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THE PROTEIN-BINDING OF C^{14}-BILIRUBIN IN HUMAN AND MURINE SERUM *

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Although at physiologic pH small amounts of unconjugated bilirubin are soluble in protein-free aqueous solutions (1), in plasma, virtually all pigment is bound to protein (2–4). In this interaction of bilirubin with protein, albumin plays a major role (3–13), but some authors have suggested that, in addition, "specific" bilirubin-carrying proteins may exist in the α- or β-globulins (14–18). These reports disagree, however, about the precise globulin fraction involved, and other investigators (3, 5, 7–10, 12) have denied that bilirubin is bound by globulins. Resolution of this question is important because this transport process is believed to be one of the factors regulating the distribution of bilirubin between the blood and other organs, and governing the rate of pigment removal by the liver (19).

Because of difficulties in identifying and quantitating small amounts of bilirubin (20, 21), previous investigations of this problem had to be conducted at greatly increased pigment concentrations (3–6, 8–18), using either icteric sera (3–6, 8–10, 12–14, 16–18) or specimens with bilirubin added *in vitro* (3–6, 8, 9, 13, 15–17). This may have prevented detection of globulin fractions with high affinity but limited carrying capacity for pigment. Such minor fractions would become evident only at lower pigment concentrations, at which albumin, because of its lesser affinity, would bind a smaller percentage of the total bilirubin (22).

With the aid of isotopically labeled bilirubin of high specific activity (23) and continuous-flow electrophoresis (24), it has been possible to analyze serum samples with bilirubin concentrations within or close to the physiologic range. These studies have demonstrated that in both human and murine serum, bilirubin is bound solely to albumin (25).

MATERIAL AND METHODS

Fasting human serum was obtained from three healthy men without hepatobiliary or hematological disease and with normal serum bilirubin concentrations of 0.4 to 0.6 mg per 100 ml. A second group of sera was collected from patients with nonconjugated hyperbilirubinemia of various etiologies. This group included: two men with "constitutional hepatic dysfunction" (Gilbert's disease) (26), a woman in an acute hemolytic crisis due to sickle-cell anemia, two normal, full-term, newborn infants, and a child with severe congenital nonhemolytic jaundice (Crigler-Najjar syndrome) (27). The child's serum was studied both after *in vitro* addition of C^{14}-bilirubin (see below) and after iv injection of C^{14}-bilirubin dissolved in 5% human albumin (28). A third group of sera was obtained from two patients with hereditary abnormalities in serum albumin; one had congenital analbuminemia [1] (29), and the other, hereditary bis-albuminemia (30). In the latter instance, the study was done on 250 mg of lyophilized Cohn fractions IV and V [2] (31), corresponding to approximately 5 ml of serum. These protein fractions, dissolved in dilute Tris buffer at pH 8.6 (see below), contained no detectable bilirubin, and they were processed in the same way as whole serum. Clinical details of patients are presented in the Appendix.

Murine serum was obtained from blood drawn from the inferior vena cava of adult, male, Sprague-Dawley rats. In some instances, studies were done on serum from Gunn rats with hereditary, nonconjugated hyperbilirubinemia (32, 33) and from their nonicteric, heterozygous littermates. In one experiment, a female, homozygous, Gunn

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[1] Serum kindly supplied by Dr. Robert S. Gordon, National Heart Institute, Bethesda, Md.

[2] The authors are grateful to Dr. Karl Schmid, Department of Medicine, Massachusetts General Hospital, Boston, Mass., and to Dr. Edward L. Sarcione, Roswell Park Memorial Institute, Buffalo, N. Y., for making these samples available.

rat with an unconjugated bilirubin concentration of 2.8 per 100 ml was injected intravenously with 200 μg C^{14}-bilirubin (0.15 μc) dissolved in 2.0 ml serum from a heterozygous littermate. Serum obtained 45 minutes later was subjected to continuous-flow electrophoresis without further addition of C^{14}-bilirubin.

Two male, icteric, Gunn rats were injected intravenously with a saline suspension of C^{14}-labeled rat erythrocytes prepared by *in vivo* administration of glycine-2-C^{14} and then sensitized *in vitro* with incomplete antibodies against rat red cells (34).[3] Virtually all of the administered erythrocytes, equivalent in amount to 2 ml of whole blood, were cleared from the recipients' circulation within 2 hours of the injection (34). After an additional hour, when serum bilirubin levels had reached 7.2 and 10.8 per 100 ml, respectively, blood was withdrawn from each recipient, and the serum was subjected to continuous-flow electrophoresis without further addition of labeled pigment.

C^{14}-bilirubin was prepared and its specific activity determined as previously described (23). Tris-maleate buffer (22), 0.073 M at pH 8.6, or 0.067 M phosphate (Na_2HPO_4-KH_2PO_4) buffer at pH 7.4 (35) was diluted with 7 vol distilled water, and the pH was readjusted by addition of a few drops 1.0 N NaOH or 1.0 N H_3PO_4. These diluted buffers will be referred to as Tris or phosphate buffer, respectively. The preparation, dialysis, and electrophoresis of serum samples were performed in a darkroom at 4° C (23) to minimize deterioration of the bilirubin.

Continuous-flow electrophoresis of human and murine serum. Crystalline C^{14}-bilirubin, 17 to 6,000 μg, representing a total of 0.02 to 0.05 μc was rapidly dissolved in 0.2 to 0.5 ml 0.1 N NaOH and diluted with buffer to a final volume of 10 ml. Under constant stirring, this solution was added to 5.0 ml serum. A 1.0-ml sample of this diluted serum was frozen for later analysis, while the remaining 14 ml was dialyzed in a cellophane bag against 800 ml buffer for 16 hours. After another sample was removed for later analysis, the dialyzed serum was processed through a Beckman-Spinco model CP continuous-flow electrophoresis apparatus (24) for 16 to 18 hours at a constant current of 28 to 36 ma and a voltage of 650 to 750 v, depending on the buffer used. The sample was applied at a rate of 0.6 to 0.7 ml per hour and required approximately 4 hours to descend the curtain. Upon completion of the experiment, each of the 32 eluted fractions was diluted to 10 ml with buffer and analyzed for radioactivity, bilirubin, and protein concentration. The samples removed before and after dialysis and the residual sample remaining in the feeder tube of the electrophoresis apparatus were similarly analyzed.

