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Studies in the mechanism of the diazotization of bilirubin

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Studies in the Mechanism of the Diazotization of Bilirubin

by

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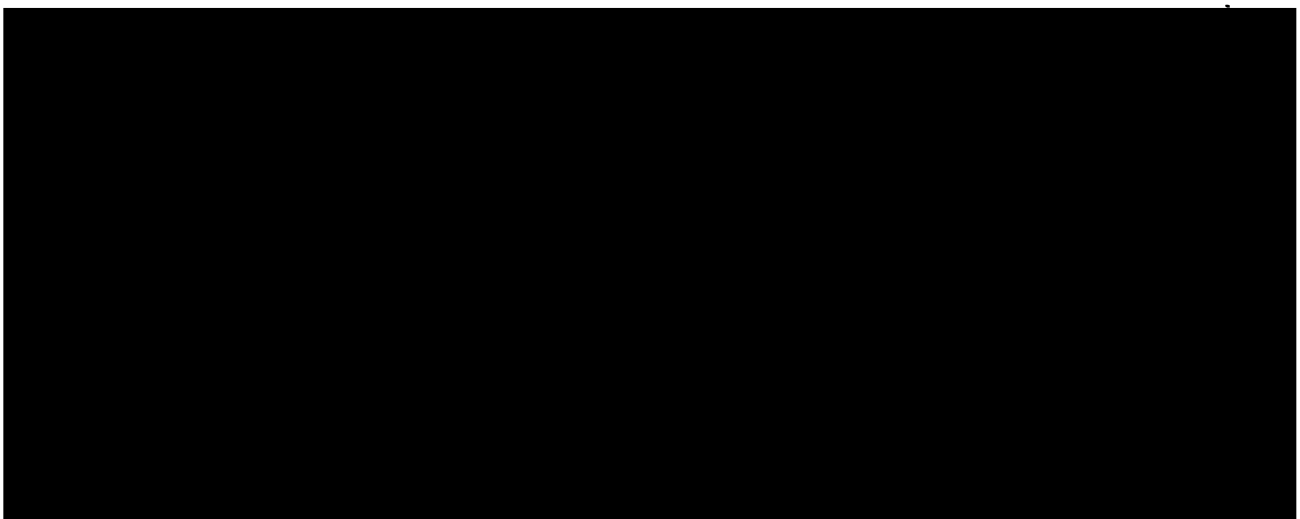

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ABBREVIATIONS

UCB	Unconjugated Bilirubin
BMG	Bilirubin Monoglucuronide
BDG	Bilirubin Diglucuronide
UDP	Uridine Diphosphate
LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
AMHPLC	Alkaline Methanolysis followed by HPLC analysis of bilirubins
TLC	Thin Layer Chromatography

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I HISTORICAL

The association of jaundice with liver disease has been known since antiquity. The diagnostic and prognostic aspects of jaundice were discussed by Hippocrates (1). Galen (2) was the first to link jaundice with bile and gallstones. By isolating bilirubin from old blood extravasations Virchow (3) in the mid-nineteenth century provided the first experimental proof that bile pigment was derived from blood. Fischer (4,5) in the late thirties and forties elucidated the chemical structure of heme and bilirubin and showed that they have closely related tetrapyrrole structures. The culmination of this line of inquiry was the isotope labelling studies (6) which proved that bilirubin is derived from the prosthetic group of hemoglobin.

Study of the clinical chemistry of bilirubin began with Ehrlich's discovery, (7) in 1883, that bilirubin reacts with diazotized sulphanilic acid to give a colored product. He used the reaction to show the presence of bile pigments in urine. In 1913 van den Bergh (8) adapted Ehrlich's diazo reaction for use in the estimation of bilirubin in serum and bile. A mistake in van den Bergh's laboratory lead to the discovery that bilirubin in bile did not require the addition of methanol to react with diazotized sulphanilic acid (9). Subsequently, it was found that characterizing bilirubin in serum as direct or indirect reacting (requiring methanol) could help in the differential diagnosis of several diseases (10,11). The difference between direct and indirect reacting bilirubins was not known

until the discovery in the early fifties that bilirubin is converted to glucoronide conjugates in the liver and that the conjugates are the direct reacting bilirubins (12,13,14).

