Blue Light and Bilirubin Excretion

Antony F. McDonagh, Lucita A. Palma, David A. Lightner

Summary. Blue light converts bilirubin in the skin of jaundiced rats to metastable geometric isomers that are transported in blood and excreted in bile. The same reaction probably occurs in jaundiced babies exposed to light, particularly during treatment with phototherapy. Excretion of unisomerized bilirubin is prevented by intramolecular hydrogen bonding, and the pigment has to be metabolized to more polar derivatives to be excreted efficiently.

Each year thousands of newborn babies with neonatal jaundice are treated with phototherapy, that is, prolonged irradiation with bright blue or white light (*1*). This is done to prevent brain damage caused by the cytotoxic yellow metabolite 4*Z*,15*Z*-bilirubin IXα (bilirubin). Phototherapy works because it reduces the concentration of bilirubin in the circulation and diminishes the risk that the pigment will diffuse into the brain where it can exert its toxic effect. How it works is not at all clear. The most widely held notion is that light therapy causes photooxidation or photodegradation of bilirubin to other compounds that are easily excreted (*2*). However, there is no reliable, convincing scientific evidence to back up this view. One fascinating aspect of the problem is the observation, made originally by Ostrow (*3*), that phototherapy appears to stimulate excretion of bilirubin by the liver into bile (*4*). This observation is particularly intriguing because, normally, bilirubin cannot be excreted significantly by the liver unless it is first metabolized to so-called conjugates with glucuronic acid and other sugars (*5*). How, therefore, can light, shone on the skin, trigger the excretion of unconjugated bilirubin by the liver? Several theories have been proposed to explain this phenomenon, but none has been verified experimentally, and biochemical intermediates that might be responsible have not been detected or identified in vivo. In this article we propose a mechanism for phototherapy and explain why it apparently causes excretion of unconjugated bilirubin. This mechanism involves light-induced conversion of bilirubin to unstable intermediates that can be excreted by the liver into bile and that can subsequently revert to natural bilirubin. Although we have been unable to isolate and purify these intermediates, owing to their thermal instability, we have been able to detect and identify them spectroscopically and by a novel technique in which a rat is used essentially as a chromatography unit. Curiously, it has turned out that the nature and properties of these intermediates provide fresh insight into normal bilirubin metabolism and indicate why hepatic conjugation of bilirubin is such a necessary step in mammalian metabolism.

A. F. McDonagh and L. A. Palma are staff members of the Department of Medicine and the Liver Center, 1120 HSW, University of California, San Francisco 94143. D. A. Lightner is a professor in the Department of Chemistry, University of Nevada, Reno 89557.

The Phototherapy Intermediate in vivo

Newborn babies develop hyperbilirubinemia, and often jaundice, because the hepatic system for conjugating and excreting bilirubin is functionally immature at birth and shortly afterward (*5*). Consequently, bilirubin accumulates in the circulation and extravascular tissues until the excretory apparatus becomes fully functional, which usually occurs within the first week after birth. In our experimental studies we used homozygous adult male Gunn rats (*5*, *6*). These animals are congenitally unable to conjugate bilirubin and have lifelong hyperbilirubinemia. Their bile contains no conjugated bilirubin and only a low concentration of unconjugated bilirubin (*7*). What human newborns have for but a brief span, homozygous Gunn rats have all their lives. These rats are a useful experimental model and respond to phototherapy in much the same way as jaundiced babies; their skin becomes bleached, plasma bilirubin levels slowly subside, and there is a prompt excretion of pigment in bile (*3*, *8*, *9*).

Weight-matched pairs of rats, usually littermates, with similar serum bilirubin levels were treated as follows (*10*). One rat was shaved and depilated over most of its back and a narrow plastic tube was inserted into the common bile duct and led through the abdomen to the outside. The animal was placed in a restraining cage, constructed so that most of the back and sides of the animal could be exposed to light, and warmed with an infrared heating lamp until the rectal temperature of the rat was normal. Then the rat was irradiated with four 40-watt blue fluorescent tubes situated about 5 centimeters from the rat parallel to its length with two above and one on each side. After 30 minutes the flow of bile was interrupted by sealing the end of the cannula, and irradiation was continued for three more hours. This time course was chosen because preliminary studies had revealed that the excretion of pigment in bile in response to light reaches a maximum after about 30 minutes and remained at this level for at least several hours (*11*). The animal was again anesthetized, removed from the cage, and laid under a photographic safelight with its abdomen upward and its back nestled between two 20-W blue fluorescent tubes. As quickly as possible the rat was exsanguinated through the abdominal aorta in such a way that the blood and viscera were exposed only to the safelight and only the back of the animal was exposed to the blue light. Serum was then obtained by centrifuging the clotted blood in the dark. A second rat was treated as above, except that it was not shaved or exposed to the blue lights. During the 3.5-hour "phototherapy" period, this control rat was irradiated with an infrared heating lamp to maintain body temperature. Thus, each experiment yielded two serum samples: one from an irradiated rat, the other from the unirradiated control. Throughout this article, these samples are referred to as *light* serum and *dark* serum, respectively.

To detect whether phototherapy intermediates were present in *light* serum, samples of *light* and *dark* serum were injected intravenously in sequence into a third homozygous Gunn rat kept in the dark. This third rat, the "chromatography" rat, was fitted with a catheter in the femoral vein and an indwelling biliary cannula, and then placed in a restraining

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cage. The biliary cannula was connected to the flow cell of a spectrophotometric detector set at 470 nanometers (*9*) so that changes in the absorbance of bile with respect to time could be recorded continuously. During the experiment a replacement mixture of bile salts and lipids (*9*) was infused into the femoral vein to maintain constant bile flow and prevent the bile from becoming depleted of bile salts and lipids. Our expectation was that phototherapy intermediates present in the injected *light* serum would be taken up rapidly by the liver in the recipient rat and excreted concentrated and protein-free in bile, making them easier to detect and identify.

When *dark* serum (2 milliliters) was injected into the chromatography rat there was little or no detectable increase in the absorbance of bile at 470 nm (Fig. 1). In contrast, when *light* serum (2 ml) was injected or when the chromatography rat was irradiated briefly with blue light there followed a pronounced peak on the absorbance recorder. Bile samples corresponding to these peaks were collected in ice-cooled tubes in the dark, and pigments were extracted quantitatively from them and analyzed (*12*). The main pigment in the extracts of bile collected after injecting *light* serum and in the extracts of bile collected after irradiation of the rat was identified by thin-layer chromatography (*13*), high-performance liquid chromatography (*14*), and absorption spectrophotometry as 4*Z*,15*Z*-bilirubin IXα, the naturally occurring isomer of bilirubin (*15, 16*). The same compound also was present in extracts of control bile and in extracts of bile collected after injecting *dark* serum, but the amounts were relatively small (Fig. 1).