Analytical methods. Direct spectral absorption was determined in the 450-mμ band. Bilirubin concentration was measured spectrophotometrically by Fog's diazo method (36). Protein concentrations were estimated spectrophotometrically by the method of Lowry, Rosebrough, Farr, and Randall (37), and the observed optical density was corrected for the small absorbance resulting from the bilirubin present in some of the fractions.

Three to four ml of each eluted fraction, or 0.1 ml of diluted or dialyzed serum was lyophilized, dissolved, and counted by methods previously reported (38). When vials had a counting rate exceeding 3 SD above the mean counting rate of similarly prepared background samples, they were considered to contain significant radioactivity. In each eluted fraction, it was possible to detect as little as 0.05 to 0.12% of the combined total radioactivity recovered in all 32 fractions. After addition of 4 μg C^{14}-bilirubin to each of the 32 eluted protein fractions, recovery of radioactivity averaged 98 ± 2 (SD)%.

Starch-block electrophoresis of human serum. With serum from two normal men and from a subject with constitutional hepatic dysfunction, proteins were separated by starch-block electrophoresis[4] (39). Forty μg C^{14}-bilirubin, representing 0.03 μc, was dissolved in 0.1 ml 0.1 N NaOH and mixed with 0.5 ml 0.1 M borate buffer at pH 8.6. Two-tenths ml of this solution was added to 0.5 ml of each serum, and the mixture was dialyzed against 200 ml of the borate buffer for 16 hours. A 0.2-ml sample of the dialyzed serum mixture was used for determination of radioactivity; the remaining 0.5 ml was subjected to electrophoresis on a 1.5- × 12-inch starch block prepared from hydrolyzed starch[5] in the borate buffer. After electrophoresis for 20 hours at 200 v and 25 ma, the protein contained in each 0.5- to 1.0-cm segment of the block was eluted with two 5-ml washes of 0.02 M borate buffer, at pH 8.6. In each eluate, protein concentration was estimated from the spectral absorption at 280 mμ (40), and 4 to 5 ml were assayed for radioactivity as described above. The composition of the major protein peaks was identified by immunoelectrophoresis in agar gel[6] (41).

Dialysis experiments at pH 8.6. Human serum of known bilirubin and albumin concentration was diluted with nine parts Tris buffer. C^{14}-bilirubin, 0.8 to 1.0 mg, was dissolved in 0.5 ml 0.1 N NaOH and diluted with Tris buffer to a final volume of 5.0 ml. A 0.1- to 1.0-ml volume of this solution of labeled bilirubin was added to 1.0 ml of the diluted serum, and the volume was adjusted to 2.0 ml with 0.01 N NaOH in Tris buffer. Samples of 1.0 ml of these diluted serum samples containing C^{14}-bilirubin were dialyzed in a Visking cellophane bag (0.002-inch wall thickness) against 30 ml Tris buffer for 24 hours with agitation at 100 cycles per minute in a Dubnoff metabolic shaking incubator. Bilirubin and protein concentrations and total radioactivity were determined before and after dialysis. Pigment concentrations were expressed in relation to albumin concentration, de-

[3] The authors are grateful to Dr. James H. Jandl for his advice and help in preparing these sensitized red cells.

[4] The authors are grateful to Dr. Jack S. Remington, Thorndike Memorial Laboratory, Boston, Mass., for his help in performing this procedure.

[5] Connaught Medical Laboratories, University of Toronto, Toronto, Canada.

[6] Kindly performed by Dr. Frederick Bigelow, Thorndike Memorial Laboratory, Boston, Mass.