II PHYSIOLOGY

Two recent reviews of bilirubin physiology are those of Gollan (10) and Schmid (11). Bilirubin is the principal waste product resulting from the degradation of heme; its formation reflects the continuing turnover of heme from hemoglobin, myoglobin and cytochromes. Of the daily production of 250 to 350 mg of bilirubin (15,16) (3.8 $\pm$ 0.6 mg/kg), the latest work indicates that the non-hemoglobin sources of bilirubin range from 23 to 37 per cent (17,18). The turnover of hemoproteins in the liver is responsible for practically all of the non-hemoglobin production of bilirubin. The turnover rate of liver hemoproteins is rapid and thus, as demonstrated by the injection of labelled precursors, the production of bilirubin can be divided into two pools: an "early labelled" pool reflecting the rapid turnover of liver hemoproteins and a "late labelled" pool reflecting the slow turnover of hemoglobin.

The average life span of normal erythrocytes is approximately 120 days. At the end of this period the senescent erythrocytes are selectively removed from the circulation and destroyed at a rate of about 2 to 3 million cells per second. Sequestration takes place in the reticuloendothelial cells of the spleen, liver, or bone marrow. Lysis occurs within minutes and the degradation of hemoglobin then proceeds within the phagocyte. The small amount of hemoglobin produced by intravascular hemolysis is predominantly degraded by parenchymal cells in the liver; in cases of larger intravascular hemolysis

degradation also occurs in the kidney.

The first step in the catabolism of heme-proteins is the removal of the apo protein, which is then degraded and returned to the amino acid pool. The ferroprotoporphyrin ring (Figure 1) is cleaved by a microsomal heme oxygenase enzyme (19). The enzyme requires the presence of a central iron atom on heme and NADPH. Three moles of O_2 are consumed; a molecule of CO is produced and cleavage is specific for the α-meso bridge. The resultant biliverdin is then reduced to bilirubin by the cytosolic NADPH dependent enzyme biliverdin reductase (20). Since bilirubin is very sparingly soluble in aqueous solutions at physiological pH it must be transported to the liver by a carrier protein, albumin. Albumin has one high affinity site (reported binding constant of 3×10^7 mole/L) and at least two lower affinity sites (21).

The processing of bilirubin by the liver can be divided into three steps: 1. uptake 2. conjugation 3. secretion into bile. Hepatic uptake at the sinusoidal surface is a facilitated transport process mediated by a carrier protein which is saturable, exhibits Michaelis-Menten kinetics, and is shared by a variety of other organic anions (22). The maximum capacity of this carrier system is much larger than the normal net transfer rate of bilirubin from plasma to bile (23); consequently, it is not the rate limiting step in bilirubin metabolism. Little is known about binding and transport of bilirubin within the hepatocyte. Several cytosolic bilirubin binding proteins have been identified, Ligandin being the most extensively studied,

which may be involved in intracellular transport of bilirubin (24).

Bilirubin is conjugated by a microsomal uridine diphosphate (UDP) - glucuronosyltransferase (25) which forms a 1-O-acyl linkage between one of the propionic acid side chains and glucuronic acid. Glucuronic acid is the predominant conjugating moiety in humans with glucoside and xyloside conjugates being formed in only trace amounts (26). A transglucuronidation mechanism in which two moles of BMG react to give one mole of BDG and one mole of UCB had been proposed for the formation of diglucoronide, but recent work indicates that diglucoronide is also formed by a microsomal UDP-glucuronosyltransferase (27). It is not known whether or not one or two distinct glucuronosyltransferases are involved in bilirubin conjugation. In normal human bile bilirubin diglucuronide constitutes the major pigment with monoglucoronide representing only a minor fraction (26,28).