The results indicate that phototherapy generates a substance that either stimulates excretion of unconjugated bilirubin by the liver or that is itself excreted by the liver and converted to bilirubin in bile or during its extraction from bile. This substance is not bilirubin itself, since large quantities of that were present in both *light* and *dark* serums, but it could, of course, be an isomer of the natural pigment.

We used a similar method to detect the intermediate in serum from irradiated Gunn rats that had not been manipulated surgically. Shaved, homozygous Gunn rats were irradiated for 3.5 hours in restraining cages, and serum was collected as described above. Control rats were simply kept in restraining cages under an infrared lamp for 3.5 hours before serum was collected from them. Serum samples (3 ml) from irradiated and nonirradiated rats were injected, in the dark, into a homozygous Gunn rat as described earlier, and the biliary output of pigment at 470 nm was monitored at a sensitive, expanded absorbance scale setting [0.08 absorbance (*A*) units, full-scale deflection]. Invariably, the peak obtained after injecting serum from the irradiated rat was substantially larger than that ob-

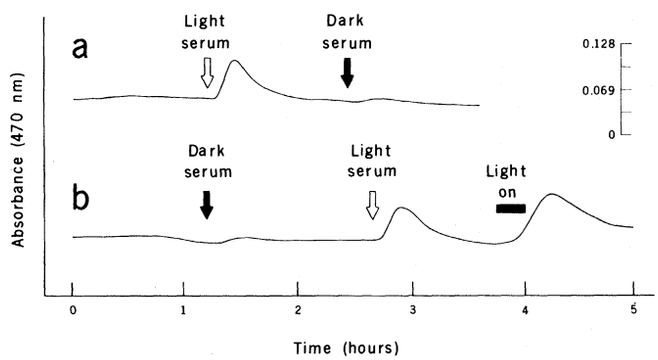

Fig. 1. Detection of phototherapy intermediates in vivo. *Light* and *dark* serum samples were obtained from irradiated and unirradiated bile-blocked homozygous Gunn rats, respectively. These samples were injected (arrows) via the femoral vein into restrained homozygous Gunn rats kept in the dark, and the absorbance of their bile was monitored continuously at 470 nm with the use of a flow cell (0.5 mm, path length; 2 μl, capacity) at a detector setting of 0.32 absorbance units, full-scale deflection. Data from two separate experiments (a and b) are shown. In (b) the chromatography rat had been shaved along the center of its back and at the point indicated was irradiated for 15 minutes with a horizontal light fixture containing eight 20-W Westinghouse Special Blue fluorescent tubes located 15 cm above the rat. In each experiment bile samples were collected at 30-minute intervals from the flow-cell effluent (i) before any injections had been made, (ii) after each injection, and (iii) (lower experiment only) after irradiation of the rat, with allowance being made for the hold-up time of the apparatus. In (a) the serum bilirubin concentrations in the *light* serum, *dark* serum, and chromatography rat were 0.11, 0.11, and 0.13 m*M*, respectively, and the quantities of bilirubin isolated from the bile samples were 0.9 μg (before injection), 5.6 μg (after injection of *light* serum), and 0.8 μg (after injection of *dark* serum). In (b), serum bilirubin concentrations were 0.11 m*M* (*dark* serum), 0.09 m*M* (*light* serum), and 0.12 m*M* (chromatography rat), and the quantities of bilirubin isolated from the bile were 1.5 μg (before injection), 1.2 μg (after injecting *dark* serum), 2.6 μg (after injecting *light* serum), and 6.2 μg (after irradiation).

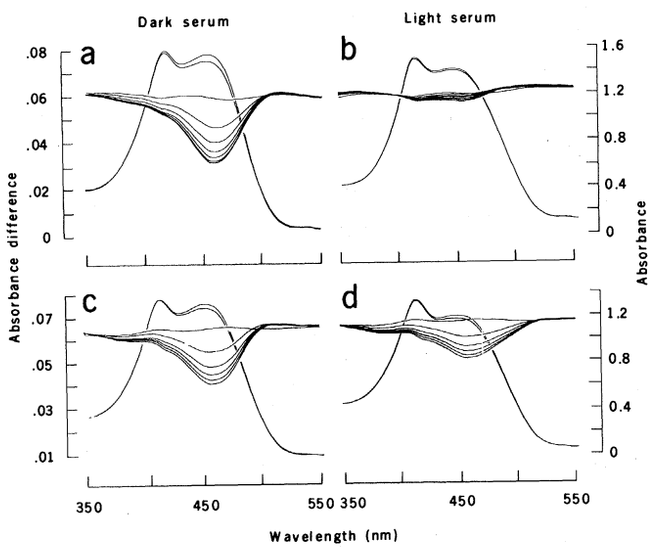

Fig. 2. Absorbance and absorbance difference spectra of *dark* and *light* serum samples before (a and b) and after (c and d) incubation at 37°C for 15 hours. The double-humped curves are absorbance spectra. For the absorbance difference spectra serum was placed in both reference and sample cuvettes (1-millimeter path length), and the baseline was obtained. The sample cuvette was then irradiated with blue light (Westinghouse Special Blue 20-W) and spectra were recorded at 5-second intervals up to a total irradiation time of 30 seconds (a and b) or 25 seconds (c and d). Absorbance spectra were determined with air as the reference.

tained after injecting serum from the control rat. This result was independent of the sequence of injection and the serum bilirubin concentrations of the irradiated and control animals, as measured by a diazo procedure (*15*). Thus, serum from irradiated jaundiced Gunn rats that have not had prior surgery seems to contain detectable amounts of an excretable intermediate not present in control serum from unirradiated rats. But the concentration of this intermediate is low, much lower than in serum from irradiated bile-blocked rats.