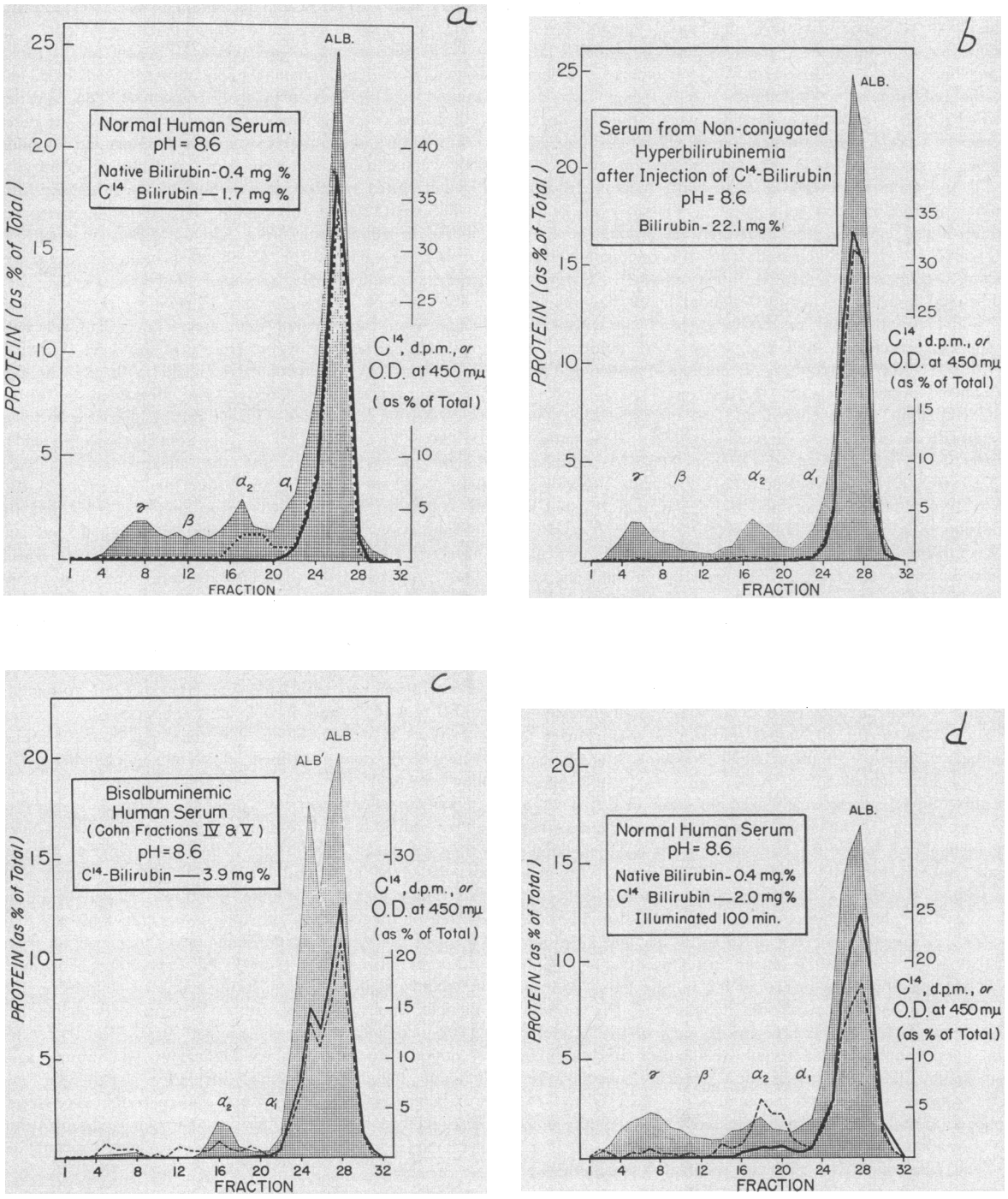


FIG. 1. CONTINUOUS-FLOW ELECTROPHORESIS OF HUMAN SERUM CONTAINING C^{14}-BILIRUBIN. Shaded area represents protein content; dotted line, spectral absorption (OD) at 450 mμ; and solid line, C^{14}-radioactivity (dpm) in each eluted fraction. Values are plotted as percentage of the total combined recovery in all 32 eluted fractions. In a, c, d, and e, C^{14}-bilirubin was added *in vitro;* in b, C^{14}-bilirubin was injected intravenously; c was obtained with Cohn fractions IV and V.

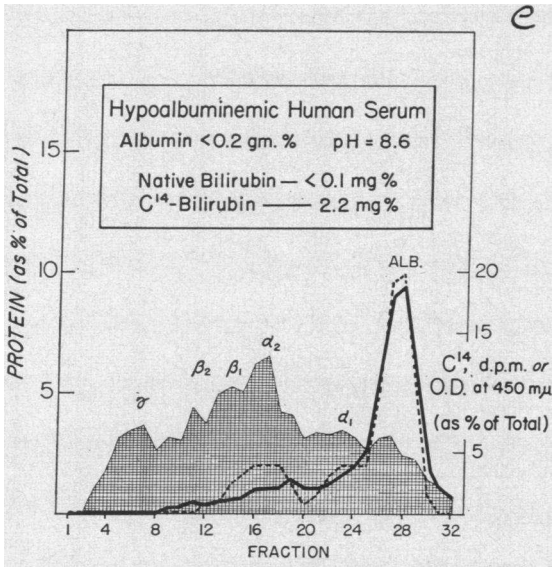


FIG. 1.—(*Continued*).

termined by paper strip electrophoresis (39), in order to permit comparison of the results obtained with different serum samples and to adjust for the slight volume changes that occurred during dialysis.

In some instances, electrophoresis or dialysis was carried out with C¹⁴-bilirubin subjected to partial decomposition before its addition to the serum samples. For the dialysis experiments, 0.2 mg C¹⁴-bilirubin dissolved in 0.5 ml 0.1 N NaOH and 10 ml Tris buffer was exposed for 120 minutes to unfiltered illumination from a General Electric mercury lamp at a distance of 25 cm (23). Before and at frequent intervals during illumination, 1.0-ml samples were removed and added to equal volumes of serum, and the resulting solutions were dialyzed and analyzed as above.

RESULTS

Five electrophoretic analyses were carried out at pH 8.6 and two at pH 7.4 with normal human sera, supplemented *in vitro* with C¹⁴-bilirubin to a total pigment concentration ranging from 0.8 to 72 mg per 100 ml. In these sera, 99.6 to 100% of the recovered radioactivity migrated with the albumin fraction. The ratio of C¹⁴-activity to protein was highest in tubes with the highest albumin concentrations (Figure 1a). Similar observations were made with the serum samples exhibiting naturally elevated levels of unconjugated bilirubin and with serum obtained after iv administration of C¹⁴-bilirubin to the child with congenital nonhemolytic jaundice (Figure 1b). With Cohn fractions IV and V from bis-albuminemic serum, two large peaks of radioactivity were observed, each coinciding with one of the two albumin fractions (Figure 1c). In every instance, the albumin band was visibly yellow, exhibited positive diazo (36) and Gmelin (42) reactions, and showed maximal spectral absorption at 450 mμ. Total recovery of radioactivity and protein in the 32 eluted fractions ranged from 75 to 80% of the amount delivered from the feeder tube, while the rest remained on the electrophoresis curtain. There was virtually no loss of label into the circulating buffer, and the ratio of total radioactivity to protein in the combined eluates ranged from 97 to 103% of that in the dialyzed serum.

When C¹⁴-bilirubin of known specific activity was added to serum samples, the bilirubin concentration as measured by the diazo reaction was on the average 4% lower than that predicted from the isotope concentration. The concentration of diazo-reacting pigment decreased an additional few per cent during dialysis before the electrophoretic separation, but only minute amounts of radioactivity appeared in the dialysate (Figure 5). On subsequent electrophoresis, a trace of isotope, in addition to the label in the albumin fraction, was sometimes found in the α-globulin zone, migrating usually between the α_1 and α_2 bands. The amount of label in this location never exceeded 0.4% of the total recovered radioactivity and bore no relationship to the nature of the serum used, or to the concentration of C¹⁴-bilirubin. Furthermore, if several electrophoretic separations were performed with multiple samples from a single serum specimen, radioactivity was present in the α-globulin band on some occasions, but was absent on others. This isotopic material associated with the α-globulins was colorless, never exhibited positive diazo or Gmelin reactions, and displayed no absorption maximum in the visible range. A yellow, benzidine-positive pigment also detected in the α-globulins contributed optical absorbance at 450 mμ, but was not associated with radioactivity (Figure 1a).