As in the intracellular movement of unconjugated bilirubin, little is known about the intracellular movement of conjugated bilirubins and the potential role of the Golgi apparatus and microtubular system in secretion. The secretion of bilirubin conjugates into the bile is an active transport process which occurs against a large concentration gradient. It exhibits competitive inhibition (i.e. bilirubin and other comparable organic anions share the same carrier or carriers), saturation and appears to be the rate limiting step in the clearance of bilirubin from blood (22,29). The canalicular

transport of bilirubin is functionally distinct from the transport of bile acids (30,31); however, it is not totally independent of bile acid secretion. The formation of mixed micelles in the canalicular lumen has been proposed as a "sink" which reduces the luminal concentration of bilirubin on the non-micellular phase of the bile, thus enhancing the secretion of bilirubin (22). The role of micellar secretion of organic anions within the canalicular lumen is not yet clear. A second proposal is that the increase in the rate of bilirubin secretion seen upon infusion of bile acids is due to the recruitment of centrilobular hepatocytes which are not normally involved in secretion (22).

The absorption of conjugated bilirubins in both the gall bladder and intestine is negligible because of their polar nature and molecular size; therefore, there is no appreciable enterohepatic circulation of bilirubin. The conjugates traverse the small intestine largely intact; however, in the terminal ileum and colon they are reduced by the action of colonic bacteria to a series of colorless tetrapyrroles collectively called urobilinogens. Urobilinogens undergo an enterohepatic circulation. Less than 20% of the daily production is re-absorbed and approximately 90% of the reabsorbed urobilinogens are immediately re-excreted by the liver. The remaining urobilinogens reaching the systemic circulation are excreted by the kidneys (approximately 2% of the daily production). Normal adult feces contain a mixture of urobilinogens and their colored oxidation products, urobilins (32).

III PATHOPHYSIOLOGY

Under normal physiologic conditions the plasma bilirubin concentration varies directly with bilirubin turnover and inversely with hepatic clearance. The normal range is generally considered to be 5.1 to 17.1 μMole/L (0.3 to 1.0 mg/dl), and is slightly higher for males than females (33). Recent work with improved techniques suggests that the presence of conjugates, which are undectable in normal serum (34,35), is an abnormal finding which indicates hepatobiliary dysfunction.

Hyperbilirubinemia is defined as plasma bilirubin levels exceeding 17.1 μMole/L (1mg/dl)(10,11). Jaundice (icterus), the yellow coloration of the sclerae, mucous membranes and skin, usually becomes apparent at bilirubin levels greater than 50 μMole/L (3 mg/dl). When hyperbilirubinemia develops abruptly the appearance of jaundice may lag one to two days behind the rise in plasma bilirubin levels. Hyperbilirubinemia itself rarely results in tissue damage; the only exception is the encephalopathy (Kernicterus) that occurs in severe unconjugated hyperbilirubinemia of the newborn, and occassionally in patients with hereditary unconjugated hyperbilirubinemias. Encephalopathy is observed at levels of 342 to 600 μMole/L (20 to 35 mg/dl) which suggests that once the high affinity site of albumin is saturated, significant amounts of unbound bilirubin start to appear and diffuse across the blood brain barrier (36,37). Since the blood brain barrier is impermiable to conjugated bilirubins, encephalopathy is never a complication of conjugated hyperbilirubinemias. Plasma bili-

