of serum under phenobarbital treatment is several times longer than would be expected if the fractional turnover rate (λ_1) determined during phenobarbital administration had been established soon after introduction of the drug (see Appendix II). The long interval required to reach the lower level of serum bilirubin concentration appears more consistent with an action of phenobarbital governed by a slow, rate-limiting step rather than by one involving only a rapid process such as a change in the binding constant of albumin to bilirubin (45, 46). Second, the rate at which the serum bilirubin concentration *increases after withdrawal* of phenobarbital is essentially the same rate at which it *falls after initiating* therapy (Figs. 1 and 2 and Appendix I).

[5] Schmid and Hammaker (22), using bilirubin-^{14}C, have previously demonstrated a normal turnover of bilirubin in a $4\frac{1}{2}$ year old boy with congenital, nonhemolytic, hyperbilirubinemia. Since bilirubin turnover was essentially unaltered in our patient during phenobarbital treatment, it is assumed that any increase in bilirubin production from nonerythroid sources, claimed to occur in this patient during phenobarbital administration (44), was small in comparison to the total bilirubin turnover.

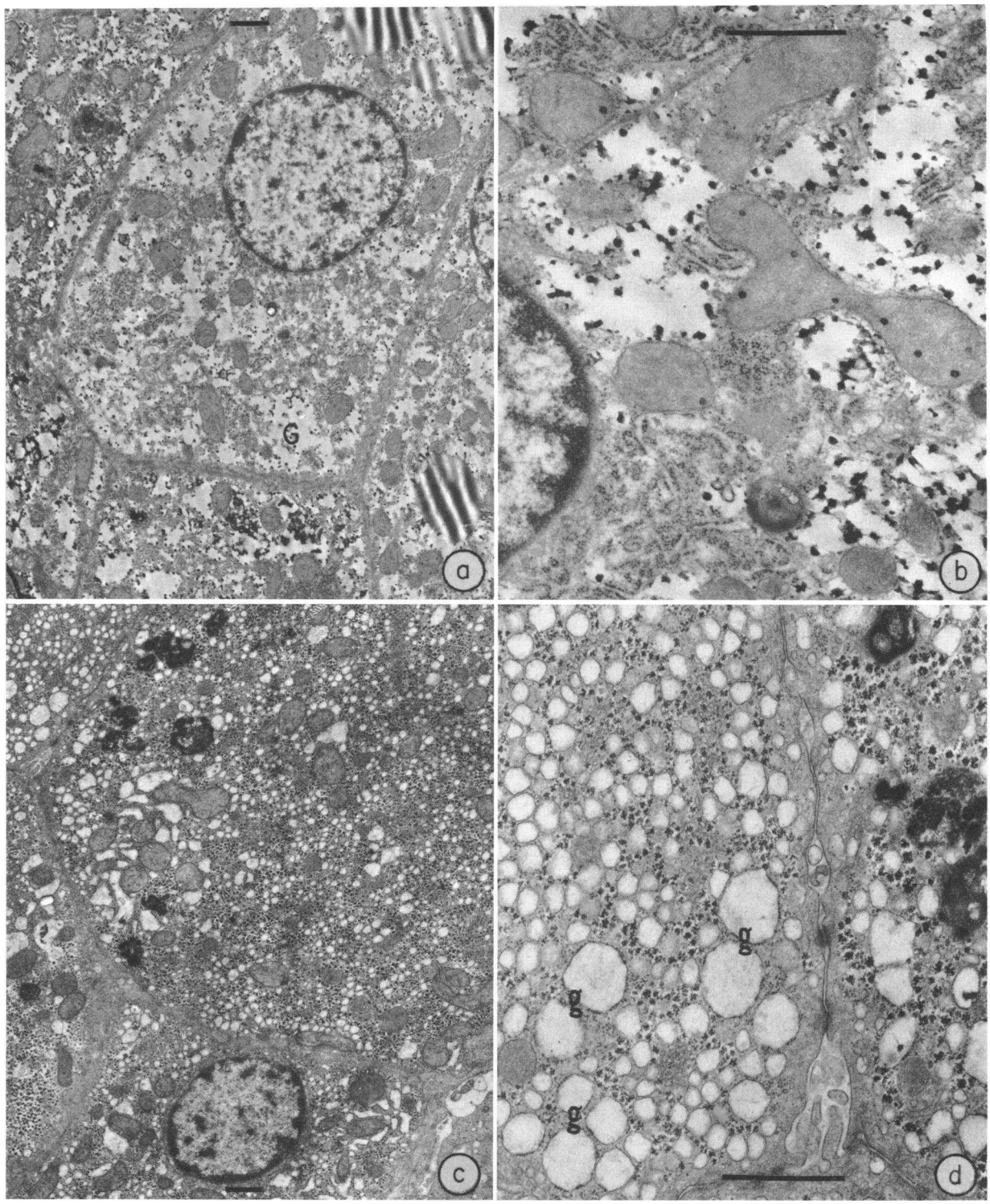

FIGURE 7 Electron micrographs of liver biopsies of patient T. U. obtained before (*a* and *b*, age 551 days) and during (*c* and *d*, age 677 days) phenobarbital administration. Black bars represent 1 μ. Fig. 7*a*, ×5800; *b*, ×20,000; *c*, ×5800; *d*, ×20,000. Photomicrographs *a* and *b*, obtained *before* phenobarbital administration, show hepatocytes from central portion of lobule selected by trimming block after phase microscopic examination of adjacent thick section. Note large, "empty" spaces

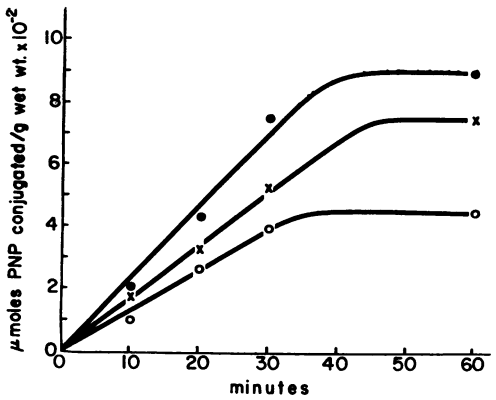

FIGURE 8 Conjugation of *p*-nitrophenol (PNP) by homogenates of liver obtained surgically from patient T. U. before (age 132 days receiving 0.075 mg/day of L-triiodothyronine [T_3]) and during phenobarbital (age 677 days) administration and from a 10 month old girl, P. M., with idiopathic hypoglycemia (control, no phenobarbital). The homogenates were the supernatants obtained by homogenizing 1 volume of tissue with 2 volumes of 0.25 M