A CLINICAL METHOD FOR THE MICRODETERMINATION OF FREE AND PROTEIN-BOUND BILIRUBIN

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
MARTHA TALBOTT THOMAS
1971

~~JUL 27 1974~~ R13

~~JUL 8 1974~~ R10

~~JUL 27 1974~~ R10

~~JUL 17 1974~~

~~OCT 1 1978~~ 244

22 046

ABSTRACT

A CLINICAL METHOD FOR THE MICRODETERMINATION

OF FREE AND PROTEIN-BOUND BILIRUBIN

By

Martha Talbott Thomas

A thermoprecipitation method for the separation of free and protein-bound bilirubin has been developed. The validity of the separation has been established by comparing experimental results with predictions based on mass action law. The identity of the components measured has also been established by demonstration of their spectral absorbance characteristics. The data obtained on heat-precipitated specimens suggests that there is an average of two binding sites for bilirubin on each molecule of albumin. Heating hastens the loss of carbon dioxide from the plasma specimens so that by the time precipitation has occurred the pH usually rises to about 9.0 or more. Although the bilirubin binding of albumin shifts with pH changes, the stoichiometric relationships are stable, and no difficulty should be anticipated in deriving an appropriate correction factor for clinical use.

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Martha Talbott Thomas

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1971

ACKNOWLEDGEMENTS

I wish to express my appreciation and gratitude to Dr. A. E. Lewis, my major professor, for his support, counsel and advice throughout my course of study.

I thank Dr. Joan Mattson and Dr. Arthur Kohrman for serving on my committee and for their helpful comments and suggestions.

My thanks also go to the faculty, staff and fellow students of the Department of Pathology for their words of encouragement and assistance. I particularly wish to thank Dr. C. C. Morrill, Chairman of the Department of Pathology, for his continued moral support.

I am grateful for the financial assistance of the Allied Health Professions Advance Training Grant, AHT-69-049, which was available to me during the first part of my graduate study.

For Ebert,

Ralph, Meg and Gail

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INTRODUCTION

The purpose of this study was to investigate heat precipitation as a clinically useful means of separating free bilirubin from protein-bound bilirubin. To establish the validity of the proposed method, the data were tested to see if they were consistent with predictions based on the law of mass action.

The key problem in the management of neonatal jaundice is the determination of the time at which an exchange transfusion is necessary to forestall the development of kernicterus. Neurological damage in infants who survive severe damage in the neonatal period may result in mental deficiency, spasticity or nerve deafness. Exchange transfusion has been found to be effective in the prevention of bilirubin encephalopathy. Since exchange transfusion is not an innocuous procedure, the selection of the high risk infant who is in need of therapy is still a problem. The relationship between the incidence of brain damage and total serum bilirubin concentration has been established in hemolytic disease of the newborn. This relationship is less well defined in non-hemolytic jaundice.

The nature of the bile pigment as well as the total quantity responsible for the hyperbilirubinemia is of both diagnostic and prognostic significance. Bilirubin in the plasma can be separated into 2 forms; conjugated (direct) and non-conjugated (indirect). Both of these are carried by albumin. The non-conjugated form maintains an equilibrium between that fraction carried by albumin and a fraction free in the

plasma. Under normal circumstances the free fraction is very small, and no significant amounts appear outside the circulation. In cases of hyperbilirubinemia, however, the albumin binding sites for bilirubin may become saturated. If this occurs, a significant concentration of free bilirubin will develop. This form of bilirubin is diffusible and is highly cytotoxic. It is responsible for the development of kernicterus. As there are many variables affecting the binding capacity of the serum albumin, it may be more desirable to know the concentration of free bilirubin in a given serum than the total bilirubin concentration.

Formation of bilirubin:

When erythrocytes are destroyed in the reticuloendothelial system the released hemoglobin undergoes metabolic degradation. One of the products of this degradation is bilirubin. Oxidative cleavage of the protoporphyrin ring and removal of both the globin fraction and the iron yields a green pigment, biliverdin, which is subsequently reduced to bilirubin. Biliverdin is not normally present in human plasma.

Bilirubin transport:

The principal bile pigment entering the circulation from the reticuloendothelial tissue is bilirubin which is transported in the serum as a complex with albumin. This unconjugated bilirubin is almost insoluble in water and, as long as it is in complex with the albumin, is nontoxic. The unconjugated bilirubin is transferred from the plasma into the liver cell. Little is known about the mechanisms responsible for this transfer except that it is too rapid to be linked to albumin. Presumably, an exchange occurs at the lateral cell membrane facing the hepatic sinusoid; unconjugated bilirubin enters the

cytoplasm of the parenchymal liver cell and is bound by unidentified receptor molecules. The bilirubin is then conjugated with glucuronic acid in the endoplasmic reticulum. Conjugation converts bilirubin from a lipid-soluble aglycone to a water-soluble conjugate which can be excreted. In man the major conjugate formed is an ester glucuronide.

Bilirubin conjugation:

In the formation of bilirubin glucuronide, uridine metabolism is linked to carbohydrate metabolism. Adenosine triphosphate (ATP) is required as a source of high energy bonds in the initiation of the sequence. Uridine diphosphate (UDP) is converted to uridine triphosphate (UTP) by ATP in the presence of nucleoside diphosphokinase.

$$(1)\quad \text{ATP} + \text{UDP} \xrightleftharpoons{\text{nucleoside diphosphokinase}} \text{UTP} + \text{ADP}$$

UTP then reacts with glucose-1-phosphate to form UDP-glucose (UDPG) and pyrophosphate (PP). This step requires UDPG-pyrophosphorylase.

$$(2)\quad \text{UTP} + \text{glucose-1-phosphate} \xrightleftharpoons{\text{UDPG pyrophosphorylase}} \text{UDPG} + \text{PP}.$$

Subsequently, the UDPG undergoes oxidation to UDP glucuronic acid (UDPGA) in the presence of UDPG dehydrogenase which is found in the soluble fraction of the liver.

$$(3)\quad \text{UDPG} + 2\ \text{NAD}^{+} \xrightarrow{\text{UDPG dehydrogenase}} \text{UDPGA} + 2\ \text{NADH} + 2\ \text{H}^{+}$$

The final step, which is rate limiting, involves the enzymatic transfer of glucuronic acid from UDPGA to bilirubin. This reaction is catalyzed by glucuronyl transferase, an enzyme found in the microsomal fraction of liver homogenates.

$$(4)\quad \text{UDPGA} + \text{bilirubin} \xrightarrow{\text{glucuronyl transferase}} \text{bilirubin diglucuronide} + \text{UDP}$$

The UDP which accompanies glucuronide formation reenters the cycle by phosphorylation to UTP. Since the glucuronic acid used in glucuronide biosynthesis is derived from glucose, not from exogenous glucuronic acid, this carbohydrate must be available for the proper functioning of the system.

Bilirubin excretion:

Bilirubin diglucuronide is subsequently transferred from the cytoplasm of the liver cell into the bile. Almost all of the bilirubin present in mammalian bile is conjugated. These conjugates are subsequently hydrolyzed in the intestine. Bilirubin is partially reabsorbed in the enterohepatic circulation but primarily undergoes a series of reductions to colorless compounds called urobilinogens. A portion of these is reabsorbed into the enterohepatic circulation and re-excreted by the liver. Normally a small fraction is excreted in the urine.

The metabolism of the bilirubin produced by the fetus is not fully understood. However, it is evident that the fetus depends to a large extent on active transplacental transport for removal of bilirubin. Bilirubin obviously does not diffuse freely in both directions across the placental membranes since jaundiced mothers do not produce jaundiced infants.