Light and *dark* serums from bile-blocked rats were not distinguishable on the basis of their absorption spectra, which had overlapping peaks at 417 and 454 nm, due to oxyhemoglobin and bilirubin, respectively. But their response to blue light and to heat was remarkably different, as revealed by absorbance difference spectrophotometry (Fig. 2). *Dark* serum showed a rapid loss of absorbance at 459 to 461 nm on brief irradiation as previously reported (*17*) (Fig. 2a). In contrast, *light* serum was almost unaffected by similar irradiation, an indication that the sample was at a photostationary state (Fig. 2b). This singular difference between *light* and *dark* serums was not due to unavoidable differences between samples in the concentration of bilirubin or hemoglobin. We observed it consistently in more than a dozen experiments, regardless of whether the *light* serum or the *dark* serum had the highest bilirubin or hemoglobin concentration. A further distinction between the *light* and *dark* serums was revealed by incubating the samples in the dark at 37°C for 15 hours. This caused only a small decrease in the concentration of bilirubin and hemoglobin in each sample. Incubating the *dark* serum had little effect on its photoreactivity (Fig. 2, a and c). Incubating the *light* serum had a marked effect (Fig. 2, b and d). Serum that had formerly been at a photostationary equilibrium and insensitive to brief irradiation now showed a loss of absorbance in the difference spectrum at 459 nm when exposed briefly to blue light (Fig. 2d).

To reveal the intermediate (or intermediates) more clearly, samples of *light* and *dark* serums were washed in the dark with chloroform and then with petroleum ether (*18*). This procedure removed most of the bilirubin and left clear aqueous solutions. The effect of light on these solutions was then examined by difference spectrophotometry with the use of a sensitive absorbance scale. Again, there was a pronounced difference between the *dark* and *light* samples (Fig. 3). The *dark* sample (Fig. 3a)

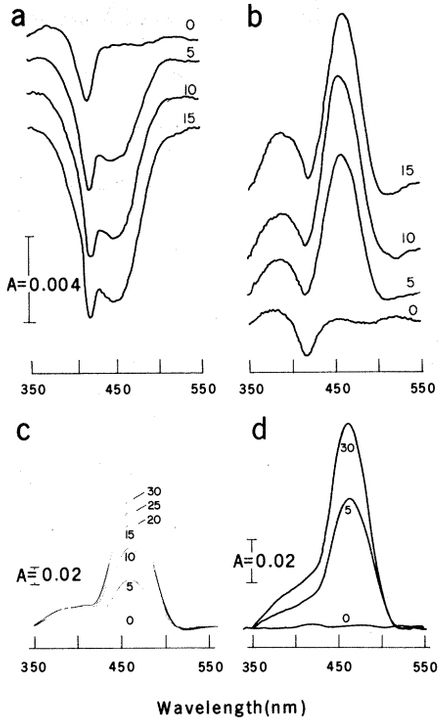

Fig. 3. Absorbance difference spectra obtained on irradiating (a) washed *dark* serum, (b) washed *light* serum, (c) photobilirubin in serum, and (d) a washed solution of photobilirubin in serum. Spectra were recorded as irradiated minus nonirradiated. Numbers on the curves are cumulative irradiation times in seconds. Difference spectra were run in 1-mm (a, b, and c) or 5-mm (c) path length cuvettes, and a Westinghouse Special Blue 20-W tube was used for irradiation. The trough near 417 nm in (a) and (b) is an anomaly in the baseline.

showed a loss of absorbance centered at about 450 nm, which was probably largely due to photooxygenation of trace amounts of bilirubin still present. The *light* sample (Fig. 3b) showed a distinct increase in absorbance centered at 462 nm close to where endogenous bilirubin in serum from the Gunn rat absorbs. The absorbance increased to a maximum after 10 to 15 seconds and then decreased on longer irradiation. The final washed solution from the *light* serum sample was unstable even in the dark and the well-defined peak shown in Fig. 3b was only detectable when the extractions were performed quickly and the spectra were obtained without delay.

These observations demonstrate the difference in photochemical reactivity between *dark* and *light* serums, a difference that is not readily apparent from their absorption spectra. This difference was abolished by irradiating the *dark* serum in vitro. When *dark* serum from the unirradiated rat was irradiated in a quartz cuvette of 1-cm path length between two 20-W blue lights for 10 minutes (which was not long enough to photooxidize more than 7 percent of the bilirubin), it became qualitatively similar to the *light* serum from the irradiated rat. The *dark* serum then showed no further significant change by difference spectroscopy on brief irradiation (5 to 30 seconds). After extraction with chloroform and petroleum ether and subsequent irradiation, the *dark* serum no longer showed a loss of absorbance in the difference spectrum as it had done originally (Fig. 3a), but yielded an increase in absorbance of 463 nm like that previously observed with the serum from the irradiated rat.

These spectroscopic measurements support the main finding of the in vivo study—namely, that serum from the irradiated rats contains a substance not present in serum from unirradiated control animals. Taken together, the spectroscopic observations suggest that light converts bilirubin in vivo and in vitro to a substance with a very similar absorption band. This new substance is stable in serum at 4°C but slowly reverts to bilirubin at 37°C, it is less lipophilic than bilirubin since it is not extractable from serum with chloroform, and it regenerates bilirubin on brief irradiation.

Identity of the Intermediate

Irradiation of bilirubin in organic solvents produces a photoequilibrium mixture containing bilirubin and a material we have named photobilirubin (*19*). In aerobic solutions, this reaction is much faster than the competing photooxygenation reaction of bilirubin and is readily detectable by absorbance difference spectrophotometry (*19*). Photobilirubin is itself a mixture of compounds. It is believed to contain 4*Z*,15*E*-bilirubin IXα, 4*E*,15*Z*-bilirubin IXα, and 4*E*,15*E*-bilirubin IXα (Fig. 4), all of which are geometric isomers of the naturally occurring, more thermodynamically stable 4*Z*,15*Z* isomer. The visible absorption bands of all four isomers overlap, and, at photoequilibrium under blue light, the composition of the mixture is 4*Z*,15*Z* >>> 4*E*,15*Z* ≃ 4*Z*,15*E* >> 4*E*,15*E* (*19*). Although linear representations of these structures are similar, their three-dimensional structures probably are different. The natural isomer exists predominatly as the intramolecularly hydrogen-bonded structures **5** and **6** (Fig. 5) (*20–22*) which accounts for its lipophilicity. Molecular models show that such extensive hydrogen-bonding is sterically impossible for the three other isomers (for example, see structure **7** in Fig. 5). Consequently the

components of photobilirubin are more polar and more hydrophilic than the natural isomer (*19*). In addition, photobilirubin, being thermodynamically less stable than bilirubin, reverts readily to bilirubin when warmed or irradiated in solution (*19*).