rubin levels of 17 to 68 μMole/L (1 to 4 mg/di) could be due to hemolysis, abnormal hepatic function, or both. The normal liver has a large reserve capacity for eliminating bilirubin; consequently, elevated levels due to hemolysis seldom exceed 68 μMole/L (4 mg/dl)(38). Excluding unconjugated hyperbilirubinemias due to hereditary syndromes, plasma bilirubin concentrations persistently above 68 μMole/L imply reduced hepatic function irrespective of the presence of hemolysis (11). Unconjugated hyperbilirubinemias may be caused by accelerated bilirubin formation associated with hemolysis or ineffective erythropoiesis, by impared hepatic blood flow or by defective hepatic uptake or conjugation of bilirubin. Conjugated hyperbilirubinemias may be due to cholestasis resulting from impaired bile flow at the canalicular level (hepatocellular disease) or bile duct level (biliary obstruction). Reverse pinocytosis from the liver, reflux from damaged canaliculi, or reflux from damaged canaliculi via intercellular space are all proposed mechanisms for conjugated hyperbilirubinemia due to cholestasis (11). In patients with conjugated hyperbilirubinemias due to reduced hepatic function, approximately 60% of the total serum bilirubin is conjugated and 60 to 70% of the conjugates are bilirubin monoglucuronides (34,35). Neither the fraction of total plasma bilirubin present as conjugates nor the proportion of individual bilirubin conjugates can distinguish biliary obstruction from hepatocellular disease (11,35).

Urinary excretion of bilirubin is believed to arise from

glomerular filtration of non-protein bound bilirubin. Bilirubin conjugates, as opposed to unconjugated bilirubin, are filtered to a much greater extent reflecting their greater solubility and probable decreased binding to serum proteins (39). This filtered fraction is largely reabsorbed by the tubules (40), with the residual non-reabsorbed component giving rise to the bilirubinuria which is seen with conjugated hyperbilirubinemias. Urine bilirubin consists predominantly of bilirubin diglucuronide (35), which suggests that it is less tightly bound to serum proteins than both mono and unconjugated bilirubin. Urinary urobilinogen is increased in hemolytic disorders, in patients with hepatocellular disease and/or portasysmetic shunts, but not in patients with biliary obstruction (41). Urinary urobilinogen excretion is related to urine pH, higher values being recorded in alkaline than in acid urine, and a diurnal variation has also been demonstrated (39).

Several hereditary syndromes resulting in hyperbilirubinemia have been associated with defects in specific aspects of bilirubin metabolism. Crigler-Najjar Syndrome (Type 1) (42) is a familial form of chronic severe unconjugated hyperbilirubinemia resulting from the absence of hepatic UDP - glucuronosyltransferase activity. Death usually occurs during the first year of life. Crigler-Najjar Syndrom (Type 2) (42) results from a deficiency of hepatic UDP - glucuronosyltransferase activity. Neurologic damage is rare for this milder syndrome. Dubin-Johnson Syndrome(43) is a chronic benign conjugated hyperbilirubinemia caused by a congenital defect in hepatic

excretion of bilirubin, cholecystographic agents, and certain organic anions. Rotors Syndrome (44) is a rare, chronic, familial, non hemolytic jaundice with predominantly conjugated hyperbilirubinemias. The primary defect in Rotors Syndrome is in hepatic uptake and storage.

IV BILIRUBIN STRUCTURE AND STABILITY

Two recent reviews of bile pigment chemistry are those of McDonagh (45,46). The common skeletal structure, numbering system, and the β substituents of several isomers of bilirubin are shown in Figure 2. As previously mentioned, in nature, the cleavage of the protoporphyrin IX ring is specific for the α meso bridge resulting in the formation of the IX α isomer. The isomers formed by splitting the ring at the β, δ, and γ positions are referred to as the IX α, IX β, and IX γ isomers. Each bilirubin isomer can also exist as one of four geometric isomers about the 4-5 and 15-16 double bonds, the trans (Z-Z) configuration being the most stable. Bilirubin IX α is much less soluble in water at neutral or acidic pHs than the β, δ, and γ isomers (47). The X-ray crystallography of bilirubin IX α crystals (48) has shown that bilirubin IX α forms an involuted hydrogen bonded structure which buries the polar groups. It thus appears that the role of conjugation is to perturb this intramolecular hydrogen bonded structure (47) resulting in greater water solubility. This is supported by the fact that the IX β, δ, and γ isomers, which can not form the intramolecular hydrogen bonded structure, are excreted unconjugated by Gunn rats (49) (rats which are incapable of conjugation). It has also been proposed that the efficacy of phototherapy treatment for hyperbilirubinemic infants is due to the photoisomerization of bilirubin from the Z-Z to the E-E or E-Z configurations which are no longer capable of forming

the involuted hydrogen bonded structure (50).