Conjugation and transport of bilirubin in the newborn:

Bilirubin conjugation in the newborn is less efficient than in the adult. Glucuronyl transferase is much less active in the livers of the newborn than in adults. In addition to the decreased activity of

glucuronyl transferase, the amount of UDP glucuronic acid is decreased. This is a reflection of the limited activity of UDPG dehydrogenase which is necessary for the formation of UDP glucuronic acid. Interestingly, the activity of UDPG pyrophosphorylase is only slightly decreased in the liver of the newborn as compared with the adult liver. This enzyme, however, is also essential in glycogen synthesis. Since the enzyme mechanisms in the liver for the formation of glucuronide are not fully developed at birth, the infant is predisposed to some degree of jaundice. These functions can be depressed further by limitations in related areas of metabolism, such as carbohydrate metabolism, which might determine the availability of glycogen for the formation of UDPG. Such factors can influence the actual degree of jaundice in any one infant.

Even under optimal circumstances the hepatic ability of the newborn to conjugate bilirubin seems to be limited to the physiologic load imposed by normal degradation of hemoglobin. With these limitations even a small increase in the amount of the pigment presented to the liver will result in hyperbilirubinemia. The hepatic capacity to metabolize bilirubin during the first few days of life increases rapidly. Just as the appearance of jaundice on the second or third day indicates a failure to conjugate a normal load of bilirubin, the disappearance of that jaundice after about the third day is an indication that the infant is not only clearing the load presented by daily hemoglobin degradation but is also metabolizing the accumulated bilirubin. In many premature infants there is an increasing accumulation of bilirubin beyond the third day. This usually indicates slower development of the hepatic capacity for conjugation.

The amount of bilirubin which can be transported in the blood by albumin is subject to limitations, the first of which is the concentration of albumin itself. If the serum albumin concentration is low, as in prematurity, the albumin becomes saturated with bilirubin at lower pigment concentrations. The binding capacity of albumin for bilirubin is decreased by other organic anions such as salicylates, sulfa drugs and fatty acids, which are either bound by albumin at the same locus as bilirubin or which alter the ability of this locus to bind bilirubin. Albumin binding of bilirubin shows a marked pH sensitivity. Acidosis increases the unbound bilirubin concentration both in serum and tissues.

If the albumin-binding capacity of the blood is exceeded, free diffusible bilirubin increases. This form, which freely permeates cell membranes, is cytotoxic and is the pigment which causes kernicterus or staining of the nuclei of the basal ganglia, the hypothalamus and the floor of the fourth ventricle. Other tissues are also affected. Necrosis of testicular epithelium and the islets of Langerhans has been observed in severely jaundiced infants. Characteristic papillary necrosis or "bilirubin infarcts" in the kidney have also been described. Bilirubin crystals have been found in many tissue cells.

Bilirubin toxicity:

Bilirubin exerts its toxic effects on cells by the uncoupling of oxidative phosphorylation. It also inhibits the synthesis of heme. The mode of inhibition is not yet clearly understood but this activity may be very important in explaining the effect of bilirubin on cerebral tissue. Heme is a prosthetic group of many enzymes which are necessary for biologic oxidation. Any interference in carrying out these processes could readily lead to cellular damage, particularly in areas which are sensitive to mild anoxia.

Very little is known about the factors which determine the distribution of bilirubin in the tissues. Adipose tissue seems to have a relatively high affinity for unconjugated bilirubin which is lipid soluble. A large quantity of adipose tissue in the newborn may influence the distribution of the pigment, the manifestation of jaundice, the plasma level and perhaps may also protect cerebral tissue from the toxic effect of bilirubin. It is not known what determines cell susceptibility. However, if cells which exhibit higher metabolic activity have higher requirements in terms of oxygen availability, there may be a correlation between the commonly involved areas of the brain and the susceptibility to damage. The areas involved are phylogenetically older regions which probably begin to function earlier than the cortex. Therefore, they may be expected to have greater metabolic demands at the time of birth. On this basis they could be more susceptible to injury by an agent which uncouples oxidative phosphorylation.

There is extensive evidence for the existence of a blood-brain barrier which excludes bilirubin. Many other tissues stain when the concentration of plasma bilirubin is only moderate but brain tissue does not usually stain except at high levels of bilirubin. There is a specific difference in the readiness with which bilirubin crosses the barrier in the newborn as compared with the adult. The occurrence of kernicterus in the newborn is evidence that the blood-brain barrier, at least for bilirubin, is less efficient at birth than later in life. As there is no good evidence about the age at which the blood-brain barrier actually matures in the newborn, the time when the danger of bilirubin encephalopathy has passed is not known.

Kernicterus is most commonly associated with erythroblastosis fetalis but may develop as a result of other forms of hemolytic anemia in the newborn. It has also been described in nonhemolytic conditions which are associated with severe neonatal jaundice, such as sepsis or the Crigler-Najjar syndrome. In the absence of hemolytic or hepatocellular disease, kernicterus is rare in the normal full-term infant in contrast to its incidence in the premature.

Relationship of prematurity to kernicterus:

The premature infant is more susceptible to kernicterus than the full-term infant for a number of reasons. Severe hyperbilirubinemia may result from slow accumulation of bilirubin in the blood. This is associated with delayed development in the conjugating capacity of the liver which is common in prematurity. The premature infant is more likely to have a low serum albumin level. He has less adipose tissue to serve as a protective buffer. The most vulnerable premature infant is the one with respiratory distress. Hypoxia, through its interference with cell metabolism, could contribute to a further delay in maturation of hepatic function. More directly, the decrease in pH which accompanies respiratory distress decreases the affinity of albumin for bilirubin and increases vascular permeability. Under these circumstances the propensity for bilirubin to move into the tissues is increased. An increased incidence of kernicterus in prematurity has also been traced to iatrogenic factors stemming from practices in the management of the premature infant. High doses of some vitamin K preparations and sulfonamides have been shown to increase the movement of bilirubin into the tissues.

The number of variables involved in the development of kernicterus, particularly in the premature infant, is high. Therefore, the arbitrary allowable limit of 20 mg./dl. of total bilirubin, below which damage will not occur in the average infant, may not reflect the needs of the individual. The concentration of free diffusible bilirubin is much more pertinent.

LITERATURE REVIEW

The state of unconjugated bilirubin in serum has been studied by many investigators using various techniques. *In vitro* studies have demonstrated the cytotoxicity of bilirubin to such fundamental cellular processes as cell respiration (Day, 1954), oxidative phosphorylation (Zetterstrom, 1956) and electron transport (Bowen, 1958). The toxic concentration of bilirubin in these studies was about 20 mg./dl., a plasma concentration associated at times with kernicterus. As these experiments were all conducted with either cell fragments or isolated cells, only limited conclusions can be drawn about the relationship of this concentration to the level at which toxicity appears. Since kernicterus has been observed in infants with serum bilirubin concentrations considerably below 20 mg./dl. and concentrations above this level have been innocuous, it is apparent that factors other than plasma concentration alone are important.

Silverman *et al.* (1956) and Harris *et al.* (1958) noted that premature infants treated with sulfisoxazole developed kernicterus six times more frequently than comparable control infants but that the serum bilirubin concentrations were higher in the unaffected control infants. Odell (1958) demonstrated the displacement of bilirubin from albumin by sulfonamides *in vitro*. On the basis of results obtained in further investigations (1959a, 1959b) Odell postulated that small concentrations of free bilirubin exist in extracellular fluids and that such concentrations can be increased independently of the total pigment

concentrations in the blood plasma. He suggested that the high incidence of kernicterus in premature infants who had been given sulfisoxazole occurred as a sequel to the action of this drug in displacing bilirubin from albumin. In this same group of experiments Odell showed that salicylates and caffeine sodium benzoate displace bilirubin even more readily than sulfa drugs. Josephson and Furst (1966), after a further exploration of the effect of sulfa drugs on bilirubin, stated that free bilirubin appears at a total bilirubin concentration of 20 mg./dl. only if the sulfa level is 25 mg./dl. or more. They suggested that the serum concentration of sulfonamide which might jeopardize a premature infant for kernicterus is above 15 mg./dl.