These observations suggested that in the process of dissolving and dialysis, a small and variable proportion of the added C¹⁴-bilirubin deteriorated to diazo-negative derivatives (23); these remained in part attached to protein and migrated in the α-globulin fraction. The behavior of these bilirubin derivatives was further studied by supplementing serum with labeled bilirubin previously

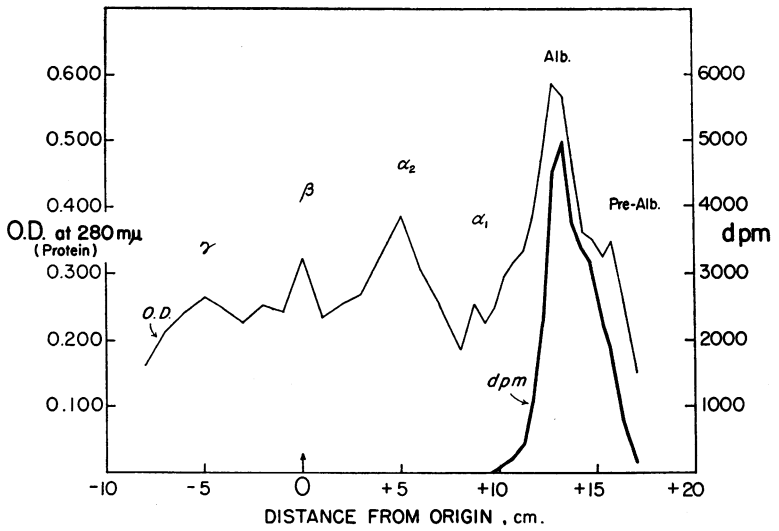


FIG. 2. STARCH-BLOCK ELECTROPHORESIS AT pH 8.6 OF NORMAL HUMAN SERUM SUPPLEMENTED *in vitro* WITH 2.6 MG PER 100 ML C^{14}-BILIRUBIN. The light line represents protein content per centimeter segment determined by direct spectral absorption (OD) at 280 mμ; the dark line, C^{14}-activity (dpm).

subjected to partial photo-oxidative decomposition in alkaline solution (23). Under these conditions, a much larger fraction of the radioactivity appeared in the dialysate, all in the form of colorless, diazo-negative derivatives of bilirubin (Figure 6). On electrophoresis, the ratio of radioactivity to protein in the combined eluates was only 73% of that in the dialyzed sample, indicating that much of the labeled material was lost into the circulating buffer. Most of the label remaining attached to protein was associated with albumin, but 5.2% migrated in the α-globulin zone (Figure 1d), entirely in the form of diazo-negative compounds. The albumin fraction contained all the unaltered C^{14}-bilirubin and an additional amount of labeled diazo-negative derivatives, as shown by the fact that the concentration of diazo-reacting pigment in this fraction did not account for all radioactivity present.

Similar observations were made when the concentration of C^{14}-bilirubin in serum exceeded 2 moles of pigment per mole of albumin (see below). Thus, in normal serum supplemented with 165 mg C^{14}-bilirubin per 100 ml, approximately one-fifth of the added label appeared in the dialysate in the form of bilirubin derivatives. Moreover, on subsequent electrophoretic analysis, the ratio of radioactivity to protein in the combined eluates was reduced by one-third as compared to the dialyzed serum, and 4.7% of the recovered label migrated with the α-globulins. In hypo(an)albuminemic serum supplemented with 2.2 mg C^{14}-bilirubin per 100 ml, 28% of the recovered radioactivity appeared in the globulin zones, while most of the unchanged C^{14}-bilirubin migrated in the usual location of the albumin band (Figure 1e), which was virtually absent in this instance. In both these sera and in contrast to all other sera studied, however, a weak diazo reaction was elicited in the α-globulin fraction, although it was too weak to account for more than a small portion of the total radioactivity present in this zone.

On starch-block electrophoresis at pH 8.6 where the prealbumin and α_1-globulin bands were more clearly separated from the albumin fraction (Figure 2), 83 to 85% of the applied radioactivity was recovered in the combined eluates. In the three serum specimens studied by this method, all detectable radioactivity migrated with the albumin fraction, except for a small amount of label migrating in the prealbumin zone; this zone was shown by immunoelectrophoresis to include small amounts of contaminating albumin.

Electrophoresis of rat serum. In contrast to human serum, rat serum contains only negligible concentrations of native bilirubin (43) and on electro-

phoretic analysis, it exhibits an "f" protein fraction that migrates anodally to the albumin (44) (Figure 3). Nonetheless, in 9 experiments with normal rat serum with 0.2 to 41.6 mg per 100 ml C^{14}-bilirubin added, electrophoresis at pH 8.6 (Figure 3a) and pH 7.4 (Figure 3b) revealed a distribution of pigment comparable to that observed in human serum. As with human serum, only minute amounts of radioactivity diffused into the dialysates, almost all of the isotopic material migrated with the albumin, and the combined electrophoretic eluates exhibited a ratio of total label to protein similar to that in the dialyzed samples. In the rat serum, however, a larger discrepancy was noticed between the concentrations of diazo-reacting pigment and C^{14}-activity, indicating that decomposition of bilirubin occurred more rapidly in murine than in human material. Therefore, it was not surprising that on electrophoretic analysis, a proportionally larger fraction of the added C^{14}-bilirubin appeared in the form of labeled protein-bound oxidation products. Thus, 89.8 to 97.7% of the recovered radioactivity migrated with the albumin fraction, while 2.3 to 10.2% appeared in the α-globulin zone (Figure 3a, b). In contrast to the albumin fraction, the radioactive material in the α-globulins exhibited neither positive diazo or Gmelin reactions, nor the spectral absorption properties of bilirubin. Similar electrophoretic patterns were obtained after *in vitro* addition of C^{14}-bilirubin to sera of homozygous jaundiced and heterozygous Gunn rats, and after iv administration of labeled pigment to a homozygous Gunn rat (Figure 3c). Furthermore, in Gunn rats injected with C^{14}-labeled, sensitized red cells, all of the diazo-positive pigment derived from endogenous breakdown of hemoglobin became attached to serum albumin.

Electrophoretic analyses were conducted with four samples of a single serum pool, each supplemented with a different quantity of the same C^{14}-bilirubin sample. Under these conditions, a progressive 200-fold increase in C^{14}-bilirubin concentration resulted in a proportional rise in the *absolute* amount of radioactivity associated with the α-globulin fraction, indicating that the binding capacity of this protein fraction had not been reached. This was further demonstrated by enriching sera with labeled pigment previously subjected to partial photo-oxidation (23). With C^{14}-bilirubin illuminated for 35 minutes, the α-globulins contained 19.6% of the recovered radioactivity (Figure 3d). After illumination for 270 minutes, 24.6% of the radioactivity was found in this protein fraction, and additional isotopic material migrated anodally to the "f" fraction, apparently without attachment to protein (Figure 3e). Since in the latter instance illumination had caused decomposition of more than 90% of the initial diazo-reactive pigment, the presence of 40% of the radioactivity in the albumin band revealed that the diazo-negative derivatives interact with this protein fraction as well as with α-globulins.