The "Achilles heel" of bilirubin is the central bridge, which is susceptible to electrophilic and oxidative attack. The reactions of bilirubin can be divided into two broad categories: thermal and photochemical. Thermal reactions contributing to instability are acid catalyzed isomerization, free radical isomerization, and autoxidation. In acid catalyzed isomerization electrophilic attack by protons at the α carbon of a pyrrole ring adjacent to the central methylene bridge leads to the formation of pairs of dipyrrylmethenes which can recombine in a random manor to give a mixture of IIIα, IXα, and XIIIα isomers (51). For bilirubin IXα, acid catalyzed isomerization occurs rapidly upon treatment with strong acid and slowly with weaker acids. In aqueous solutions within the pH range 7.4 to 12 bilirubin IXα undergoes a rapid free radical isomerization (52) also yielding a mixture of the same three isomers. The reaction requires oxygen or free radical initiators, and in the presence of oxygen can be inhibited by ascorbic acid, glutathione, thiourea, and by binding to albumin. In aqueous solutions at alkaline pH in the presence of oxygen, bilirubin IXα is also susceptible to autoxidation (53). The mechanism of autoxidation is unknown but seems to involve trace metal catalysis. Autoxidation leads to the formation of varible amounts of biliverdin and a complex mixture of dipyrrolic and pyrrolic degradation products. Autoxidation can be in-

hibited by EDTA and by binding to albumin. Bilirubin is sensitive to light and must be protected from it. In deoxygenated aqueous solutions irradiation results in isomerization and gradual decomposition (46). The photoisomerization reaction is the photochemical analogue of the "dark" free radical isomerization. In the presence of oxygen bilirubin also undergoes photoxidation yielding a mixture of biliverdin and breakdown products (54). Conjugation of bilirubin IXα (i.e. esterification of the propionic acid side chains) disrupts the intramolecular hydrogen bonds exposing the central methylene bridge and rendering it more reactive (53), especially in organic solvents. This increased reactivity of bilirubin conjugates has presented formidible problems in their isolation and storage.

V BILIRUBIN DETERMINATION

The most common method for measuring bilirubin in biological fluids is colormetric quantitation of the azopigment formed after reaction with diazotized aromatic amines. The electrophilic diazonium salt attacks bilirubin at the central methylene bridge. Sulphanilic acid is the most commonly used aromatic amine. At first the product of this reaction was thought to be a disazobilirubin, but Fischer and Haberland (55), in the thirties, demonstrated that bilirubin was split at the central bridge with the formation of an azodipyrrole and a yellow intermediate. They further hypothesized that the intermediate was a hydroxypyrromethene carbinol which then condensed to reform bilirubin. Titration of bilirubin with diazotized sulphanilic acid showed that the intermediate reacts with a second diazo molecule (56,57). Formation of the second mole of azodipyrrole is accompanied by the release of formaldehyde (58). The generally accepted mechanism for the overall reaction is shown in Figure 3. The net result is that one mole of bilirubin produces two moles of azodipyrrole pigments.

The lag between the observation that bilirubins could be divided into direct and indirect diazo reacting fractions and its chemical explanation lead to innumerable random modifications of bilirubin procedures. The fact that UCB does react under conditions used for measurement of direct reacting bilirubins (conjugates) and that the amount that reacts is greatly influenced by pH, time of reaction, con-

centration of sulphanilic acid, concentration of diazo reagent, and bile acids was not appreciated. The situation became so confusing that for a time the reality of direct reacting bilirubins and the usefulness of testing for them was questioned (59). Following the discovery that direct reacting bilirubins are bilirubin conjugates, there were several systematic studies of reaction conditions for both total and conjugated bilirubins, most notable those of Nosslin (60) and Michaelsson (61). These studies led to the development of the present recommended procedures for the determination of total and conjugated bilirubin (62). Conditions in which conjugated bilirubins react and UCB does not react at all have not yet been found.