sucrose and centrifuging at 0°C for 15 min at 9000 *g*. See Methods for details of the incubation. O——O, T. U., 132 days, T_3, no phenobarbital; ×——×, P. M., no phenobarbital; ●——●, T. U., 677 days, phenobarbital, 5 mg/kg per day. The millimicromoles of PNP conjugated per gram of wet weight of liver per minute was greater during phenobarbital, 232, than in the two control incubations, T. U. (132 days), 130, and P. M., 175.

This suggests that the same, slow, rate-limiting step may also be the dominant factor in controlling the rate of increase in serum bilirubin concentration upon withdrawal of phenobarbital.

Observations in Gunn rats and in patients with congenital nonhemolytic jaundice suggest that the elevated level of serum bilirubin is the result of a deficiency in the enzymatic system converting bilirubin to its readily excretable glucosiduronate (47). These considerations, together with the findings that phenobarbital and similar drugs can stimulate or induce the in vivo formation of enzyme systems accelerating various aspects of the metabolism of drugs and hormones, including conjugation as glucosiduronate (9, 10, 41, 48, 49), imply that the action of phenobarbital has been to induce the formation of a bilirubin-metabolizing enzyme system. Such a concept is supported by the findings of Yaffe et al. (14) that phenobarbital therapy was able to return a deficient glucosiduronate-conjugating capacity for salicylamide to normal in their patient, and by the observations of increased AER (Fig. 7) and rate of glucosiduronate conjugation of PNP (Fig. 8) of liver obtained from our patient during phenobarbital administration. Recently, several infants with marked congenital, nonhemolytic, unconjugated hyperbilirubinemia have been reported to show no change in serum bilirubin concentration with phenobarbital treatment.[6] In addition, DeLeon, Gartner, and Arias (49) have shown that homozygous rats (Gunn) genetically incapable of forming bilirubin glucosiduronate have no change in bilirubin metabolism when phenobarbital is given in amounts sufficient to increase AER and to induce drug-metabolizing enzymes. The latter authors have suggested that such differences in responsiveness to phenobarbital may help delineate the extent of the inherited deficiency of UDPGA transferase.