An attempt was made to protect bilirubin against oxidation by adding 0.04% ascorbic acid (45) to the systems used for dissolving, dialysis, and electrophoresis of the pigment. Even with this precaution, however, complete stabilization of the added C^{14}-bilirubin could not be achieved in murine serum, and traces of labeled derivatives still appeared in the α-globulin zone.

Dialysis of labeled pigment in serum. When human sera containing various concentrations of C^{14}-bilirubin were dialyzed for 24 hours at pH 8.6, the proportion of radioactivity remaining in the dialyzing bag depended upon the initial pigment concentration (Figure 4). At concentrations *below* 2 moles of bilirubin per mole of albumin, the regression line, calculated by the method of least squares (46), did not differ significantly from that which would have pertained if all bilirubin had remained bound to protein in the bag. With higher pigment concentrations, on the other hand, approximately 40% of the label present in excess of 2 moles of bilirubin per mole of albumin was lost during the dialysis. The two regression lines had significantly different slopes ($p < 0.001$) and intersected at a predialysis pigment concentration of 2.05 moles per mole of albumin. Serum from a normal person and from a subject with constitutional hepatic dysfunction yielded virtually identical results (Figure 4). Two-thirds of the radioactivity was lost from the bag content in the absence of serum protein.

At bilirubin concentration below 2 moles per mole of albumin, approximately 2% of the label within the bag appeared in the dialysates, while at pigment concentrations in excess of 2 moles,

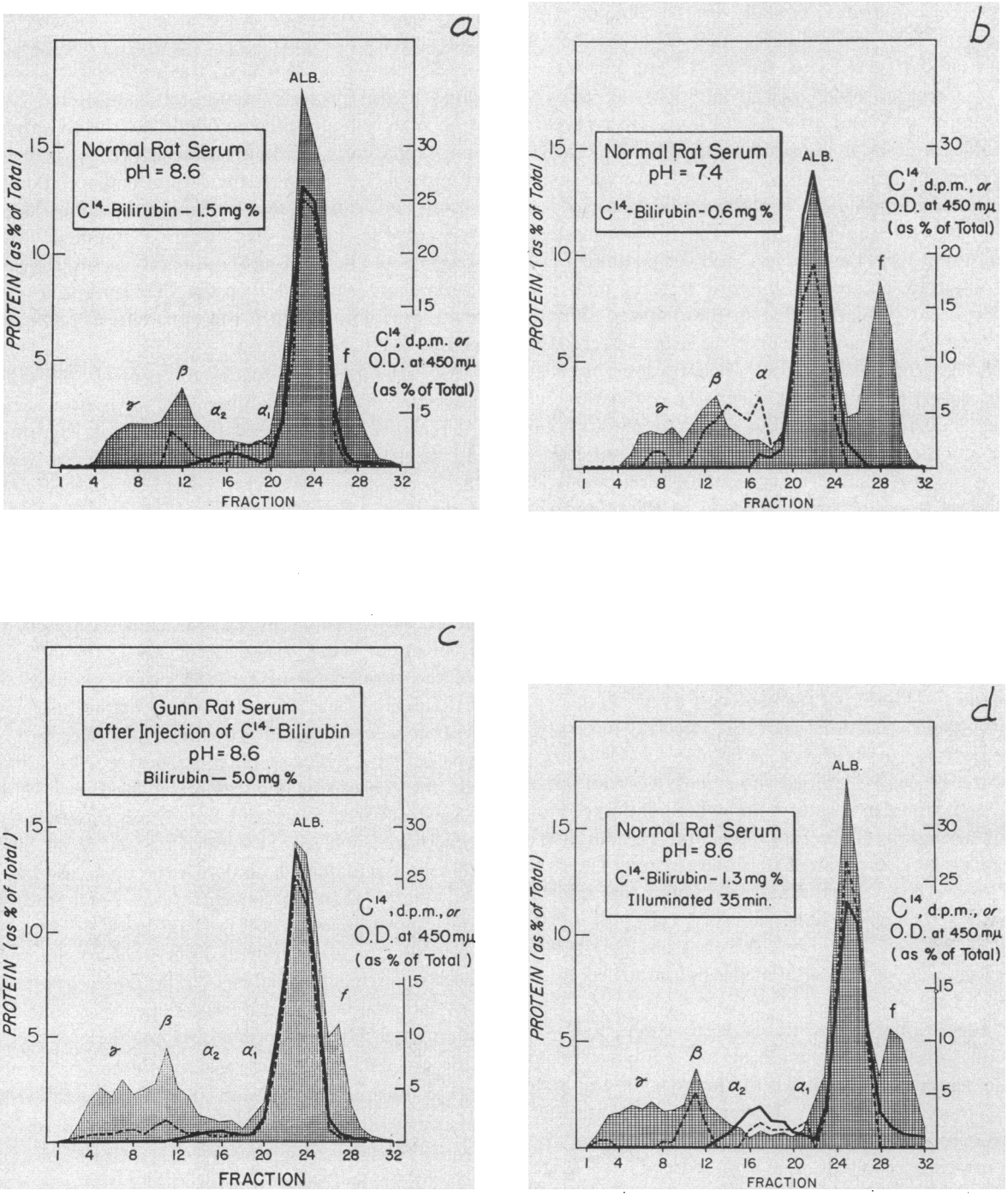
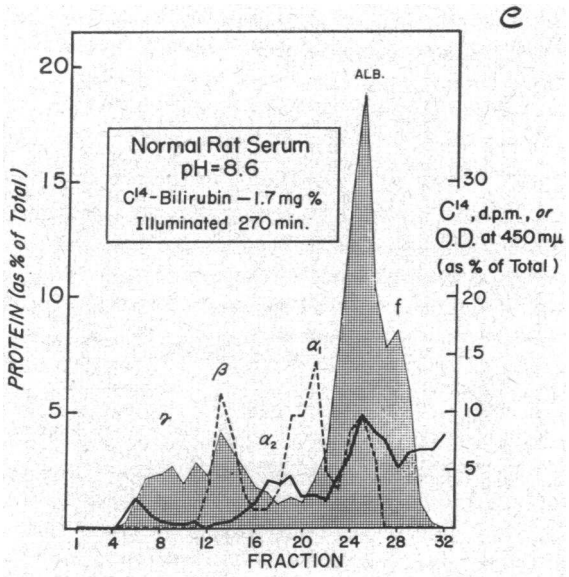


FIG. 3. CONTINUOUS-FLOW ELECTROPHORESIS OF RAT SERUM CONTAINING C^{14}-BILIRUBIN. Data are plotted as in Figure 1. In a, b, d, and e, the labeled pigment was added *in vitro;* in c, C^{14}-bilirubin was injected intravenously. In d and e, C^{14}-bilirubin had been subjected to partial photo-oxidative decomposition before its addition to serum.