Nosslin studied the direct reaction only with BDG. He assumed his preparation was 100% BDG, and thus if the direct reaction goes to completion it should equal the total reaction. He observed that the direct reaction gave 80-90% of the total reaction (60), and thus concluded that the direct reaction does not go to completion. More recent work (63) (presumably with a purer preparation) has shown the direct reaction for BDG to be nearly complete. All work with BMG (63,64) so far has shown incomplete reaction in the direct reaction. The important features of the Nosslin-Michaelsson (62) modifications of the Jendrassik-Grof (65) procedures for the total and direct bilirubin determinations are as follows:

1. A pH of approximately 2 in the direct procedure

to minimize the reactivity of UCB.

2. A concentration of diazonium salt in the diazo reagent of 1.8 - 2.0 μMole/L. Higher concentrations would increase the reaction of UCB in the direct reaction, and lower concentrations do not allow the reaction to go to completion.

3. A concentration of sulphanilic acid in diazo reagent of approximately 25 μMole/L. An excess of sulphanilic acid is required as a catalyst for the second step.

4. The accelerator for the total reaction is a caffeine-benzoate buffer or a dyphylline-acetate buffer. Reaction is complete in no longer than ten minutes (with methanol it takes thirty minutes or longer) and they protect the azopigment from destruction by excess diazo reagent.

5. The reaction is stopped with ascorbic acid which destroys excess diazo reagent. This prevents reaction of UCB in the direct reaction upon addition of the alkaline color developer. Also ascorbic acid significantly reduces the fading after alkalinization in hemolytic samples.

6. After stopping the reaction, alkaline color developer is added immediately to prevent ascorbic acid destruction of the azopigment. The accelerator is added to the direct reaction after stopping the reaction so that the same blank can be used for both total and direct reactions.

7. The measurement of the azopigment is taken after alkalinization. This shifts the peak from the red (λ_{max} = 530 nm) to the more intense blue form (λ_{max} = 600 nm). Slight changes in pH in the acid region can shift the azopigment spectra and cause errors. In addition, hemoglobin interferes more at 530 nm than at 600 nm.

8. The blank is the same as the total except ascorbic acid is added before the diazo reagent. In this manner the error caused by the colored product between ascorbic acid and the diazo reagent is substracted. The use of dyphylline instead of caffeine reduces turbidity problems (precipitation of caffeine).

Pure conjugated bilirubin standards are not available. Consequently, conjugated and unconjugated azopigments have been assumed to have the same molar extinction and all results are expressed in terms of UCB. Lucassen (57) has reported that the acid forms (λ_{max} = 530 nm) of the conjugated and unconjugated azopigments have the same molar extinction.

The use of aromatic amines other than sulphanilic acid as azo coupling reagents has not been extensively studied. The most common application has been to employ azopigments which are more lipophilic (66,67,68) and can be extracted into organic solvents from which isolation and chromatography are possible. Claims that only conjugates react with ethylanthranilate at pH 2.7 (67,69) have recently been disputed (70).

Direct spectrometry (λ_{max} = 463 nm) can be used to measure bilirubin concentrations in sera of newborns or in amniotic fluid where interference from lipochromes such as carotene is minimal and the bilirubin present is entirely unconjugated. Direct spectrometry also has the advantage of requiring only a small amount of sample. Most methods (72,73) measure absorbances at two wavelengths in order to correct for the absorbance of hemoglobin.

Other methods which are seldom (if ever) used include spectropolarimetry (74), fluorimetry (75) (after treatment with phosphoric acid), oxidation to biliverdin (76), direct spectroscopy after extraction into an organic solvent (77) (supposedly only unconjugated bilirubin is extracted), direct spectroscopy after precipitation with acetone (78) (proteins and supposedly unconjugated bilirubin are precipitated), direct spectroscopy before and after bleaching (79,80) and radioisotope dilution methods for total (34) and conjugated bilirubin (81).