If phenobarbital has induced the formation of a bilirubin-metabolizing enzyme system, presumably UDPGA transferase, then we propose that the rate of increase of serum bilirubin concentration upon phenobarbital withdrawal was limited by the slow rate of degradation of the induced enzyme(s) (or rapid rate of degradation with limited capacity) (50).[7] In turn, this can imply that the fall in bilirubin concentration with phenobarbital administration is the result of a rapidly and maximally induced increase in a bilirubin-metabolizing enzyme characterized by a slow rate of degradation (or a rapid rate of degradation with limited capacity), the latter being the rate-limiting reaction which makes the rates of increase and decrease in serum bilirubin similar. Further experimental evidence is needed to elucidate and to establish the validity of these speculations on the mechanism of action of phenobarbital.

APPENDIX

I. Similarity in the absolute values of the slopes of Fig. 1 (onset of phenobarbital therapy) to that of Fig. 2 (withdrawal of phenobarbital therapy) can be shown by the following statistical evaluation:

A. Determination of the *pooled* residual variances about the slopes of Figs. 1 *a*, 1 *b*, and 2.

$$s^2 = \frac{[Y^2] - [XY]^2/[X^2]}{N - 2}$$

[6] Discussion at the presentation of a portion of this work (12).

[7] The turnover of phenobarbital is assumed to be rapid (51) compared to the degradation of the bilirubin-metabolizing enzyme system.

(*G*) due to partial loss of glycogen. Granular endoplasmic reticulum in the cytoplasmic aggregates in *b* is cut in profile or obliquely. Little agranular endoplasmic reticulum (AER) is present.

Photomicrographs *c* and *d*, obtained *during* phenobarbital administration, show hepatocytes from the peripheral portion of the lobule selected by trimming block after phase microscopy of adjacent thick sections. Note separation of the glycogen rosettes into small masses by a large population of agranular microsomal vesicles. Profiles of dilated granular endoplasmic reticulum (*g*) are intermixed with AER.

where s^2 = residual variance of values about a single slope, and $[Y^2] = \Sigma Y^2 - (\Sigma Y)^2/N$

$[X^2] = \Sigma X^2 - (\Sigma X)^2/N$

$[XY] = \Sigma XY - (\Sigma X)(\Sigma Y)/N$

Y = log serum bilirubin concentration; X = days; and N = number of samples defining a single slope.

Let subscripts *a*, *b*, and *c* refer to values from the Figs. 1 *a*, 1 *b*, and 2, respectively, and A, B, and C = $(N - 2)s^2$ for the same respective figures.

Then $s_p^2 = \frac{A + B + C}{N_a + N_b + N_c - 6}$, where s_p^2 = *pooled* residual variances for the three studies.

B. Student's *t* test applied to the slopes of Figs. 1 *a* and 1 *b*.

$$t = \frac{\text{slope}_a - \text{slope}_b}{\sqrt{s_p^2\left(\frac{1}{[X^2]_a} + \frac{1}{[X^2]_b}\right)}} = 0.17 \ (P > 0.5).$$

This result indicates no significant difference between slopes.

C. Student's *t* test applied to the slopes of Fig. 2 and the mean of the slopes of Figs. 1 *a* and 1 *b*.

let m = mean of Fig. 1 slope $= \frac{[XY]_a + [XY]_b}{[X^2]_a + [X^2]_b}$.