(FIG. 3.—*(Continued)*.

much more radioactivity was dialyzable (Figure 5). In both instances, the dialysates were colorless and failed to exhibit positive diazo or Gmelin reactions. The extrapolated regression line obtained with the higher pigment concentrations intersected the abscissa at 1.95 moles of bilirubin per mole of albumin (Figure 5), a value not significantly different from the pigment-binding capacity determined by analysis of the contents of the dialyzing bag (Figure 4).

When rat serum was supplemented with labeled pigment previously subjected to increasing degrees of photo-oxidation, the pattern of dialyzable radioactivity was more complex (Figure 6). With up to one-fourth of the added pigment decomposed as determined by the diazo reaction, the percentage of dialyzable label did not differ significantly from that found with native bilirubin, indicating that small amounts of the oxidative derivatives are bound to protein. As the proportion of decomposed pigment further increased, the percentage of dialyzed label rose progressively until, with nearly all C¹⁴-bilirubin oxidized, more than 50% of the radioactivity escaped into the dialysates (Figure 6).

DISCUSSION

By the use of C¹⁴-bilirubin with high specific activity (23), the present study has demonstrated that, at pigment concentrations ranging from 0.8

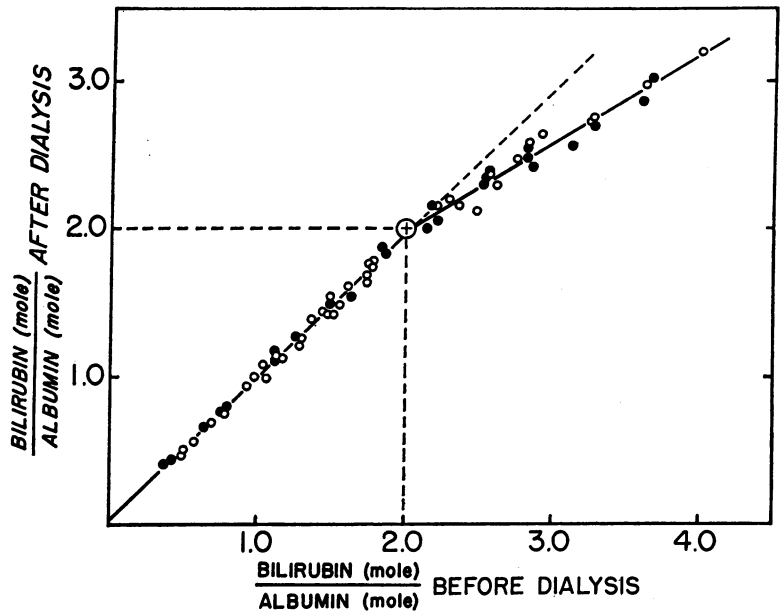


FIG. 4. DIALYSIS FOR 24 HOURS AT pH 8.6 OF HUMAN SERUM SUPPLEMENTED *in vitro* WITH C¹⁴-BILIRUBIN. The ratio of labeled bilirubin to albubin after dialysis is plotted against the initial pigment to albumin ratio. Open circles; normal serum; closed circles: hyperbilirubinemic serum from a patient with constitutional hepatic dysfunctions.

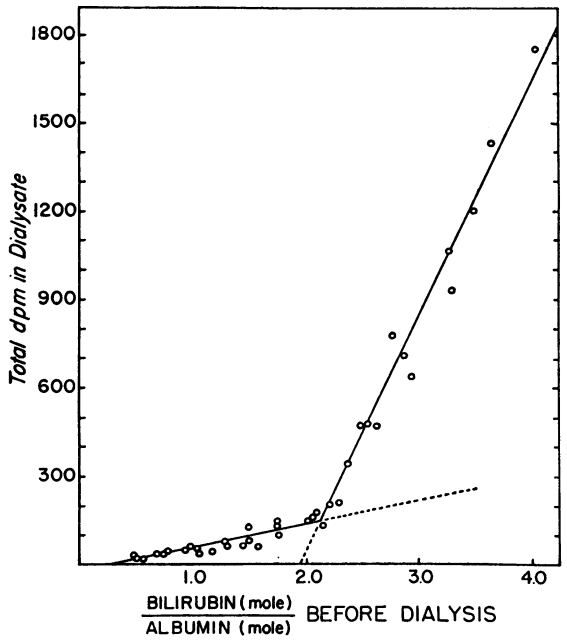


FIG. 5. DIALYSIS FOR 24 HOURS AT pH 8.6 OF NORMAL HUMAN SERUM SUPPLEMENTED *in vitro* WITH C^{14}-BILIRUBIN. Total radioactivity appearing in the dialysate is plotted against the initial ratio of C^{14}-bilirubin to albumin.

mg per 100 ml up to the binding capacity of serum protein, albumin is the only protein fraction in human serum that binds bilirubin (3, 6, 10, 11, 13). Similar observations were made with murine serum containing as little as 0.2 mg bilirubin per 100 ml. No evidence was found for the presence of globulin fractions possessing a transport function for bilirubin. Since similar patterns were obtained with continuous-flow electrophoresis at pH 8.6 and 7.4 and with starch-block electrophoresis, the absence of detectable binding of bilirubin to globulins could not be attributed to the pH, the type of buffer used, or the nature of the supporting medium employed for the protein fractionation. Moreover, it could not have resulted from the *in vitro* addition of C^{14}-bilirubin to the serum samples, since identical results were obtained after *in vivo* injection of labeled pigment into a human subject and into rats with congenital nonconjugated hyperbilirubinemia (Figures 1b, 3c).