Since the plasma bilirubin concentration is an unrealiable indicator of when kernicterus is a hazard, efforts have been made to measure unbound bilirubin and the reserve binding capacity on the assumption that unbound bilirubin is a major determinant in the development of kernicterus. Techniques used to estimate the unbound fraction include thin layer chromatography or column chromatography on sephadex (82,83,84), oxidation with peroxides plus peroxidase (85),

cellulose acetate electrophoresis (86), and fluorimetry (87). The measurement of binding capacity is usually done by titrating the plasma with unconjugated bilirubin and looking for the point where a sudden increase in unbound bilirubin occurs. Erythrocyte binding (88), dye binding (89), and salicylate binding (90) methods have also been proposed for measuring binding capacity. It is not clear that these techniques actually measure free, unbound bilirubin or binding capacity and none are in general use.

The most common method for detecting bilirubin in urine is diazotization under acidic conditions with the stable diazonium salt p-nitrobenezenediazonium p-toluene sulfonate (ictotest) (91). This appears to be the most specific test, although it is only semi-quantitative. It has the lowest interference from non-bilirubin diazo positive material and can detect bilirubin in the range of 0.05 to 0.1 mg per 100 ml of urine. An older screening method for bilirubin in urine is Fouchet's test (91) which detects bilirubin by oxidation to biliverdin with ferric chloride. Attempts at developing quantitative methods for urine bilirubin include: diazotization after coprecipitation with calcium phosphate (92), formation of azopigment Zn^{++} or Cu^{++} complexes (61,93) and selective oxidation (94).

VI KINETICS

Several kinetic studies of the diazo reaction, in both aqueous-organic solvents and aqueous systems have been published. Aqueous-organic solvents have the advantage of being able to dissolve UCB, BMG, BDG and the diazo reagent at both acidic and basic pH. In a solvent system consisting of 60% ethanol, 30% chloroform, and 10% water and containing 0.006 molar HCl, Overbeek and Deenstra (56) found the reaction for UCB to be two consecutive second order reactions with rate constants $k_1=8X10^3$ $mole^{-1}l$ min^{-1} and $k_2=1.2x10^3$ $mole^{-1}l$ min^{-1}. They did not study the reaction with conjugates or investigate the influence of sulphanilic acid on the reaction (their diazo reagent was prepared from recrystallized diazonium salt). Luccassen (57) studied the reaction for both UCB and BDG in several solvent systems. In the chloroform ethanol water system (30:60:10) he found k_1 to be constant in the pH range 1 to 4.5, and then to increase steadily for both UCB and BDG, with the increase for UCB being sharper and greater. Experiments above pH 6.5 were not done. Below pH 4.5 the k_1 for BDG was $1.6x10^3$ $mole^{-1}l$ sec^{-1} and for UCB was 100 $mole^{-1}l$ sec^{-1}. Sulphanilic acid did not have any influence on k_1, but it had a marked influence on k_2. In the absence of sulphanilic acid k_2 was very small. Lucassen concluded that the presence of an aminophenyl group is necessary for the second step. The rate constant for the second step was the same for both UCB and BDG and increased with

increasing pH. At pH values higher than 5, the second step reaction constant, k_2, could not be measured because of decomposition of azobilirubin by excess diazo reagent. At pH 2.3 and a sulphanilic acid concentration of 10^{-3}, k_2 was 31 $mole^{-1}lsec^{-1}$. In acetone-water (1:1) only BDG was studied and the pH and sulphanilic acid dependence of k_1 and k_2 were the same as those in chloroform ethanol water. In aqueous systems at pH values lower than 6 Lucassen was unable to resolve the kinetics of both BDG (data deviated from a bimolecular course) and UCB (precipitated) . At a pH of 7 for UCB k_1 was $4.0x10^4$ $mole^{-1}lsec^{-1}$ and for BDG k_1 was $1.6x10^4$ $mole^{-1}lsec^{-1}$ and increased for both as the pH was raised from 7 to 8.5. For BDG, increasing the albumin concentration from 0 to $4x10^{-6}M$ caused k_1 to decrease from $16x10^3$ to $4x10^3$ $mole^{-1}lsec^{-1}$ at a pH of 7.5. A deviation from second order reaction and a side reaction between diazo reagent and albumin prevented determination of k_1 at high albumin concentrations.