$[X^2]_c = [X^2]$ for Fig. 2.

$$\text{Then } t = \frac{m - (-\text{slope}_c)}{\sqrt{s_p^2\left(\frac{1}{[X^2]_a + [X^2]_b} + \frac{1}{[X^2]_c}\right)}} = -1.46$$

($P > 0.1$).

This result indicates no significant difference between the mean slope of Fig. 1 and that of Fig. 2.

II. The time required to decrease total body bilirubin pool to half the control value assuming that the maximum phenobarbital effect on bilirubin half-life is induced immediately can be calculated in the following manner:

Let N = bilirubin pool size at any time, t, then $dN/dt = k_t N + 33$ (see Table II for values) where $k_t = 0.45$/day = turnover constant during phenobarbital therapy and 33 = bilirubin turnover in milligrams per day, a value which was essentially unchanged by phenobarbital therapy.

Integrate, setting N = 200 mg (control pool of bilirubin) when t = 0, to obtain the following equation:

$$t = \frac{1}{k_t} \ln \frac{33 - 200k_t}{33 - k_t N}.$$

For N = 100 mg, half value of the control bilirubin pool, t = 3.5 days. However, from Fig. 1, about 25 days were required to reduce serum bilirubin concentrations (presumably reflecting total body bilirubin pool) to half-value.

It, therefore, appears unreasonable to assume that bilirubin half-life was changed immediately to the value observed in the kinetic study done during phenobarbital therapy.

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REFERENCES

1. Conney, A. H., and J. J. Burns. 1962. Factors influencing drug metabolism. *In* Advances in Pharmacology. S. Garattini and P. A. Shore, editors. Academic Press Inc., New York. **1**: 31.
2. Gillette, J. R. 1963. Metabolism of drugs and other foreign compounds by enzymatic mechanisms. *In* Progress in Drug Research. E. Jucker, editor. Birkhäuser Verlag, Basel. **6**: 13.
3. Gillette, J. R. 1966. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. *In* Advances in Pharmacology. S. Garattini and P. A. Shore, editors. Academic Press Inc., New York. **4**: 219.
4. Remmer, H. 1965. The fate of drugs in the organism. *In* Annual Review of Pharmacology. W. C. Cutting, R. H. Dreisbach, and H. W. Elliott, editors. Annual Reviews Inc., Palo Alto. **5**: 405.
5. Conney, A. H. 1965. Enzyme induction and drug toxicity. *In* Drugs and Enzymes. Proceedings of the Second International Pharmacology Meeting. B. B. Brodie and J. R. Gillette, editors. Pergamon Press Ltd., Oxford. **4**: 277.
6. Fouts, J. R., and L. G. Hart. 1965. Hepatic drug metabolism during the perinatal period. *Ann. N. Y. Acad. Sci.* **123**: 245.
7. Catz, C., and S. J. Yaffe. 1962. Pharmacologic modification of bilirubin conjugation in the newborn. *Amer. J. Dis. Child.* **104**: 516.
8. Arias, I. M., L. Gartner, M. Furman, and S. Wolfson. 1963. Effect of several drugs and chemicals on hepatic glucuronide formation in newborn rats. *Proc. Soc. Exp. Biol. Med.* **112**: 1037.
9. Burns, J. J., S. A. Cucinell, R. Koster, and A. H. Conney. 1965. Application of drug metabolism to drug toxicity studies. *Ann. N. Y. Acad. Sci.* **123**: 273.
10. Conney, A. H., K. Schneidman, M. Jacobson, and R. Kuntzman. 1965. Drug-induced changes in steroid metabolism. *Ann. N. Y. Acad. Sci.* **123**: 98.
11. Crigler, J. F., Jr., and N. I. Gold. 1966. Sodium phenobarbital-induced decrease in serum bilirubin in an infant with congenital non-hemolytic jaundice and kernicterus. *J. Clin. Invest.* **45**: 998.
12. Crigler, J. F., Jr., and N. I. Gold. 1967. Effect of sodium phenobarbital on the metabolism of bilirubin-^{3}H and ^{14}C in an infant with congenital non-hemolytic jaundice and kernicterus. *J. Clin. Invest.* **46**: 1047.
13. Burstein, S., and E. L. Klaiber. 1965. Phenobarbital-induced increase in 6β-hydroxycortisol excretion: clue to