In order to obtain optimal separation of the protein fractions, most electrophoretic analyses were performed at slightly alkaline pH, at which bilirubin is known to decompose progressively into yellow, water-soluble oxidation products that ultimately become colorless (23). Since these products do not exhibit positive Gmelin or diazo reactions, they remained undetected in previously reported studies. Recognition of these derivatives became of primary importance in the use of radioactive bilirubin because they were easily detected by the label and had to be distinguished by spectroscopic and chemical means from the labeled parent pigment. Oxidation of bilirubin is hastened by increased pH or temperature (47) and by exposure to light (23), but is retarded by attachment to protein (47) and by addition of antioxidants such as ascorbic acid (45). Therefore, all analytical and preparative procedures were carried out in the dark at 4° C, and except for the brief period between dissolving the C^{14}-bilirubin in 0.1 N NaOH and its addition to the serum samples, the pigment was not exposed to alkaline solutions unless bound to protein. Despite these precautions, deterioration of bilirubin could not be completely avoided, as manifested by a 4% decrease in the concentration of diazo-reacting pigment as compared with the radioactivity present, and by the appearance in the dialysates of traces of colorless, diazo-negative, labeled derivatives of bilirubin (Figure 5). On electrophoretic analysis, a small but variable amount of these oxidized pigment derivatives was often found in the α-globulins, located primarily between the α_1- and α_2-globulin peaks. The absolute amount of radioactivity in the α-globulin zone was roughly proportional to the total content of C^{14}-bilirubin in the serum; with human serum, it never exceeded 0.4% of the total label, whereas with murine serum, where decomposition of the added C^{14}-bilirubin was more marked, it ranged from 2 to 10%. In no instance did the globulin-bound material exhibit the spectral or chemical characteristics of bilirubin. These observations are inconsistent with the postulated existence of a specific protein fraction in the α-globulins that exhibits high affinity, but limited binding capacity for bilirubin.

The binding properties of these pigment derivatives were further elucidated by analysis of sera supplemented with partially photo-oxidized C^{14}-bilirubin. In these instances, a much larger fraction of the label appeared in the dialysates (Figure 6), and on electrophoretic analysis (Fig-

ures 1d, 3d, 3e), the proportion of radioactivity in the colorless, diazo- and Gmelin-negative α-globulin zone was greatly increased, especially with murine serum. Similar results were obtained with normal or hypoalbuminemic human serum with C^{14}-bilirubin added in amounts exceeding the binding capacity of the albumin present (Figure 1e). In these excessive concentrations, bilirubin appeared to be less stabilized and to suffer more extensive decomposition during dialysis and electrophoresis. The oxidative derivatives thus formed behaved electrophoretically like the pigment subjected to partial decomposition before its addition to the serum. In addition, however, a small portion of the excess bilirubin was found intact in the α-globulin fraction, as manifested by a weak diazo reaction in this zone. This suggested that α-globulins may transport bilirubin to a limited extent, but only in a secondary capacity when the primary binding sites on albumin have been fully saturated (6, 11, 13). The association between bilirubin and *purified* globulin fractions reported by others (8, 13, 15–17) may thus be explained, but within the range of bilirubin concentrations encountered under clinical circumstances, this is probably of no physiological significance.

The dialysis experiments revealed that with human serum at pH 8.6, 1 mole of albumin was capable of binding 2 moles of bilirubin (3, 10, 13, 15–17, 47) in such tight association that it became virtually nondialyzable (Figures 4, 5). This strong interaction with albumin also provided a high degree of protection against oxidation (47), as shown by the decrease during dialysis of the ratio of diazo-reacting pigment to radioactivity by only a few per cent. Nonetheless, in order to minimize decomposition of bilirubin, dialyses were usually terminated after 24 hours without an attempt to achieve complete equilibration of the two phases. Although this rendered impossible a mathematical approach to the binding equilibrium (48), the results demonstrated that with pigment concentrations below 2 moles per mole of albumin, the fraction of unbound or "free" bilirubin must be very small. Since dialysis was performed at pH 8.6 to assure pigment solubility (49), it could not be determined whether the molar binding ratio between albumin and bilirubin would be the same at physiologic

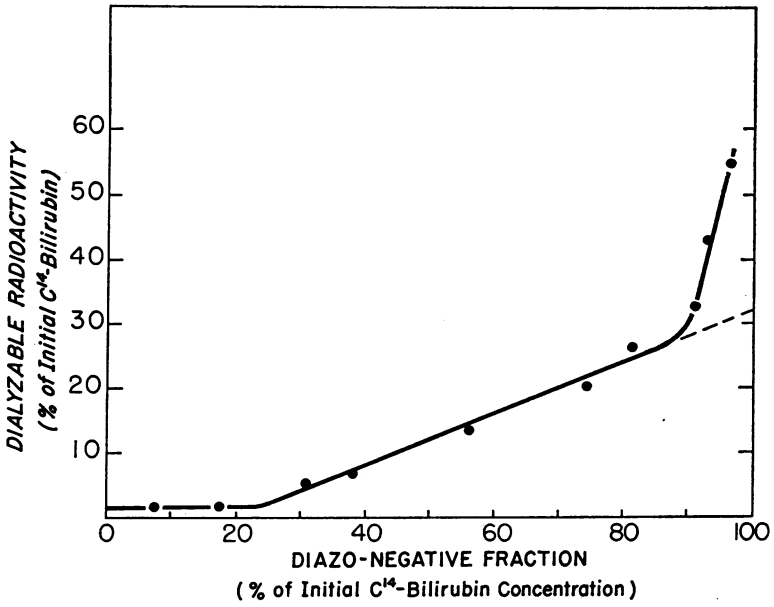


FIG. 6. DIALYSIS FOR 24 HOURS AT pH 8.6 OF NORMAL RAT SERUM, SUPPLEMENTED *in vitro* WITH C^{14}-LABELED PIGMENT. Dialyzable radioactivity is plotted against the diazo-negative fraction of the added C^{14}-pigment. The abscissa represents the photo-oxidized portion as percentage of the initially added 0.5 mg per 10 ml C^{14}-bilirubin. For methodologic details, see text.

pH. In comparable studies with bilirubin (13), however, and with other anions of similar nature (50), their interaction with albumin has been observed to be little affected by pH changes in this range.

Bilirubin in excess of 2 moles per mole of albumin appears to be rapidly converted to oxidation products, which in part remain attached to albumin and α-globulins and in part are lost into the dialysates (Figure 5). Structural differences in these pigment derivatives may be responsible for their different modes and sites of protein attachment; this may account for the complex electrophoretic (Figures 3 a, d, e) and dialytic (Figure 6) patterns observed as progressively larger proportions of the added C^{14}-bilirubin were subjected to prior decomposition. It is possible that the initially formed yellow, diazo-negative derivatives of bilirubin (23) partly maintain their affinity for albumin, whereas the colorless, more polar compounds obtained on further decomposition exhibit less protein interaction and associate preferentially with globulins. These observations also suggest that the spectral shifts observed in hyperbilirubinemic serum after addition of sulfasoxazole or salicylate (51), as well as the yellow, diazo-negative pigments that appear in the ultrafiltrates of these serum samples, may be the result of oxidation of bilirubin rather than of its displacement from binding proteins, as has already been suggested by Blondheim, Lathrop, and Zabriskie (52).