Stern and Denton (1965) reported six cases of kernicterus in premature infants with no common history of medication. These infants developed only moderate bilirubin levels. Their only common feature was respiratory acidosis. According to Odell (1965) any decrease in pH promotes the dissociation of the bilirubin-albumin complex.

Subsequent research has been directed for the most part into clarification of the relationship between bilirubin and albumin in both cellular and acellular environments. Kaufmann *et al.* (1967) measured the uptake of bilirubin from serum by washed red cells. The uptake increased sharply at a bilirubin-albumin molar ratio of one. This corresponds to a bilirubin concentration of 24.8-26.4 mg./dl. in an infant with 3.1 gm./dl. of albumin. This is fairly close to the critical concentration of 20 mg./dl. above which exchange transfusion is indicated in erythroblastotic infants. It is even closer to the 28-30 mg./dl. criterion for exchange transfusion in infants with idiopathic hyperbilirubinemia. These results are in complete accord with those of Odell (1966) who found in mitochondrial suspensions that the distribution

of bilirubin is confined primarily to the albumin space until the bilirubin-albumin molar ratio exceeds one.

The first report of the application of Sephadex gel filtration to the diagnosis of neonatal jaundice was made by Jirsova *et al.* (1967). Seventeen patients were studied, five of whom were in poor clinical condition. Visible bilirubin remained on the column only with the latter group and it was proposed that this method might be of value for improvement of clinical diagnosis. Kaufmann and his associates (1969) adsorbed bilirubin from artificially jaundiced sera using specially prepared small columns of Sephadex. The amount of free bilirubin adsorbed from sera with bilirubin-albumin molar ratios between 1 and 2 was linearly related to the total bilirubin concentration of the serum. They were unable to demonstrate adsorption of bilirubin by the column at ratios below 1. They suggested that the use of Sephadex columns to separate bilirubin might aid in determining the time when exchange transfusions should be performed to prevent kernicterus.

Starinsky and Shafrir (1970) used Sephadex to investigate the interaction of bilirubin, linoleate and human plasma albumin. Their purpose was to evaluate the importance of free fatty acid competition with bilirubin in neonatal hyperbilirubinemia. They found that bilirubin is only displaced from albumin by concentrations of free fatty acids that far exceed the physiological levels seen in neonates. According to Chunga and Lardinois (1971), who combined a Sephadex extraction technique with an ultramicro diazo reaction to measure free bilirubin, a decrease in pH not only decreases the binding of bilirubin by albumin but it also unbinds bilirubin already attached to albumin.

They observed that the unbinding occurred in proportion to the degree of acidosis. Zamet and Chunga (1971) using this method were unable to demonstrate any correlation between free and total bilirubin levels in jaundiced newborns.

Variations exist in the estimates of the number of bilirubin molecules bound by 1 albumin molecule. The quantitative data of Martin (1949) suggest that the composition of the complex is of the order of 3 moles of bilirubin to 1 mole of purified albumin. Martin also examined a mixture of bilirubin and albumin in a 2:1 molar ratio over a pH range of 8-3.5 and noted a gradual dissociation as the pH decreased. The bilirubin began to precipitate near a pH of 4.5. Watson (1962) found a 3.3:1 ratio using purified human albumin and a pH range of 7.25-8.85. He stated that it was impossible to dialyze free bilirubin unless a 3:1 ratio were exceeded. Ostrow and Schmid (1963), dialyzing isotopically labelled bilirubin from human and murine serum, found 2 moles of pigment to bind tightly to 1 mole of albumin. Schmid and associates subsequently (1965) used Sephadex gel filtration to separate albumin-bound bilirubin from unbound pigment at a pH of 8.3. Their results indicated that bilirubin applied in excess of 2 moles per mole of albumin remained on the column, whereas with ratios of less than 2:1, virtually all the pigment appeared in the effluent bound to the protein. When bilirubin-albumin ratios were just less than 2:1, traces of unbound pigment were detectable on the column but the amounts were too small for them to measure. Rutkowski (1967) demonstrated that calcium carbonate selectively adsorbs unbound bilirubin from bilirubin-albumin solutions. Using this method with purified albumin, he found a molecular binding ratio of 3:1. He found variable but consistently higher ratios with serum (4.1-5.7) than he found with purified albumin. However,

these estimates were made using dilute solutions and he reported that this binding capacity decreased dramatically when the concentration was increased, perhaps as a result of increased steric effects. Keenan *et al.* (1969) used Sephadex columns to study the binding characteristics of albumin and bilirubin in pooled cord sera, sera of individual infants, adult sera and albumin solutions. Infant sera bound significantly less bilirubin (0.9:1) than adult sera (1.12:1) or albumin solution (1.5:1) at pH's slightly less than physiologic pH. Jacobsen (1969), reporting on an indirect kinetic method for quantitative determination of unbound bilirubin, found that 3 moles of bilirubin bind reversibly to 1 mole of purified albumin. He demonstrated 2 classes of binding sites for bilirubin, each with a characteristic dissociation constant. In the first class 1 mole of bilirubin is firmly bound to 1 mole of albumin with a dissociation constant of 7×10^{-9} M. Two moles are bound less strongly in the second class. The dissociation constant of this secondary binding is 2×10^{-6} M. Jacobsen and Fedders (1970) subsequently reported on a similar series of studies done on normal cord serum, normal adult serum and serum from exchange transfusions of icteric newborns. They concluded that the concentration of free bilirubin in serum is extremely low as long as the first binding site of the albumin is not fully occupied. However, it increases sharply when this site is saturated. In the icteric newborns the capacity of the first site seemed much lower than expected from the total concentration of albumin.

The nature of anion binding to plasma proteins has been the subject of many studies. Most attention has been focussed on the binding by albumin of various indicator dyes. According to Blondheim (1955) the dye-binding capacity of serum varies directly with the albumin

concentration except in cases of jaundice. He found a difference in dye-binding behavior between whole serum as opposed to dilutions of serum. Whole serum, below a critical albumin concentration of about 1.5 gm./dl., did not bind dye to a significant extent under the conditions of his experiment whereas diluted serum bound dye in the proportion expected from the dilution. Serum, whose albumin content was above 1.5 gm./dl., bound dye only in proportion to that amount of albumin which was in excess of that concentration. This critical concentration may represent that amount of albumin whose total binding sites are equal in number to those bound to naturally-occurring substances such as bilirubin, carotene, vitamins, bile acids and fatty acids. It may also reflect the presence of an albumin of very low binding capacity present in the serum of both patients and normal subjects.

Waters (1967) measured both the total bilirubin and the phenolsulfonphthalein-binding capacity in serum from 150 hyperbilirubinemic infants. He found neurological damage in these infants to be associated only with sera which met two criteria: viz., a bilirubin level above 20 mg./dl., and a dye-binding capacity of less than 50 µg./ml. Lucey *et al.* (1967) in a similar study were unable to demonstrate any difference between the dye-binding capacity of infants with neurological damage and those without. They concluded that phenolsulfonphthalein and bilirubin act competitively rather than in an additive fashion and that the method was of doubtful value.

Porter and Waters (1966) determined the binding capacity of albumin in human serum for both 2-(4'-hydroxy-benzene-azo)-benzoic acid (HBABA) and bromphenol blue. They concluded that the reserve binding capacity

for these dyes may be a better criterion for exchange transfusion than the concentration of total unconjugated bilirubin. Applegarth *et al.* (1970) reported that the HBABA-binding capacity of sera from newborn infants compared well with the theoretical unbound albumin concentration which was calculated for each infant from both the minimum and maximum published binding ratios of bilirubin and albumin.

Bjerrum (1968) used gel filtration to investigate the binding of bromphenol blue to bovine and human serum albumin at a physiological pH. His experiments showed the binding to be divided into 2 classes, each having its own binding constant. By competition with bilirubin bound to albumin before the dye-binding experiment, he demonstrated that 1 mole of bilirubin is bound in each of the 2 classes.