Photobilirubin, therefore, is an obvious candidate for the unidentified intermediate in the serum of the irradiated rats (*23*). To ascertain whether this is so, we prepared photobilirubin from bilirubin and dissolved it in rat serum (*24*); the effect of light on this solution was then examined by difference spectroscopy before and after extraction with chloroform and petroleum ether (*18*). In both cases, there was a rapid increase in absorbance, which reached a photostationary state after about 30 seconds (Fig. 3, c and d). The maximum of this increased absorption band was at 462 nm, the same position as the peak observed with washed *light* serum from irradiated rats (Fig. 3b) and washed irradiated *dark* serum from unirradiated control rats. This indicates that photobilirubin is the unstable intermediate present in the serum from irradiated rats (*25*). Consistent with this, we have also found that photobilirubin binds to serum albumin, is stable in serum for at least 48 hours at 4°C in the dark but undergoes about 50 percent reversion to bilirubin after 15.5 hours at 40°C, and is rapidly excreted in bile when injected intravenously into Gunn rats, yielding bilirubin on extraction (*26*). We estimate that the concentration of photobilirubin in the serum from the irradiated bile-blocked rats was of the order of 12 μM. Unfortunately, we have been unable so far to separate photobilirubin from the serum and identify it unambiguously owing to its thermal reconversion to bilirubin during isolation and purification.

Mechanism of Phototherapy

We propose the following mechanism for the apparent excretion of bilirubin during phototherapy (Fig. 6). Photochemical excitation of bilirubin near the skin surface yields an excited state species that decays to ground-state bilirubin or, after isomerization, to ground-state photobilirubin. Both of these substances can migrate through the plasma membrane into the blood, where they become bound to albumin, and both are extracted from blood into hepatocytes. When the bilirubin conjugating system is not functioning, only the more polar water-solvated photobilirubin, which does not require conjugation for excretion, passes onward and outward into bile.

The first step in this process is formation of photobilirubin, which is a reversible unimolecular reaction (as opposed to the irreversible bimolecular reactions that are sometimes invoked to explain phototherapy). We do not know the multiplicity of the excited-state bilirubin species involved in this reaction, but a singlet mechanism is most likely in view of (i) the low triplet yield of bilirubin in solution and (ii) recent studies of model systems (*27*). The reaction probably occurs mainly in extravascular tissue near the skin surface since exogenous photobilirubin, given intravenously, is excreted in bile sooner than endogenous photobilirubin generated by irradiating the animal. Thus, when *light* serum or solutions of photobilirubin in serum were injected intravenously into a Gunn rat in the dark, the rise in pigment concentration in bile began almost at once, whereas when the same rat was irradiated there was a delay and the rise was not detected until about 3 to 5 minutes after the light was turned on (*9*). This delay suggests that photobilirubin is largely formed extravascularly and then shifts to the circulation, in consistence with previous observations on the bleaching of skin during phototherapy (*28–30*).

The second step in the mechanism, therefore, is movement of photobilirubin from peripheral extravascular tissues to blood. Most likely this is simply a passive diffusion-controlled process. It seems to be bidirectional and quite rapid because the circulating isomeric pigments in irradiated obstructed rats

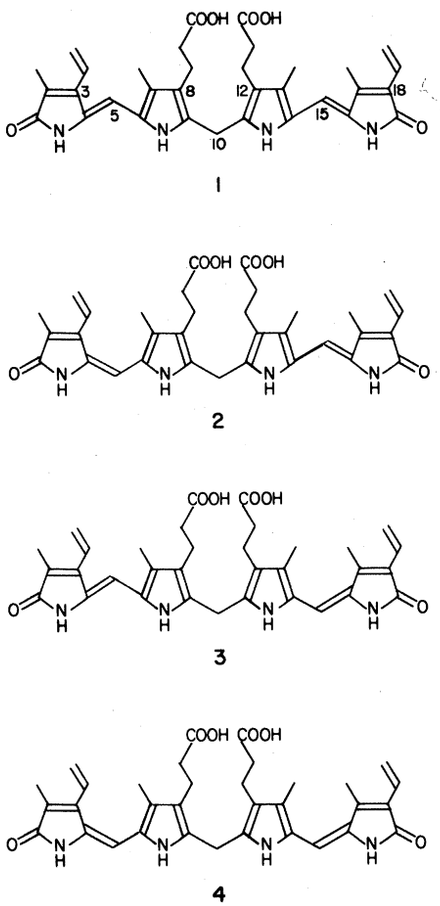

Fig. 4. The four configurational isomers of bilirubin IXα. Structure **1** is 4*Z*,15*Z*- (the naturally occurring most stable isomer); **2** is 4*E*,15*Z*-; **3** is 4*Z*,15*E*-; and **4** is 4*E*,15*E*- .

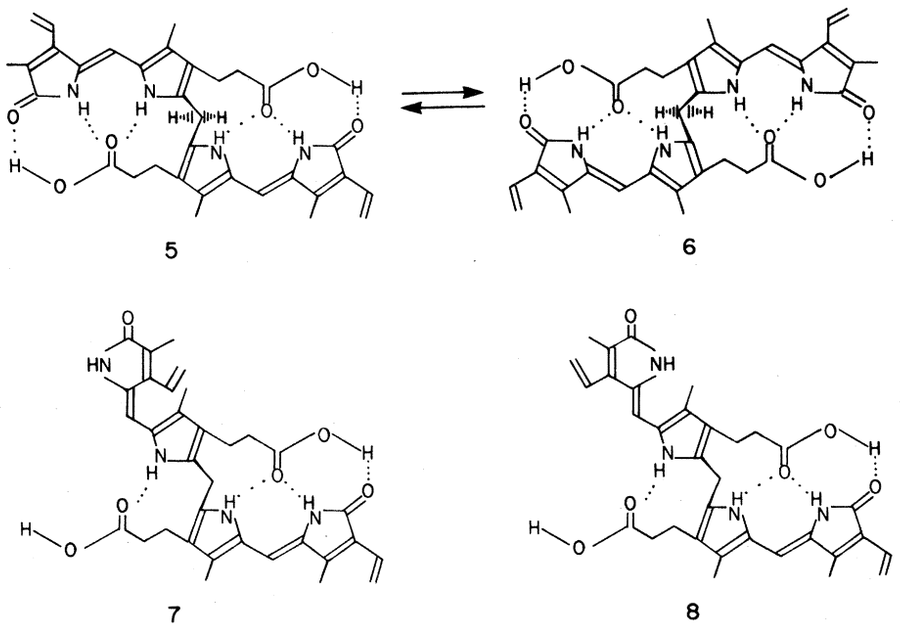

Fig. 5. Simplified two-dimensional representations of bilirubin IXα conformers. Enantiomeric (mirror image) structures **5** and **6** are the preferred *syn*, *syn* conformers of bilirubin (4*Z*,15*Z*-bilirubin IXα), **7** is a *syn*, *syn* conformer of 4*E*,15*Z*-bilirubin IXα, and **8** is an *anti*, *syn* "non-preferred" conformer of bilirubin (4*Z*,15*Z*-bilirubin IXα).

reached photoequilibrium concentrations within 3.5 hours.