its significance in human urine. *J. Clin. Endocrinol. Metab.* **25**: 293.
14. Yaffe, S. J., G. Levy, T. Matsuzawa, and T. Baliah. 1966. Glucuronide-conjugating capacity in a hyperbilirubinemic infant due to apparent enzyme induction by phenobarbital. *N. Engl. J. Med.* **275**: 1461.
15. Wallace, G. B., and J. S. Diamond. 1925. The significance of urobilinogen in the urine as a test for liver function with a description of a simple quantitative method for its estimation. *Arch. Intern. Med.* **35**: 698.
16. Malloy, H. T., and K. A. Evelyn. 1937. Determination of bilirubin with the photoelectric colorimeter. *J. Biol. Chem.* **119**: 481.
17. Kingsley, G. R. 1939. The determination of serum total protein, albumin, and globulin by the biuret reaction. *J. Biol. Chem.* **131**: 197.
18. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**: 751.
19. Gellis, S. S., and D. Y.-Y. Hsia. 1955. Liver function tests in infants and children. *In* Pediatric Clinics of North America. Symposia on unusual infections of childhood; laboratory tests and special procedures. W. B. Saunders Company, Philadelphia. 177.
20. Bodansky, A. 1933. Phosphatase studies. II. Determination of serum phosphatase. Factors influencing the accuracy of the determination. *J. Biol. Chem.* **101**: 93.
21. Schmid, R., and L. Hammaker. 1963. Metabolism and disposition of C^{14}-bilirubin in congenital nonhemolytic jaundice. *J. Clin. Invest.***42**: 1720.
22. Wilzbach, K. E. 1957. Thitium-labeling by exposure of organic compounds to tritium gas. *J. Amer. Chem. Soc.* **79**: 1013.
23. Clarke, J. T. 1965. Purification and analysis of bilirubin. *Clin. Chem.* **11**: 681.
24. Ostrow, J. D., L. Hammaker, and R. Schmid. 1961. The preparation of crystalline bilirubin-C^{14}. *J. Clin. Invest.* **40**: 1442.
25. Weber, A. Ph., and L. Schalm. 1962. Quantitative separation and determination of bilirubin and conjugated bilirubin in human serum. *Clin. Chim. Acta.* **7**: 805.
26. Okita, G. J., J. J. Kabara, F. Richardson, and G. V. Leroy. 1957. Assaying compounds containing H^3 and C^{14}. *Nucleonics.* **15**: 111.
27. Oliverio, V. T., C. Denham, and J. D. Davidson. 1962. Oxygen flask combusion in the determination of C^{14} and H^3 in biological materials. *Anal. Biochem.* **4**: 2054.
28. Conway, W. D., A. J. Grace, and J. E. Rogers. 1966. Simplification of oxygen-flask combustion procedure for preparation of samples for liquid scintillation counting. *Anal. Biochem.* **14**: 491.
29. Solomon, A. K. 1960. Compartmental methods of kinetic analysis. *In* Mineral Metabolism. C. L. Comar and F. Bronner, editors. Academic Press Inc., New York. **IA**: 138.
30. Uzman, B. G., G. E. Foley, S. Farber, and H. Lazarus. 1966. Morphologic variations in human leukemic lymphoblasts (CCRF-CEM cells) after long-term culture and exposure to chemotherapeutic agents. *Cancer.* **19**: 1725.
31. Burger, P. C., and P. B. Herdson. 1966. Phenobarbital-induced fine structural changes in rat liver. *Amer. J. Pathol.* **48**: 793.
32. Becker, F. F., and B. P. Lane. 1968. Regeneration of the mammalian liver. VI. Retention of phenobarbital-induced cytoplasmic alterations in dividing hepatocytes. *Amer. J. Pathol.* **52**: 211.