Odell *et al.* (1969) described a method for estimating the relative concentration of free bilirubin in serum by measurement of the saturation of albumin for bilirubin. He used salicylate as a competitor to determine the amount of bilirubin displaced. Since the association constants of salicylate and bilirubin for albumin are of the same order of magnitude, the amount of bilirubin displaced is proportional to the initial saturation of albumin with bilirubin. In his study infants without hemolytic disease showed a direct correlation between the saturation of their albumin with bilirubin and the bilirubin-protein concentration ratio of their serum. Infants with hemolytic disease did not show such a correlation until after they had had an exchange transfusion. This was probably due to competition for albumin binding sites by hematin.

General discussions of bilirubin metabolism may be found in summaries listed in the bibliography by Brown (1962), Claireaux (1960), Sunderman (1967) and With (1968).

MATERIALS AND METHODS

Source of Specimens

Experimental model sera were prepared as follows: 25 mg. of bilirubin* was dissolved in 1.0 ml. of 0.1 M Na_2CO_3 and 0.75 ml. of 0.1 N NaOH and diluted to 50 ml. with pooled normal serum. Dilutions were subsequently made from this stock solution, using pooled normal serum as the diluent, to cover a range of 10 mg./dl.-50 mg./dl. All procedures were carried out in subdued light.

One and five-tenths milliliter aliquots of each dilution were placed in 12 x 75 mm. tubes which were stoppered tightly and placed in a boiling water bath for 1.5 minutes. After quick cooling in a cold water bath, 1.5 ml. of M/15 phosphate buffer, pH 7.4, was added. Each clot was thoroughly broken up and mixed with the buffer by means of specially prepared, footed, stirring rods and a vortex mixer. All tubes were spun at 34,000 rpm for a minimum of 5 minutes. The supernatant extracts were then decanted into clean tubes.

Bilirubin

Total bilirubin analysis of the extracts was done according to the ultramicro method of Chunga and Lardinois (1971) with two modifications. In order to use glassware most readily obtainable in our laboratory at this time all volumes were doubled. Caffeine sodium benzoate was

*Matheson, Coleman and Bell, Norwood, Ohio.

substituted for diphylline as the activating agent in the analysis. Caffeine is less soluble than diphylline and a slight turbidity has been reported to develop occasionally when caffeine is used (Michaelson, 1961). We did not encounter this problem in the course of the experiments.

Bilirubin and diazobenzene-p-sulfonic acid yield a colored coupling product which is red in acid and neutral solutions and blue in alkaline solutions. Unconjugated bilirubin acts very slowly with the diazo reagent unless an accelerating substance such as caffeine sodium benzoate is added. Ascorbic acid is used to destroy the diazo reagent. It also eliminates the effect of any hemolysis in the sample by stabilizing the azo product. The red azo pigment is then converted into blue by alkalinization with Fehling II reagent.

Method. Five-tenths milliliter of extract is added to 0.4 ml. of caffeine sodium benzoate. Two-tenths milliliter of diazo reagent is then added and the reaction allowed to proceed for 10 minutes. At this time 0.02 ml. of ascorbic acid is added. This is followed promptly by the addition of 0.3 ml. of Fehling II solution. The resultant color is stable for 1 hour. It is necessary to prepare a blank for each tube. Blanks are required with this method because a colored reaction product results from the combination of ascorbic acid and the diazo reagent. Blank tubes differ from the unknowns only in that the extract is not added until after the ascorbic acid.

The spectrophotometric absorption of the samples was measured at 600 nm. in 100 µl. silica microcells using a Beckman DU spectrophotometer*

*Beckman Instruments, Palo Alto, California.

fitted with a micro aperture plate. The total bilirubin of each was calculated mathematically from the equation for the standard curve and multiplied by 2 to compensate for the doubling of volume by buffer.

Standardization. A standard bilirubin curve was prepared for use in this experiment. Versatol-Pediatric,* with a known bilirubin concentration of 20.4 mg./dl., was mixed with human serum albumin, 5 gm./dl. in pH 7.4 phosphate buffer to give dilutions ranging from 1:10-1:200. Total bilirubin analysis of each dilution was made using the method described. The equation for the line was calculated by the method of least squares and subsequently used to calculate the bilirubin concentration of the extracts.

Precision studies. The precision of the Chunga and Lardinois method was estimated on two different concentrations of bilirubin in the normal range and the 95% confidence limits of the standard deviations calculated by application of the Chi square distribution. A precision study was also made on the extraction technique itself using paired specimens taken from bilirubin-serum mixtures which ranged from 12-50 mg./dl. of total bilirubin.

Total Protein

Total protein concentrations of the extracts were determined using the method of Lowry *et al.* (1951). This method is based on a preliminary interaction of protein and Cu^{++} in alkaline solution followed by the

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*Commercial control serum from General Diagnostics Division, Warner-Chilcott, Morris Plains, New Jersey.

reduction of phosphotungstic and phosphomolybdic acids to molybdenum blue and tungsten blue. Both the copper-protein complex and the tyrosine and tryptophan of the protein participate in this reduction. The method is sensitive to very small concentrations of protein.

Method. The details for making reagents A, B, C and E are described in the Appendix. One milliliter of Reagent C is added to 0.2 ml. of extract, diluted 1:10 in phosphate buffer, pH 7.4. The tubes are mixed and allowed to stand for 10 minutes at room temperature. One-tenth milliliter of Reagent E is added rapidly and mixed immediately using a vortex mixer. After a minimum of 30 minutes the samples are read in a Beckman DU spectrophotometer at 750 nm.

The protein concentrations were calculated from a standard curve previously prepared in our laboratory from bovine albumin.

Spectral Absorbance Curves

A spectral absorbance curve over a range of 400-475 nm. was done on an aliquot of pooled extract. Another curve was done on the same extract to which one drop of human albumin was added. All readings were made in 100 µl. silica microcells using a Beckman DU spectrophotometer. pH 7.4 phosphate buffer was used as the blank.

Total Protein and Albumin

The total protein and albumin concentrations of the pooled serum were determined by the biuret method using 28% sodium sulfite as the globulin precipitant. The biuret reaction is given by all compounds with two or more amide or peptide bonds linked directly or through an intermediate carbon atom.

Method. Four-tenths milliliter of serum is added to 9.6 ml. of sodium sulfite and mixed. Two milliliters of this mixture is added to 5 ml. of biuret reagent for the total protein analysis. Three milliliters of anesthetic ether is then added to the remainder. The tubes are stoppered, shaken 40 times in 20 seconds and centrifuged for 5 minutes. Two milliliters of the resulting subnatant are mixed with 5 ml. of biuret reagent for albumin analysis. Color development is complete in 10 minutes.

Readings were made on a spectrophotometer against a reagent blank at 545 nm. using Monitrol I as a standard and Monitrol II* as a control.

Calculations:

$$\text{Albumin (gm./dl.)} = \frac{(\text{Absorbance of x})(\text{Albumin conc. of std.})}{\text{Absorbance of std.}}$$

pH Measurements

pH measurements of the mixtures of bilirubin and pooled serum were made using a Beckman Expandomatic pH meter.**

The results obtained are listed in the following tables.

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*Normal and abnormal commercial control sera from American Monitor Corporation, Indianapolis, Indiana.