The third step in the overall process is rapid hepatic removal of photobilirubin from the circulation. This was demonstrated by the prompt excretion of pigment after intravenous injection of *light* serum or photobilirubin into Gunn rats in the dark, and by the increase in circulating photobilirubin in bile-blocked Gunn rats compared to normal Gunn rats when both were irradiated in the same way.

The last step in the mechanism is secretion of photobilirubin from the hepatocyte into the biliary canaliculus. This step does not require enzymatic conjugation since the main pigment isolated from bile of irradiated Gunn rats, or Gunn rats injected with photobilirubin (*26*), was unconjugated bilirubin and the methods used to isolate it were mild (*12*) and would not have hydrolyzed pigment conjugates.

At present we do not know whether photobilirubin reverts to bilirubin once it has been excreted into bile. However, the instability of the pigment in protein-free aqueous solutions (*19, 26*) suggests that reversion will occur soon after it has passed through the canalicular membrane. This point could have some clinical relevance in view of the suggestion that the reversion product, bilirubin, causes diarrhea by inhibiting intestinal lactase (*31*). Bilirubin might also be reabsorbed into the circulation from the intestine (*32*), which would reduce the overall efficiency of its removal. In summary, we propose that phototherapy of unconjugated hyperbilirubinemia causes bilirubin to be transported from skin to bile. The departed bilirubin is replaced in the skin by bilirubin from the blood, resulting in a decrease in the concentration of circulating bilirubin.

The initial effectiveness of phototherapy will depend on several factors. These include the wavelength and intensity of the light, the surface area exposed, and the rates at which photobilirubin is removed from the skin and the blood (*33*). At low light intensities, formation of photobilirubin probably is the rate-limiting step. But, at relatively high intensities, formation of photobilirubin could exceed its rate of removal permitting rapid formation of a photostationary equilibrium between bilirubin and photobilirubin. If this occurred, the system would be unaffected by a further increase in the light intensity. Therefore, there may be an optimal maximum light intensity for phototherapy beyond which there will be no increase in the phototherapeutic effect. Whether this intensity is within safe or practical limits is not known. In contrast, there is no reason to believe that there is a lower light intensity limit for photobilirubin formation. Photobilirubin will be formed, not only in treated infants, but also in all jaundiced infants insofar as they are exposed to visible light. Since physiologic jaundice of the newborn is very common, photobilirubin may be considered to be, in a sense, a normal metabolite.

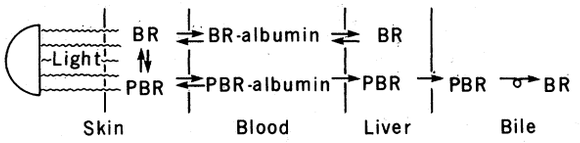

Fig. 6. The mechanism of bilirubin excretion during phototherapy of neonatal jaundice. (*BR*, 4*Z*,15*Z*-bilirubin IXα; *PBR*, photobilirubin).

The proposed mechanism accounts satisfactorily for the apparent biliary excretion of bilirubin during phototherapy, but it does not completely explain all of the effects of the treatment on bilirubin metabolism. During phototherapy, substances derived from bilirubin (but not identical or isomeric with it) are excreted in bile and urine (*3*). The nature of these is obscure. Usually they are assumed to be photooxidation or photodegradation products of bilirubin. However, they could also include secondary products formed by thermal degradation of photobilirubin in vivo or artifacts formed during the analysis of bile and urine in vitro. In view of the known photoreactivity of bilirubin (*34*), it is hard to imagine that photooxidation would not occur at all during phototherapy. Indeed, it is a likely source of the dipyrrolic propentdyopent-like compounds that are excreted, during treatment, in urine (*3, 28, 35*). Nevertheless, there is no experimental evidence that photooxidation of bilirubin plays a major role, and the rather limited quantitative data that are available (*3, 8*) point to unbound biliary excretion of intact bilirubin as the dominant process. Therefore, we suggest that the scheme shown in Fig. 6 represents the most important mechanism underlying phototherapy and the main contributor to the clinical response of lowered serum bilirubin (*36*). It can no longer be held that photodegradation of bilirubin alone is responsible for the effect of phototherapy or that light simply accelerates the breakdown of bilirubin by mechanisms similar to those normally operative in patients with the Crigler-Najjar syndrome and in Gunn rats (*37*).

Bilirubin Conjugation

The photometabolism of bilirubin in jaundiced rats provides new insight on the metabolism of bile pigments in normal animals. In normal mammals and those, such as the Gunn rat, with defective conjugating systems small amounts of unconjugated bilirubin are excreted by the liver into bile (*7, 38*). However, this pathway is inadequate to cope with the load of bilirubin that has to be shifted, and in normal mammals most of the bilirubin is metabolized to conjugates, which are excreted more readily (*5*). In contrast, biliverdin IXα (10,23-didehydrobilirubin IXα), the end product of heme catabolism in many nonmammalian vertebrates, is excreted efficiently without conjugation (*39*). It has never been adequately explained why, on the one hand, bilirubin is the only naturally occurring bile pigment that requires conjugation for efficient excretion, or, on the other, why some bilirubin is excreted in bile in unconjugated form.

Recent physicochemical studies have established that bilirubin and its dicarboxylate anion are alone among natural bile pigments in their ability to form ridge-tile-shaped structures containing multiple intramolecular hydrogen bonds (*20, 21*). Solutions of the pigment contain rapidly equilibrating equimolar quantities of two nonidentical enantiomeric (mirror image) hydrogen-bonded conformers represented by **5** and **6** (Fig. 5) (*22*). The preponderance of these two conformers in solution accounts for the curious lipophilicity of the pigment and the insensitivity of its ultraviolet-visible spectra to solvent effects (*15*). We have presented evidence in this article that photobilirubin, which contains predominantly *4E,15Z* and *4Z,15E* isomers (*19*), is excreted readily in unconjugated form. This demonstrates that configurational isomerization of bilirubin at the C-5 or C-15 bridge, which disrupts the usual hydrogen-bonded system and makes the tetrapyrrole more soluble in water, is sufficient to make it readily excretable, as predicted by theory (*20, 40*). It also provides direct evidence that it is the intramolecular hydrogen-bonding and consequent lipophilicity of the natural isomer that inhibit its excretion from the hepatocyte into bile, as postulated (*41, 42*). Since bilirubin cannot be excreted efficiently by the renal route either (*43*), further metabolism becomes obligatory for its disposal. This explains the need for conjugation, but does not explain why some bilirubin is excreted in uncon-