33. Isselbacher, K. J., M. F. Chrabas, and R. C. Quinn. 1962. The solubilization and partial purification of a glucuronyl transferase from rabbit liver microsomes. *J. Biol. Chem.* **237**: 3033.
34. Pogell, B. M., and L. F. Leloir. 1961. Nucleotide activation of liver microsomal glucuronidation. *J. Biol. Chem.* **236**: 293.
35. Jones, A. L., and D. W. Fawcett. 1966. Hypertrophy of the agranular endoplasmic reticulum in hamster liver induced by phenobarbital (with review on the functions of this organelle in liver). *J. Histochem. Cytochem.* **14**: 215.
36. Orrenius, S., J. L. E. Ericsson, and L. Ernster. 1965. Phenobarbital-induced synthesis of the microsomal drug-metabolizing enzyme system and its relationship to the proliferation of endoplasmic membranes. *J. Cell Biol.* **25**: 627.
37. Brown, H., E. Englert, and S. Wallach. 1958. Metabolism of free and conjugated 17-hydroxycorticosteroids in subjects with thyroid disease. *J. Clin. Endocrinol. Metab.* **18**: 167.
38. Gold, N. I., and J. F. Crigler, Jr. 1963. Influence of L-triiodothyronine on steroid hormone metabolism: Studies in a patient with adrenal hyperplasia (Cushing's Syndrome). *J. Clin. Endocrinol. Metab.* **23**: 156.
39. Akerren, Y. 1954. Prolonged jaundice in the newborn associated with congenital myxedema. *Acta Paediat. Scand.* **43**: 411.
40. MacGillivray, M. H., J. D. Crawford, and J. S. Robey. 1967. Congenital hypothyroidism and prolonged neonatal hyperbilirubinemia. *Pediatrics.* **40**: 283.
41. Inscoe, J. K., and J. Axelrod. 1960. Some factors affecting glucuronide formation *in vitro*. *J. Pharmacol. Exp. Ther.* **129**: 128.
42. Quinn, G. P., J. Axelrod, and B. B. Brodie. 1958. Species, strain and sex differences in metabolism of hexobarbitone, amidopyrine, antipyrine, and aniline. *Biochem. Pharmacol.* **1**: 152.
43. Booth, J., and J. R. Gillette. 1962. The effect of anabolic steroids on drug metabolism by microsomal enzymes in rat liver. *J. Pharmacol. Exp. Ther.* **137**: 374.
44. Robinson, S. H., R. Lester, J. F. Crigler, Jr., and M. Tsong. 1967. Early-labeled peak of bile pigment in man; studies with glycine-^{14}C and delta-aminolevulinic acid-^{3}H. *N. Engl. J. Med.* **277**: 1323.
45. Odell, G. B. 1959. Dissociation of bilirubin from albumin and its clinical implications. *J. Pediat.* **55**: 268.
46. Ostrow, J. D., and R. Schmid. 1963. Protein-binding of ^{14}C-bilirubin in human and murine serum. *J. Clin. Invest.* **42**: 1286.
47. Schmid, R. 1966. Hyperbilirubinemia. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson, editors. McGraw-Hill Book Company, New York. 2nd edition. 871.
48. Zeidenberg, P., S. Orrenius, and L. Ernster. 1967. Increase in levels of glucuronylating enzymes and associated rise in activities of mitochondrial oxidative enzymes upon phenobarbital administration in the rat. *J. Cell Biol.* **32**: 528.
49. DeLeon, A., L. M. Gartner, and I. M. Arias. 1967. The effect of phenobarbital on hyperbilirubinemia in glucuronyl transferase deficient rats. *J. Lab. Clin. Med.* **70**: 273.
50. Schimke, R. I. 1966. Studies on the roles of synthesis and degradation in the control of enzyme levels in animal tissues. *Bull. Soc. Chim. Biol.* **48**: 1009.
51. Lous, P. 1954. Plasma levels and urinary excretion of three barbituric acids after oral administration to man. *Acta Pharmacol. Toxicol.* **10**: 147.