**Beckman Instruments, Palo Alto, California.

Table 1. Standard curve for bilirubin using dilutions of Versatol-Pediatric and the ultramicro method of Chunga and Lardinois

Dilution	Concentration in mg./dl.	Absorbance
1:10	2.04	0.720
1:10	2.04	0.740
1:15	1.53	0.522
1:20	1.02	0.355
1:20	1.02	0.402
1:40	0.51	0.194
1:40	0.51	0.189
1:100	0.205	0.089
1:200	0.102	0.051

$Y = 2.892\ x\ -0.0531$

SEE = 0.0384

95% confidence limits of s.: 0.0254-0.0781

Table 2. Precision studies performed on the bilirubin method of Chunga and Lardinois

Pool	Sample	Absorbance	Concentration in mg./dl.
1	1	0.195	0.511
	2	0.197	0.517
	3	0.193	0.505
	4	0.192	0.502
	5	0.196	0.514
	6	0.192	0.502
	7	0.190	0.496
	8	0.189	0.493
	9	0.192	0.502
	10	0.185	0.482

$\bar{x}$ = 0.502

s = 0.0097

95% confidence limits for s.: 0.0067-0.0177

Table 2--cont'd.

Specimen	Sample	Absorbance	Concentration in mg./dl.
2	1	0.306	0.832
	2	0.311	0.846
	3	0.308	0.838
	4	0.310	0.846
	5	0.305	0.829
	6	0.309	0.841
	7	0.307	0.835
	8	0.305	0.829
	9	0.306	0.832
	10	0.305	0.829

$\bar{x}$ = 0.836

s = 0.0042

95% confidence limits for s.: 0.0029-0.0077

Table 3. Microdetermination of total protein of 1:10 dilutions of extracts by the Lowry method

Sample	Absorbance	Concentration in µg./ml.	Concentration in gm./dl.*
1	0.435	157	0.157
2	0.384	137	0.137

*Corrected for dilution

Table 4. Determination of the total protein and albumin concentrations of the serum pool by salt fractionation and the biuret method using Monitrol I as the standard and Monitrol II as the control

	Assay Values	
	Total Protein in gm./dl.	Albumin (by electrophoresis) in gm./dl.
Monitrol I	6.90	4.04
Monitrol II	5.75	3.40

Sample	%T.	Absorbance	Concentration in gm./dl.
Total protein			
Monitrol I	46.5	0.332	
	46.5	0.332	6.90
Monitrol II	53.0	0.276	
	53.0	0.276	5.74
Serum Pool	47.5	0.323	
	47.0	0.328	6.77
Albumin			
Monitrol I	70.0	0.155	
	69.0	0.161	4.04
Monitrol II	73.5	0.134	
	74.0	0.131	3.35
Serum Pool	69.0	0.161	
	68.5	0.164	4.15

Table 5. pH measurements of pooled serum bilirubin mixtures

Sample	Bilirubin conc. in mg./dl.	pH
1	10	8.45
2	20	8.80
3	30	8.90
4	40	9.15
5	50	9.10

Table 6. Results of heat precipitation studies, Series I, using bilirubin mixtures from 10-50 mg./dl.

Total Bilirubin		Free Bilirubin
mg./dl.	M./l. x 10^{-4}	M./l. x 10^{-4}
50	8.547	0.252
		0.204
		0.254
		0.224
		0.214
45	7.692	0.195
		0.185
		0.185
40	6.838	0.114
		0.105
		0.156
		0.105
35	5.983	0.091
		0.115
		0.101
		0.088
30	5.128	0.086
		0.078
		0.078
		0.078
25	4.274	0.068
		0.050
		0.046
20	3.418	0.050
		0.056
		0.044
		0.062
		0.039
		0.029
		0.049

Table 6--cont'd.

Total Bilirubin mg./dl.	Total Bilirubin M./l. x 10^{-4}	Free Bilirubin M./l. x 10^{-4}
15	2.564	0.043
		0.056
		0.023
10	1.709	0.034
		0.023
		0.025
		0.021
		0.017

Table 7. Results of heat precipitation studies, Series II, using bilirubin mixtures from 12-28 mg./dl.

Total Bilirubin		Free Bilirubin
mg./dl.	M./l. x 10^{-4}	M./l. x 10^{-4}
12	2.051	0.044
		0.027
		0.021
		0.036
		0.029
		0.024
14	2.393	0.039
		0.031
		0.027
		0.031
		0.024
16	2.735	0.040
		0.051
		0.037
		0.033
18	3.076	0.046
		0.038
20	3.418	0.050
		0.056
22	3.760	0.041
		0.066
		0.078
		0.035
24	4.102	0.052
		0.060
		0.079
		0.066
26	4.444	0.092
		0.075
		0.066
		0.069
28	4.786	0.081
		0.071
		0.102
		0.107

Table 8. Results of precision studies using duplicate extracts from bilirubin-serum mixtures

Sample #	Total Bilirubin mg./dl.	Free Bilirubin mg./dl.		Difference
		I	II	
1	12	0.1714	0.1426	0.0288
2	14	0.2292	0.1830	0.0462
3	16	0.2178	0.1946	0.0232
4	20	0.2928	0.3276	0.0348
5	22	0.2408	0.3854	0.1446
6	24	0.3044	0.3508	0.0464
7	26	0.3854	0.4028	0.0174
8	28	0.4722	0.4144	0.0578
9	32	0.5474	0.4318	0.1156
10	34	0.6862	0.6110	0.0757
11	35	0.6400	0.5474	0.0926
12	35	0.6342	0.5936	0.0406
13	40	0.7324	0.6168	0.1156
14	45	1.0622	1.2300	0.1678
15	50	1.4728	1.1952	0.2776
16	45	1.1432	1.0796	0.0636
17	40	0.6688	0.6168	0.0520
18	35	0.5300	0.6746	0.1446

Table 8--cont'd.

Sample #	Total Bilirubin mg./dl.	Free Bilirubin mg./dl. I	Free Bilirubin mg./dl. II	Difference
19	30	0.5012	0.4548	0.0464
20	25	0.3970	0.2928	0.1042
21	20	0.2582	0.4606	0.2024
22	15	0.2524	0.3276	0.0752