jugated form. That may be explained as follows. Although the free bilirubin in the hepatocyte probably exists predominantly as the enantiomeric pair **5** and **6** (or the corresponding dianions) other conformers must be present, albeit in low concentrations. Some of these, such as **8**, will not be internally hydrogen-bonded to the same degree as **5** and **6**. Consequently they will be more hydrophilic, more like the configurational isomers in photobilirubin, and presumably more readily excretable than the preferred conformers of the natural isomer. (Consider, for example, the similarity between **7**, a conformer of 4*E*,15*Z*-bilirubin IXα, and **8**, a conformer of 4*Z*,15*Z*-bilirubin IXα.) We suggest that the excretion of unconjugated bilirubin in bile is due to excretion of these less hydrogen-bonded, nonpreferred conformers of bilirubin across the canalicular membrane. According to this hypothesis, free unbound bilirubin in the hepatocyte exists as a mixture of rapidly equilibrating nonpolar (lipophilic) and polar (amphiphilic) forms. Only the latter are taken up at the bile canaliculus and excreted in bile. Since their steady-state concentration is extremely low, excretion of bilirubin by this route is inefficient (*44*). However, augmented excretion of unconjugated bilirubin would be expected whenever the total concentration of unbound bilirubin in the hepatocyte is increased, as found experimentally (*45*). Poor excretability and the need for conjugation are therefore the consequence of certain structural features of bilirubin, namely an sp^3 carbon at C-10, separating two *Z*-configuration dipyrrylmethenes, and two propionic acid side chains at C-8 and C-12. Not surprisingly, other pigments that share these features (such as mesobilirubin IXα) also require conjugation for excretion (*41*), whereas pigments that do not—for example, 4*Z*,15*E*-bilirubin IXα, bilirubin IXβ (*42*), and biliverdin IXα (*39*) are readily excreted without conjugation.