Conversion of bilirubin to water-soluble, diazo-negative derivatives exhibiting only weak protein interaction may also occur *in vivo*, but this appears to be of little physiologic significance except in instances of severe unconjugated hyperbilirubinemia (28). It may represent an alternate pathway of pigment disposition in Gunn rats (28), where bile and, to a lesser degree, urine have been shown to contain bilirubin metabolites with chemical, spectroscopic, and solubility properties (23) similar to those of the bilirubin derivatives appearing in the dialysates and in the electrophoretically separated α-globulin fraction.

Since albumin is the only serum protein possessing a transport function for bilirubin, and since 1 g of it is capable of binding tightly approximately 16 mg of pigment, the extravascular distribution (53, 54) and turnover (54, 55) of albumin may be important factors in determining the extent to which bilirubin can enter various tissues and organs. In the rat liver, however, the rates of hepatic uptake, conjugation, and excretion of bilirubin (34, 56) greatly exceed those of net uptake (57, 58) and catabolism (58–60) of albumin, and in man, bilirubin disappears from the plasma (61) far more rapidly than albumin (48, 54). Moreover, dissociation between transfer of bilirubin and albumin has previously been demonstrated in the placenta (62). These observations suggest that hepatic uptake of bilirubin may not be dependent on intracellular penetration and subsequent breakdown of the albumin to which the pigment is bound, but that, as with toluidine dyes (63), the bilirubin is transferred from albumin in the plasma or hepatic lymph to cellular acceptors (48). On the other hand, the measured uptake of albumin by the liver (57–60) may represent the *net* result of more rapid bidirectional fluxes (48, 57), so that the rate of albumin penetration into the hepatic cells may actually approximate that of bilirubin transfer. For these reasons, it cannot be decided at present whether bilirubin is dissociated from its binding site on excellular albumin before it enters the liver cells, or whether the albumin-pigment complex reaches the intracellular space intact, with subsequent rapid return of the depigmented albumin to the extracellular fluid. In either case, the kinetics of this transfer may not only determine the over-all rate of pigment clearance, but may also be responsible for differences in the average serum bilirubin level among various mammalian species (43). One may also speculate that the phylogenetic emergence of albumin (64), with its very high binding affinity for bilirubin, may have required compensatory development of a hepatic conjugating apparatus that maintains the binding equilibrium in favor of the liver and permits continued pigment excretion. Indeed, in lower animals with little or no serum protein resembling mammalian albumin (64), bilirubin appears to be excreted in unconjugated form irrespective of the presence or absence of a functioning apparatus for glucuronide formation in the liver (65). If future studies should support this hypothesis, it would require modification of present concepts (66) of the role of the liver in pigment metabolism.

SUMMARY

1. Serum from humans and rats supplemented *in vitro* with C^{14}-bilirubin was analyzed by continuous-flow and starch-block electrophoresis. Similar studies were performed with serum obtained after intravenous administration of labeled bilirubin, or of labeled, antibody-sensitized red cells.

2. In all instances, at pigment concentrations ranging from physiologic levels up to the binding capacity of serum protein, C^{14}-bilirubin migrated only with albumin.

3. With human serum containing up to 2 moles of C^{14}-bilirubin per mole of albumin, the pigment was virtually nondialyzable at pH 8.6, indicating nearly complete protein binding. At higher C^{14}-bilirubin concentrations, significant amounts of label appeared in the dialysates in the form of colorless, diazo-negative, water-soluble pigment derivatives.

APPENDIX

"Constitutional hepatic dysfunction" (Gilbert's disease). The first subject was a 29-year-old former marine who first was noticed to be jaundiced during a mild febrile illness at the age of 21. Since then, unconjugated hyperbilirubinemia, ranging from 1.5 to 3.0 mg per 100 ml, has persisted. No abnormalities have been uncovered by conventional liver function tests, cholecystogram, needle biopsy of the liver, hematological studies, and estimation of his ability to form aminophenyl glucuronide (26). At the time of the present studies, his serum bilirubin was 2.7 mg per 100 ml. The second patient was a 44-year-old physician in whom unconjugated hyperbilirubinemia was first detected at the age of 15 and whose serum bilirubin has subsequently ranged from 1.2 to 4.5 mg per 100 ml. Although his liver has been palpable, repeated liver function tests, cholecystogram, determination of red-cell survival time, and estimation of his ability to form aminophenyl glucuronide (26) have yielded persistently normal results. At the time of this investigation, his serum bilirubin concentration was 1.3 mg per 100 ml.

Acute hemolytic crisis. A 21-year-old woman with proven sickle-cell anemia was admitted to the hospital because of acute onset of severe joint pains, fever, weakness, and jaundice. When blood was obtained for study 4 hours later, her hematocrit was 21.5%, sickle cells were present in routine blood smears, and the serum bilirubin level was 3.6 mg per 100 ml, of which only 0.3 per 100 ml was direct-reacting.

Newborn infants. A 4.0-ml vol of cord blood was obtained from each of two, normal, full-term newborn infants without evidence of hemolytic or hepatic disorder. The serum was pooled for study.

Crigler-Najjar syndrome. This patient was a 6-year-old boy with severe congenital nonhemolytic jaundice whose nonconjugated hyperbilirubinemia ranged from 22 to 29 mg per 100 ml. Studies reported elsewhere (28) revealed absence of conjugated bilirubin in the bile and an enzymatic defect in glucuronide formation. After iv injection of 8.26 mg of C^{14}-bilirubin containing 4.3 μc radioactivity, equilibration of the administered pigment with his total miscible bilirubin pool was achieved in approximately 30 hours (28). For continuous-flow electrophoresis, blood was withdrawn 72 hours after injection of the label, and the serum was studied without further addition of labeled pigment.

Analbuminemia. This subject with congenital analbuminemia (29) had received iv injections of human albumin. In the sample of his serum used in the present study, however, immunological techniques (67) revealed an albumin concentration of less than 0.2 g per 100 ml, and the endogenous bilirubin level was below 0.1 mg per 100 ml.

Bis-albuminemia. Details of this patient have been published previously (30).

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