$$s = \sqrt{\Sigma d^2/2n}$$

$$= \sqrt{\frac{0.266532}{44}}$$

$$= 0.07783$$

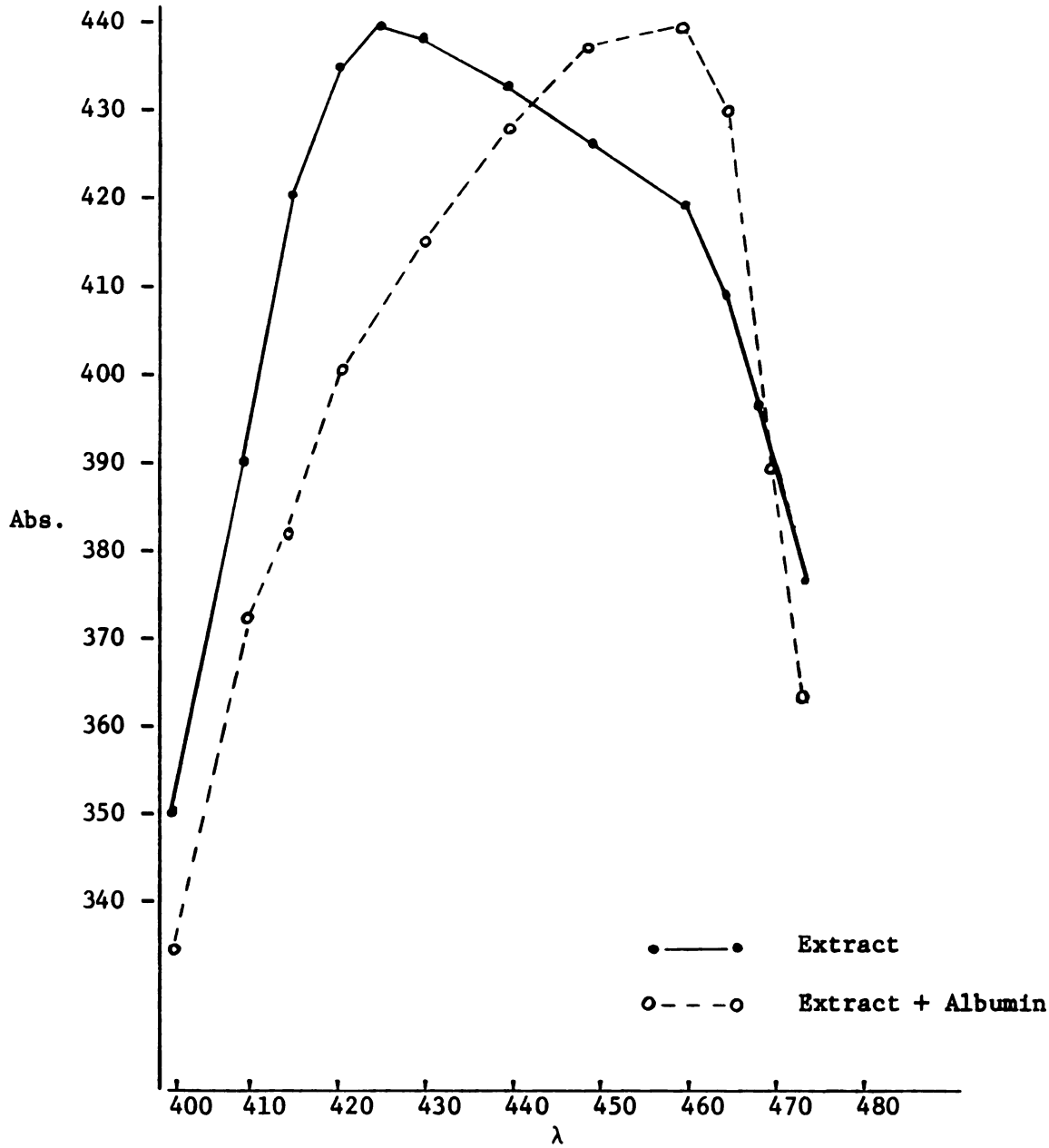

Figure 1. Spectral absorbance curves on pooled extract and pooled extract to which albumin had been added.

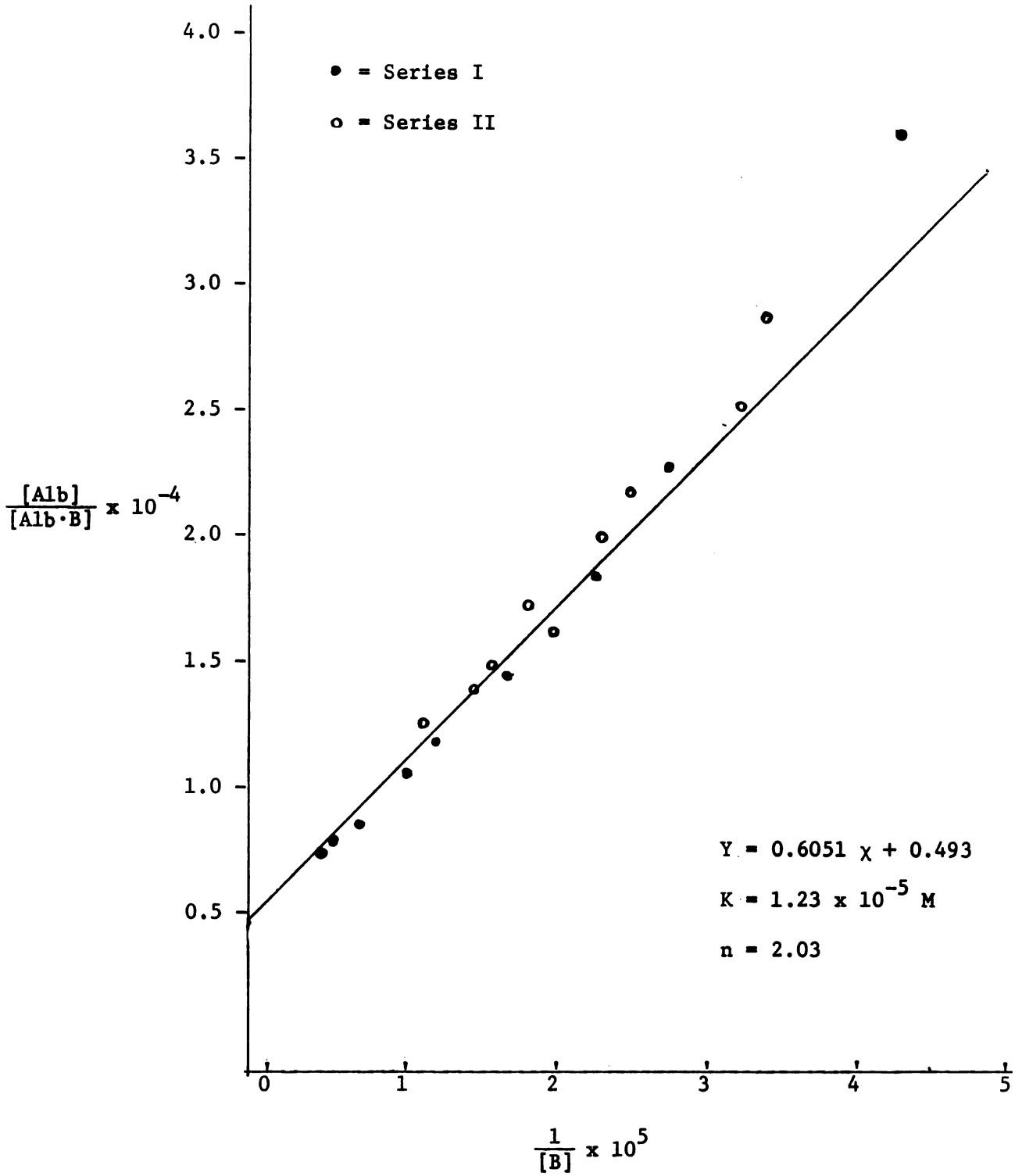

Figure 2. Mean results of heat precipitation studies.

DISCUSSION

Several procedural modifications were evaluated in developing a suitable technique for extracting bilirubin from a clot. We first, using a method developed by White (1971) in our laboratory, coagulated the protein by heating the serum for 3-5 minutes. The coagulum was whipped and the tube centrifuged to obtain a supernatant. This proved unsatisfactory both from the standpoint of volume of supernatant and concentration of bilirubin. Bilirubin appeared to be locked firmly in the clot. Subsequently we varied the boiling time, finally reducing it to the minimum required to produce a firm clot, 1.5 minutes, and whipped the coagulum with an equal quantity of buffer before centrifugation. This yielded reproducible concentrations of bilirubin, provided the tubes were tightly stoppered during heating and the boiling time was carefully controlled.

Unconjugated bilirubin is commonly described as being non-water soluble. This description stems from early studies of the behavior of bilirubin in solutions of low ionic strength. In aqueous solutions approximating physiological molarity and pH, however, the solubility of bilirubin is of the order of magnitude of 5 mg./dl. although this solubility decreases rapidly with reduction of pH. It is interesting to speculate that bilirubin crystals in tissues and bilirubin infarcts may be the result of a local decrease in pH. As the ionic strength of extracellular fluid is approximately 0.15 (Burnstine and Schmid, 1962) M/15 phosphate buffer, pH 7.4, which has

an equivalent ionic strength, was chosen as the extraction medium.

It is known that pH increases when serum is heated, as a result of CO_2 being driven off, and that maximum bilirubin binding occurs at pH's above physiologic pH. Hence, no adjustments were made in the pH of the pooled serum-bilirubin mixtures. The pH of several of the mixtures was checked, however. The observed range was from 8.45-9.15, the pH varying according to the concentration of alkaline bilirubin in the mixture. Adjustment of pH can be a cumbersome process and the purpose was to see whether, with a simple approach, free bilirubin could be extracted from a clot and measured. The two questions to be answered were whether the bilirubin being measured were indeed free bilirubin and whether by this method a realistic approximation of free bilirubin concentration could be made.