References and Notes

1. In 1974, the most recent year for which statistics are available, about 2.5 percent of all babies born in the United States were treated with phototherapy (R. M. Albrecht, U.S. Bureau of Radiological Health, personal communication).
2. M. Denker, *The Physicians* (Pocket Books, New York, 1976), p. 15; E. Furst, V. D. Sturton, F. A. Moore, T. R. Harris, *J. Pediatr.* **93**, 102 (1978); L. Blackman and W. F. Rosse, in *Current Therapy*, H. F. Conn, Ed. (Saunders, Philadelphia, 1979), pp. 265–268; S. R. Kandall and A. R. Rausen, in *Current Pediatric Therapy*, S. S. Gellis and B. M. Kagan, Eds. (Saunders, Philadelphia, 1978), pp. 263–267.
3. J. D. Ostrow, *J. Clin. Invest.* **50**, 707 (1971).
4. H. T. Lund and J. Jacobsen, *J. Pediatr.* **85**, 262 (1974).
5. R. Schmid and A. F. McDonagh, in *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, D. S. Frederickson, Eds. (McGraw-Hill, New York, ed. 4, 1978), pp. 1221–1257.
6. R. L. Swarm, in *Symposium on Animal Models for Biomedical Research IV* (National Academy of Sciences, Washington, D.C., 1971), pp. 149–160.
7. E. W. Callahan and R. Schmid, *Gastroenterology* **57**, 134 (1969).
8. A. F. McDonagh, in *Phototherapy in the Newborn*, G. B. Odell, R. Schaffer, A. P. Simopoulos, Eds. (National Academy of Sciences, Washington, D.C., 1974), pp. 56–73.
9. A. F. McDonagh and L. M. Ramonas, *Science* **201**, 829 (1978).
10. Rats were anesthetized with ketamine and acepromazine maleate (20 and 2 milligrams, in succession intramuscularly) and irradiated with Westinghouse Special Blue lights. The infrared heating lamp was a 200-W General Electric Heat-Ray lamp and the safelight was a 15-W tungsten bulb filtered through Kodak 1A or ML2 safelight filters. Serum samples were stored at 4°C in the dark for no longer than 48 hours.
11. As noted by a reviewer, it may not have been necessary to wait 30 minutes before sealing the bile duct cannula. However, we have not tested this experimentally.
12. Bile extractions and analyses were carried out as follows (*15*). Bile (1 volume) was acidified with 0.4*M* glycine-HCl buffer, *p*H 1.8 (8 volumes), and 10 percent (weight to volume) ascorbic acid in saturated saline (2 volumes) was added, and then sodium chloride (2 grams per milliliter of bile). The mixture was cooled in ice and extracted with a mixture of ethanol and chloroform (1:1 by volume, 8 ml per milliliter of bile); the organic extract was evaporated to dryness under reduced pressure at room temperature after filtration through chloroform-moistened filter paper. The residue, dissolved in a small volume (~1 ml) of chloroform, was applied to the top of a small chromatography column of silica gel H. Excess chloroform was sucked through the column under reduced pressure and the adsorbed pigment and column were washed with a further portion of chloroform (0.5 to 1.0 of the column volume). Then bilirubin was eluted under reduced pressure with chloroform containing 1 percent acetic acid. The yield of pigment was estimated spectrophotometrically (extinction coefficient = 62,600). The chloroform used in the above procedure contained approximately 1 percent ethanol.
13. A. F. McDonagh and F. Assisi, *FEBS Lett.* **18**, 315 (1971).
14. T. A. Wooldridge and D. A. Lightner, *J. Liq. Chromatog.* **1**, 653 (1978).
15. A. F. McDonagh, in *The Porphyrins*, D. Dolphin, Ed. (Academic Press, New York, 1979), vol. 6, pp. 293–491.
16. The proscripts 4*Z*, 15*Z*, 4*E*, and 15*E* are used to indicate the configuration of the molecule at its C-4 and C-15 double bonds; the postscript IXα indicates the sequence of the methyl, vinyl, and propionic acid side chains [for a more complete summary of bile pigment isomers and nomenclature see McDonagh (*15*)]. Throughout this article the word bilirubin, used alone, refers to the naturally occurring 4*Z*,15*Z*-IXα isomer.
17. R. E. Davies and S. J. Keohane, *Photochem. Photobiol.* **17**, 303 (1973).
18. Serum samples were washed in silanized tubes under a safelight (*10*) as follows. Serum (2.5 ml, *p*H 8.0) was shaken with washed chloroform (2.5 ml, washed once with 0.1*M* $NaHCO_3$, three times with water, and dried with filter paper) and centrifuged. The aqueous phase was washed with petroleum ether (boiling point 60° to 80°C, 2.5 ml), centrifuged, and used immediately for determination of difference spectra.
19. D. A. Lightner, T. A. Wooldridge, A. F. McDonagh, *Biochem. Biophys. Res. Commun.* **86**, 235 (1979); *Proc. Natl. Acad. Sci. U.S.A.* **76**, 29 (1979); A. F. McDonagh, D. A. Lightner, T. A. Wooldridge, *J. Chem. Soc. Chem. Commun.* (1979), p. 110.
20. R. Bonnett, J. E. Davies, M. B. Hursthouse, G. M. Sheldrick, *Proc. R. Soc. London Ser. B* **202**, 249 (1978).
21. A. Magnoli, P. Manitto, D. Monti, *Nature (London)* **273**, 568 (1978).
22. P. Manitto and D. Monti, *J. Chem. Soc. Chem. Commun.* (1976), p. 122.
23. The possibility that configurational isomerization of bilirubin plays an important role in phototherapy was suggested first by McDonagh [A. F. McDonagh, in *Phototherapy for Neonatal Hyperbilirubinemia*, A. K. Brown and J. Showacre, Eds. (Department of Health, Education, and Welfare, Washington, D.C., 1974), Publ. No. NIH 76-1075, p. 171; in *Bilirubin Metabolism in the Newborn (II), 1974*, D. Bergsma and S. H. Blondheim, Eds. (Excerpta Medica, Amsterdam, 1976), p. 30; _____ and L. A. Palma, in *Chemistry and Physiology of Bile Pigments*, P. D. Berk and N. I. Berlin, Eds. (Department of Health, Education, and Welfare, Washington, D.C., 1977), Publ. No. NIH 77-1000, p. 93; A. F. McDonagh, *Am. Soc. Photobiol. Abstr.* **4**, 57 (1976); *Biochem. Soc. Trans.* **4**, 219 (1976)], although clearly the same idea had occurred independently to others (*20*, *40*). The first direct experimental evidence on the photoisomerization of bilirubin in vivo was published by Ostrow [J. D. Ostrow, *Am. Soc. Photobiol. Abstr.* **4**, 60 (1976)] who at that time ruled out "the possibility that photoisomerization of the pigment, to a form incapable of internal hydrogen-bonding, accounts for the excretion of unconjugated bilirubin in bile during phototherapy." However, more recent studies suggested that photoisomerization might be important in vivo [E. A. Zenone, M. S. Stoll, J. D. Ostrow, *Gastroenterology* **72**, 1180 (1977); M. S. Stoll, E. A. Zenone, J. D. Ostrow, J. E. Zarembo, *Am. Soc. Photobiol. Abstr.* **5**, 97 (1977)]. We should add, however, that the photobilirubin and its constituents that we have detected in our studies and isolated earlier (*19*) do not appear to be the same as any of the bilirubin photoproducts described by Ostrow and co-workers. It is important to note that irradiation of bilirubin in anaerobic solution with intense light in vitro can give, depending on the reaction conditions, not only *E,Z* and *E,E* geometric isomers, but also several other polar tetrapyrroles, some of which are more hydrophilic than bilirubin [for a review, see McDonagh (*15*) or Lightner (*34*)]. Whether these other substances are formed in vivo during phototherapy to any important extent is not known, but we believe that it is unlikely, even though some of them may be readily excretable when administered to homozygous Gunn rats in the dark. Since this article was submitted two further interesting papers have been published. In one, Onishi and co-workers [S. Onishi, S. Itoh, N. Kawade, K. Isobe, S. Sugiyama, *Biochem. Biophys. Res. Commun.* **90**, 890 (1979)] have described the chromatographic separation of more than 20 photoproducts formed by prolonged irradiation of bilirubin in deoxygenated chloroform and the apparent detection of one of these products in serum from an infant treated with phototherapy. In the other paper, Stoll and coworkers [M. S. Stoll, E. A. Zenone, J. D. Ostrow, J. E. Zarembo, *Biochem. J.* **183**, 139 (1979)] have reported that irradiation of bilirubin in solution with an unfiltered intense ultraviolet light yields compounds that are isomeric with bilirubin. The substances described by Stoll and co-workers have different physicochemical properties from the bilirubins described in this article and in view of their mode of preparation their role in phototherapy is unclear.
24. Solutions of photobilirubin were prepared as follows. Crude photobilirubin contaminated with bilirubin, synthesized by a slightly modified version of an earlier method (*19*), was mixed with Sprague-Dawley rat serum, and the mixture was centrifuged to remove undissolved bilirubin. Portions of this solution were diluted to a suitable concentration with rat serum and used for difference spectra.
25. Photobilirubin formed in vivo may have a different isomer composition from photobilirubin formed in vitro in protein-free organic solvents, but we doubt that the difference is large.
26. A. F. McDonagh, L. A. Palma, D. A. Lightner, unpublished observations.
27. E. J. Land, *Photochem. Photobiol.* **24**, 475 (1976); H. Falk and F. Neufingerl, *Monatsh. Chem.* **110**, 1243 (1979).
28. S. O. Porto, in *Bilirubin Metabolism in the Newborn*, E. Bergsma *et al.*, Eds. (Williams & Wilkins, Baltimore, 1970), pp. 83–89.
29. T. P. Vogl, *J. Pediatr.* **85**, 707 (1974); T. F. Blaschke, P. D. Berk, B. F. Scharschmidt, J. R. Guyther, J. M. Vergalla, J. G. Waggoner, *Pediatr. Res.* **8**, 573 (1974).
30. Further evidence for extravascular tissue as the main site for formation of photobilirubin is provided by phototherapy experiments on homozygous Gunn rats treated with sulfadimethoxine. This drug causes a pronounced shift of bilirubin from plasma to extravascular tissue in Gunn rats, yet does not have a great influence on their biliary excretion of bilirubin in response to light (A. F. McDonagh and L. A. Palma, unpublished observations). However, we do not mean to imply that photobilirubin is formed exclusively in extravascular tissues. Some also may be formed directly in plasma as the blood circulates near the skin surface.
31. A. F. Bakken, *Acta Paediatr. Scand.* **66**, 91 (1977).
32. R. Lester and R. Schmid, *J. Clin. Invest.* **42**, 736 (1963).
33. Movement of bilirubin from blood to skin also is important and may become rate-limiting during prolonged phototherapy once the skin has be-