Since the amount of protein remaining in the extract was of interest, total protein assays were made on two of the supernatants. These showed that only 2% of the original protein was unprecipitated. No attempt was made to identify the protein fractions present. Instead a spectral absorbance curve was made on the pooled extracts from one day's run. The bilirubin remaining in the supernatant was entirely unbound bilirubin as established by the character of the spectral absorbance curve which showed a characteristic maximum at 425 nm. When albumin was added to the extract the maximum absorbance shifted to 460 nm. which is characteristic of protein-bound bilirubin (Odell, 1959a). This shift may be seen in Figure 1.

The free bilirubin obtainable from serum bilirubin mixtures, ranging from 10-50 mg./dl. of total bilirubin, was measured and the results were related to the law of mass action. Bilirubin in serum exists in at least 2 different states as shown in the simplified

stoichiometric reaction of equation (1) where Alb^+ and B^- represent albumin and bilirubin, respectively.

$$(1) \quad Alb^+ + B^- \rightleftharpoons Alb \cdot B$$

On the left-hand side of the equation the bilirubin is a free, potentially diffusible molecule. On the right-hand side it is associated with the albumin in a protein-anion complex which is not diffusible.

$$(2) \quad \frac{[Alb^+][B^-]}{[Alb \cdot B]} = K$$

Equation (2) describes the reaction in the mass action form where the molar concentration relationships are described by K, the dissociation constant. The available binding sites of albumin, $[Alb^+]$, may also be expressed as follows:

$$(3) \quad [Alb^+] = n\,[Alb] - [Alb \cdot B]$$

Here [Alb] is the molar albumin concentration, n is the average maximum number of bilirubin binding sites available per albumin molecule and [Alb·B] is the concentration of the complex. If this relationship is substituted in (2), the equation can be rewritten and rearranged as follows:

$$(4) \quad \frac{(n\,[Alb] - [Alb \cdot B])\,[B^-]}{[Alb \cdot B]} = K$$

$$(5) \quad \frac{n[Alb]}{[Alb \cdot B]} - 1 = \frac{K}{[B^-]}$$

$$(6) \quad \frac{[Alb]}{[Alb \cdot B]} = \frac{K}{n}\left(\frac{1}{[B^-]}\right) + \frac{1}{n}$$

This gives the equation for a line in which the intercept is 1/n and the slope is K/n. This equation, which was adapted from the calcium-binding studies of Moore (1970), is an oversimplification since it assumes that successive dissociation sites on the protein molecule are identical and independent. From the binding studies of Bjerrum (1968) and Jacobsen (1969) this assumption is undoubtedly incorrect. We found a plot of [Alb]/[Alb·B] against $1/[B^-]$ to be linear. This does not refute the observations of Bjerrum and Jacobsen but simply suggests that these differences are not detectable under our experimental conditions.

Figure 2 is a graphic presentation of the mean results of our thermoprecipitation studies. Free bilirubin and albumin were measured directly and the concentrations converted to molar values. Bound bilirubin was calculated as the difference between total and free bilirubin. The regression line was calculated using the results from the first series of bilirubin mixtures which ranged from 10-50 mg./dl. in increments of 5 mg./dl. as shown in Table 6. The mean results of later experiments (Table 7), which are superimposed on this graph as open circles, show continuing linearity. The greater deviation from the line as $1/[B^-]$ increases is partially an artifact inherent in a reciprocal plot. However, it may also be due to the fact that when the bilirubin-albumin molar ratio is less than 1, only one binding site is operative.

The number of binding sites was calculated to be 2.03 using a molecular weight for albumin of 69,000. This is compatible with the binding studies cited in the literature review. To summarize these, approximately 2 binding sites have been found when human serum has been used at pH's above physiologic pH. Investigations using purified albumin have resulted in the demonstration of approximately 3 binding sites. If we consider the statement of Blondheim (1955) that the first

1.5 gm./dl. of albumin in human serum does not participate in binding and recalculate our results to include only the participating albumin, the number of binding sites increases to 3.17. This is in good agreement with estimates made using purified albumin in which there were no naturally occurring anions in competition for the binding sites. The dissociation constant we found was larger than those reported by Jacobsen (1969) but this is only to be expected under the conditions of the experiment: i.e., alkaline pH and denaturation of the protein.

SUMMARY

A method has been presented here for the separation of free from protein-bound bilirubin by heat precipitation. It has been demonstrated that the bilirubin being measured is indeed free bilirubin and that the results are compatible with mass action laws. Obviously there is some difference between the estimates of free and bound bilirubin by this method and the true value which must exist *in vivo* as the bilirubin binding of albumin shifts with pH changes. However, since the stoichiometric relationships are stable, it should be possible to derive an appropriate correction factor for clinical use.

It is hoped that the method described here will make it possible to accumulate relevant and useful information which will not only establish limits beyond which exchange transfusion is mandatory but clarify those limits which may be considered safe.

From the limited data available (Zamet and Chunga, 1971) it would seem reasonable to observe, as a first approximation, that a free bilirubin concentration of less than 0.1 mg./dl. is benign but that 0.3 mg./dl. is a clear indication for immediate exchange transfusion. However, in premature infants, lower levels may be dangerous. We need to understand more clearly not only the role of prematurity in determining the levels of toxicity but the effects of duration of exposure to these levels. Then it will be possible to define these parameters more precisely.

CONCLUSIONS

The appropriate use of the method proposed here should make it obsolete to report total bilirubin alone in an infant with neonatal jaundice. It is now possible by a simple, fast, clinically acceptable method to provide the physician with a reasonably useful estimate of free bilirubin. As noted in the introduction, the level of free bilirubin is the factor determining its spread into the tissues with the result of kernicterus and brain damage. Therefore, the direct measurement of free bilirubin should provide the best index for deciding the time at which exchange transfusion is necessary.

Now that the principle of the method is reasonably well established, future efforts in this area should be devoted to adapting this principle for use with microsamples of blood. When this is done it should be possible to estimate more closely the concentration of free bilirubin which is toxic to the newborn.

BIBLIOGRAPHY

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Applegarth, D. A., Zack, P., Ross, P. M., and Israels, S.: An evaluation of the HBABA binding capacity of newborn serum. Clin. Biochem., 3, (1970): 45.

Bjerrum, O. J.: Interaction of bromphenol blue and bilirubin with bovine and human serum albumin determined by gel filtration. Scand. J. Clin. Lab. Invest., 22, (1968): 41.

Blondheim, S. H.: The relation between the albumin concentration of serum and its dye-binding capacity. J. Lab. and Clin. Med., 45, (1955): 740.

Brown, A. K.: Bilirubin metabolism with special reference to neonatal jaundice. *Advances in Pediatrics*. Chicago Yearbook Publ., Inc. (1962).

Burnstine, R. C., and Schmid. R.: Solubility of bilirubin in aqueous solutions. Proc. Soc. Exp. Biol. Med., 109, (1962): 356.

Chunga, F., and Lardinois, R.: Separation by gel filtration and micro-determination of unbound bilirubin. Acta Paediat. Scand., 60, (1971): 27.

Claireaux, A. E.: Neonatal hyperbilirubinaemia. Brit. Med. J., 1, (1960): 1528.

Day, R. L.: Inhibition of brain respiration *in vitro* by bilirubin. Proc. Soc. Exper. Biol. Med., 85, (1954): 261.

Harris, R. C., Lucey, J. F., and MacLean, J. R.: Kernicterus in premature infants associated with low concentrations of bilirubin in the plasma. Pediatrics, 21, (1958): 275.

Jacobsen, J.: Binding of bilirubin to human serum albumin. Determination of the dissociation constants. F.E.B.S. Letters, 5, (1969): 112.

Jacobsen, J., and Fedders, O.: Determination of non-albumin bound bilirubin in human serum. Scand. J. Clin. Lab. Invest., 26, (1970): 237.

Jirsova, V., Jirsa, M., Heringova, A., Koldovsky, O., and Weirichova, J.: The use and possible diagnostic significance of Sephadex gel filtration of serum from icteric newborn. Biol. Neonat., 11, (1967): 204.