come depleted of bilirubin [T. P. Vogl, H. Cheskin, T. A. Blumenfield, W. T. Speck, M. R. Koenigsberger, *Pediatr. Res.* **11**, 1021 (1977)].
34. D. A. Lightner, *Photochem. Photobiol.* **26**, 427 (1977).
35. S. Onishi, K. Shimizu, T. Yamakawa, *Jpn. J. Pediatr.* **22**, 138 (1969).
36. The presence of photobilirubin in the blood of jaundiced infants exposed to light could have practical significance. Unisomerized bilirubin binds strongly to serum albumin (*15*). The large albumin pool in the circulation acts as an effective trap for bilirubin, preventing its diffusion to the central nervous system in cases of unconjugated hyperbilirubinemia. Measurements of the free (nonprotein-bound) bilirubin concentration in serum or of the bilirubin-binding capacity of the serum albumin are coming into use in the management of neonatal jaundice to help to recognize babies in jeopardy and to determine whether further phototherapy or exchange transfusions are necessary. [See relevant papers in *Bilirubin Metabolism in the Newborn II*, D. Bergsma and S. H. Blondheim, Eds. (Excerpta Medica, Amsterdam, 1976)]. The presence of photobilirubin could potentially interfere with some of these measurements and result in misleading aberrant values [W. E. Blumberg, J. Eisinger, A. A. Lamola, R. McClead, A. Fanaroff, A. F. McDonagh, *Am. Soc. Photobiol. Abstr.* **7**, 143 (1979)]. On the other hand, the concentration of photobilirubin in serum might have some diagnostic value as an early indicator of babies with congenital cholestasis. Only in these babies, as in the bile-blocked Gunn rats, would photobilirubin levels be expected to rise precipitously after beginning phototherapy.
37. J. D. Ostrow and R. V. Branham, in *Bilirubin Metabolism in the Newborn*, D. Bergsma, D. Y-Y. Hsia, C. Jackson, Eds. (Williams & Wilkins, Baltimore, 1970), pp. 93–99; E. W. Callahan, M. D. Thaler, M. Karon, K. Bauer, R. Schmid, *Pediatrics* **46**, 841 (1970).
38. E. R. Gordon, T-H. Chan, K. Samodai, C. A. Goresky, *Biochem. J.* **167**, 1 (1977).
39. E. Colleran and P. O'Carra, in *Chemistry and Physiology of Bile Pigments*, P. D. Berk and N. I. Berlin, Eds. (Department of Health, Education, and Welfare, Washington, D.C., 1977), Publ. No. NIH 77-1000, pp. 69–80.
40. A. O. Pedersen, F. Schønheyder, R. Brodersen, *Eur. J. Biochem.* **72**, 213 (1977).
41. R. Lester, P. D. Klein, A. M. Matusen, *J. Clin. Invest.* **45**, 1839 (1966).
42. N. Blanckaert, K. P. M. Heirwegh, Z. Zaman, *Biochem. J.* **164**, 229 (1977).
43. T. K. With, *Bile Pigments* (Academic Press, New York, 1968).
44. In this hypothesis it is assumed that the rate of excretion depends on the steady-state concentration of structures such as **8**. Therefore the excretion rate depends on the position of the rapid equilibrium between, for example, **8** and **5** or **6** and not on the rate at which the equilibrium is achieved.
45. P. Berthelot, in *Bilirubin Metabolism*, I. A. D. Bouchier and B. H. Billing, Eds. (Blackwell, Oxford, 1967), pp. 189–195; L. Okolocsanyi and P. Magnenat, *Experientia* **26**, 733 (1970).
46. Supported by NSF grant CHE 74-20877 and NIH grants HD-09026, AM-11275, AM-18220, AM-18250, and assisted by gifts from the United Cerebral Palsy Research and Education Foundation and the Duro-Test Corporation. We thank Dr. R. Schmid for his cooperation and assistance and Drs. M. Bissell, J. Gollan, N. Blanckaert, and J. Gurst for critically reviewing the manuscript.

International Politics and Science: Frank Press in Venezuela

Marcel Roche and Luis A. Ordóñez

Frank Press, the noted North American geophysicist who acts as science and technology adviser to President Carter, visited Venezuela on a special mission from 7 to 10 October 1979. After Venezuela, his mission would take him to Brazil and Peru. Press was accompanied by a selected group of 30 well-known scientists and technologists, among whom were Benjamin Huberman, associate director of the President's Science and Technology Office; Richard D. Atkinson, director of the National Science Foundation; Robert Frosch, administrator of the National Aeronautics and Space Administration; Peter Bell, at the Department of Health, Education, and Welfare; Ray Chamberlain, president of Colorado State University; and Alexander Heard, chancellor of Vanderbuilt University.

During their stay, members of the North American science and technology committee visited Luis Herrera Campíns, president of the republic, and several ministers, as well as a number of Venezuelan institutions, such as the Venezuelan Technological Petroleum Institute, the National Dermatological Center, the steel plant at Puerto Ordaz, and the Instituto Venezolano de Investigaciones Científicas. It was regretted by many that the Venezuelan Association for the Advancement of Science, founded 29 years ago on British and American models and representing the scientific community, was not paid a visit.

Attitudes Toward North American Influence

The importance of Press's visit is enhanced by the fact that over the last 25 years or so there has been a significant growth of Venezuelan research—in science and, to a lesser extent, technology—so that the country is today better able to profit from foreign help in science and technology. In addition, the present President, Luis Herrera Campíns, of the Social Christian Party, appointed in March a state minister for science and technology, Raimundo Villegas, an active biophysicist and recognized member of the scientific community who was trained at Harvard under A. K. Solomon. Finally, Venezuela's 6th National Plan, which for the first time includes science and technology as a separate sector, is being prepared under the supervision of Villegas and the National Research Council (CONICIT).

On the other hand, although nearly everyone in Venezuela recognizes the global worth of North American science and technology and their impact on economic, social, and cultural aspects of U.S. life, there are grave doubts—at least among many in the academic community—about both the motivation and the efficiency of U.S. help. Previous efforts, such as President Kennedy's Alliance for Progress and, on a lesser scale, President Eisenhower's Atoms for Peace, are judged to have had little effect on the country's science and technology, much less on its social and human development. The influence of multinational corporations on the development of endogenous science (and especially, technology) is thought to have been negative. For example, the oil companies obviously did bring economic benefits, from the 1920's on, but they did little to encourage local research, preferring to perform it in their home environment. Oil is by far Venezuela's chief natural resource (providing around 92 percent of its foreign income). With a few exceptions, such as Universidad del Zulia in Maracaibo, near the oil fields, there was little research on oil by Venezuelans until 1972, when the petroleum and chemistry group of Instituto Venezolano de Investigaciones Científicas was organized. The Venezuelan Technological Petroleum Institute began functioning only in July 1976, 1 year after the industry had been nationalized on 29 August 1975. It is not implied, of course, that the oil companies

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