Josephson, B., and Furst, P.: Sulfonamides competing with bilirubin for conjugation to albumin. Scand. J. Clin. Lab. Invest., 18, (1966): 51.

Kaufmann, N. A., Kapitulnik, J., and Blondheim, S. H.: The adsorption of bilirubin by Sephadex and its relationship to the criteria for exchange transfusion. Pediatrics, 44, (1969): 543.

Kaufmann, N. A., Simcha, A. J., and Blondheim, S. H.: The uptake of bilirubin by blood cells from plasma and its relationship to the criteria for exchange transfusion. Clin. Sci., 33, (1967): 201.

Keenan, W. J., Arnold, J. E., and Sutherland, J. M.: Serum bilirubin binding determined by Sephadex column chromatography. J. Pediat., 74, (1969): 813.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, (1951): 265.

Lucey, J. F., Valaes, T., and Doriadis, S. A.: Serum albumin reserve phenolsulfonphthalein dye-binding capacity in infants with kernicterus. Pediatrics, 39, (1967): 876.

Martin, N. H.: Preparation and properties of serum and plasma proteins, XXI: Interactions with bilirubin. J. Amer. Chem. Soc., 71, (1949): 1230.

Michaelsson, M.: Bilirubin determination in serum and urine. Scand. J. Clin. Lab. Invest., 13, (1961): Suppl. 56.

Moore, E. W.: *Symposium on Ion Selective Electrodes*. ed. R. Durst, National Bureau of Standards (1970): 215.

Odell, G. B.: *In vitro* studies of the effects of sulfonamides on bilirubin. Amer. J. Dis. Child., 96, (1958): 535.

Odell, G. B.: Studies in kernicterus, I. The protein binding of bilirubin. J. Clin. Invest., 38, (1959a): 823.

Odell, G. B.: The dissociation of bilirubin from albumin and its clinical implications. J. Pediat., 55, (1959b): 268.

Odell, G. B.: Influence of pH on distribution of bilirubin between albumin and mitochondria. Proc. Soc. Exp. Biol. Med., 120, (1965): 352.

Odell, G. B.: The distribution of bilirubin between albumin and mitochondria. J. Pediat., 68, (1966): 164.

Odell, G. B., Cohen, S. N., and Kelly, P. C.: Studies in kernicterus, II. The determination of the saturation of serum albumin with bilirubin. J. Pediat., 74, (1969): 214.

Ostrow, J. D., and Schmid, R.: The protein binding of C^{14} bilirubin in human and murine serum. J. Clin. Invest., 42, (1963): 1286.

Porter, E. G., and Waters, J. W.: A rapid micromethod for measuring the reserve albumin binding capacity in serum from newborn infants with hyperbilirubinemia. J. Lab. Clin. Med., 67, (1966): 660.

Rutkowski, R. B.: The determination of maximum bilirubin binding capacity of human albumin and serum by a calcium carbonate adsorption technique. Clin. Chem. Acta, 17, (1967): 31.

Schmid, R., Diamond, I., Hammaker, L., and Gundersen, C. B.: Interactions of bilirubin with albumin. Nature, 206, (1965): 1041.

Silverman, W. A., Andersen, D. H., Blanc, W. A., and Crozier, D. N.: A difference in mortality rate and incidence of kernicterus among premature infants alloted to two prophylactic antibacterial regimens. Pediatrics, 18, (1956): 614.

Starinsky, R., and Shafrir, E.: Displacement of albumin-bound bilirubin by free fatty acids. Implications for neonatal hyperbilirubinemia. Clin. Chim. Acta, 29, (1970): 311.

Stern, L., and Denton, R. L.: Kernicterus in small premature infants. Pediatrics, 35, (1965): 483.

Sunderman, F. W., and Sunderman, F. W., Jr.: *The Clinical Pathology of Infancy*. Charles C. Thomas, Publishers, Springfield, Ill. (1967).

Waters, W. J.: The reserve albumin binding capacity as a criterion for exchange transfusion. J. Pediat., 70, (1967): 185.

Watson, D.: The transport of bile pigments; the binding of sodium bilirubinate to plasma protein. Clin. Sci., 22, (1962): 435.

White, F. J.: Thesis, Michigan State University, (1971).

With, T. K.: *Bile Pigments*. Academic Press, New York and London, (1968).

Zamet, P., and Chunga, F.: Separation by gel filtration and microdetermination of unbound bilirubin. Acta Paediat. Scand., 60, (1971): 33.

Zetterstrom, R., and Ernster, L.: Bilirubin, an uncoupler of oxidative phosphorylation in isolated mitochondria. Nature, 178, (1956): 1335.

APPENDIX

APPENDIX

Reagents for bilirubin:

1. Caffeine sodium benzoate. Dissolve the following in distilled water at 50-60 C. and make the volume up to 400 ml.

   20 gm. caffeine
   30 gm. sodium benzoate
   50 gm. sodium acetate

2. Diazo I. Dissolve 500 mg. sulfanilic acid in 15 ml. of concentrated hydrochloric acid and dilute to 1 liter with distilled water.

3. Diazo II. Dissolve 1.250 gm. sodium nitrite in distilled water and dilute to 100 ml. Make a new solution every 14 days. Store in a dark bottle.

4. Diazo reagent. Immediately before use mix 10 ml. of Diazo I and exactly 0.25 ml. of Diazo II. Use within 30 minutes.

5. Ascorbic acid solution. Dissolve 1 gm. of ascorbic acid in 5 ml. of distilled water. Renew daily. Keep in a dark bottle.

6. Fehling II. Dissolve 100 gm. of sodium hydroxide and 350 gm. of potassium sodium tartrate in distilled water and dilute to 1 liter.

Reagent for extraction of free bilirubin: M/15 Sørensen phosphate buffer, pH 7.4.

1. Dissolve 9.47 gm. anhydrous, A. R. grade, disodium phosphate in distilled water and dilute to 1 liter.

2. Dissolve 9.08 gm. anhydrous, A. R. grade, monopotassium phosphate in distilled water and dilute to 1 liter.

3. Combine 80.4 ml. disodium phosphate and 19.6 ml. monopotassium phosphate.

Reagents for total protein by the method of Lowry *et al.*

1. Reagent A. Make a 2% solution of sodium carbonate in 0.10 N sodium hydroxide.

2. Reagent B. Make a 0.5% solution of copper sulfate (pentahydrate) in 1% sodium tartrate.

3. Reagent C. Mix 50 ml. of Reagent A with 1 ml. of Reagent B. This mixture is stable for 1 day.

4. Reagent E. Make a 1:1 dilution of Folin-Ciocalteu phenol reagent (Eimer and Amend, Fisher Scientific Co., N.Y.) in distilled water.

Reagents for total protein and albumin by the biuret method.

1. 28% sodium sulfite. Dissolve 28 gm. of sodium sulfite in 100 ml. of distilled water with gentle heating. This reagent must be kept at 25 C. or higher.

2. Biuret reagent. Dissolve the following in 1 liter of distilled water:

   3 gms. copper sulfate (pentahydrate)
   12 gms. potassium sodium tartrate
   2 gms. potassium iodide

   With constant swirling, add 600 ml. 10% sodium hydroxide. Dilute to 2 liters.

VITA

The author was born in Baltimore, Maryland, on February 22, 1925. She attended Maryland State College at Towson, Johns Hopkins University in Baltimore, and Michigan State University where, in 1964, she received a B.S. degree in Medical Technology. She completed her professional training at Edward W. Sparrow Hospital in Lansing, Michigan, in July, 1965. Subsequently she was employed as a medical technologist at Sparrow Hospital until June, 1968, when she was admitted to the graduate program in Clinical Laboratory Science at Michigan State University. Since September, 1969, she has been an instructor in the Department of Pathology at Michigan State University. She is married and has 3 children.