Broderson (95) studied the reaction of icteric sera in aqueous media, at pH 2, with a large excess of diazo reagent. The reaction consisted of four separate pseudo first order phases which he believed were due to the various bilirubins present. He did not work with purified conjugates or identify which phase corresponded to which bilirubin.

The most recent work is that of Landis and Pardue (96). They report the kinetics of UCB and BDG in aqueous media over

a pH range from 4 to 12 and include data on the effects of p-diazobenzenesulfonic acid, albumin, benzoate and caffeine concentrations. In the presence of excess sulphanilic acid the rate constant for the second step approached that of the first step and the system exhibited complex kinetic behavior; therefore, they prepared the diazo reagent without any excess sulphanilic acid and limited their studies to the first step. Increasing the albumin concentration from 0 to 80 g/Liter caused a reduction of approximately 9% in the rate and was attributed to reaction of p-diazobenzenesulphonic acid with tyrosine groups of albumin, the net effect being that the amount of diazo reagent is diminished. No deviations from a bimolecular course were found. Caffeine impedes the reaction, and benzoate added in the presence of caffeine tends to counteract the effect of caffeine and enhance the reaction rate. The pH profiles of the rate constants suggests that there are three different reactive species for both UCB and BDG, corresponding to the loss of two dissociable protons. The most highly protonated species are least reactive, markedly so for UCB. For UCB they reported rate constants for the first step of 35, 1.15×10^5 and 2.75×10^6 $Mole^{-1}lsec^{-1}$ for the three different protonated states. For BDG they reported rate constants of 2.32×10^4, 3.06×10^4 and 1.01×10^6 $Mole^{-1}lsec^{-1}$.

VII EXPERIMENTAL OBJECTIVES

Two main problems have hindered the study of the chemistry of bilirubin conjugates. The first was isolating and storing them. The second, and more important, was the absence of a sensitive assay or assays capable of accurately quantitating UCB, BMG and BDG. Recently Blanckaert, et al. (34), have developed an HPLC method for the simultaneous determination of UCB, BMG and BDG (including all the isomers of the conjugates). The method involves the quantitative conversion of bilirubin conjugates to their nonpolar methyl ester derivatives, their extraction into chloroform and subsequent separation by HPLC. This method (AMHPLC) can serve as a reference method and for the first time allows bilirubin preparations to be assayed for isomerization, hydrolysis, and destruction at every stage during their manipulation. The AMHPLC method has already led to the following new findings (34,35):

1. Serum of normal adults contains virtually no conjugated bilirubins.
2. The bilirubin in sera of patients with hepatobiliary disease on the average is approximately 60% in the conjugated form and approximately 60% of the conjugates are BMG.
3. In sera from patients with hepatobiliary disease the concentration of diazo positive substances is considerably higher than that of bilirubin IXα and its

sugar conjugates.

4. The bilirubin in urine of patients with hepatobiliary disease is primarily BDG.

The nature of the non-bilirubinoid diazo positive material in sera is unknown. Blanckaret, et al. (97), have also developed a procedure for purifying BMG and BDG from enriched rat bile. The preparations are at least 90% pure in terms of contamination by UCB and the other conjugate.

An important conclusion arising from the use of these new techniques is that the appearance of bilirubin conjugates in sera of adults is probably an indication of pathology. It is also important in infants since it indicates the liver is beginning to conjugate bilirubin and may obviate the necessity of an exchange transfusion. Therefore, the development of a sensitive and specific test for low levels of bilirubin conjugates in sera might give useful clinical information. The AMHPLC method is too complex for the average laboratory, but it can be used as a reference method in the development of other procedures. This thesis will attempt a study of the basic chemistry, kinetics, reaction mechanism, and stoichiometry of the diazo reaction in the hope that the information so gained will suggest possible new methods for measuring conjugated bilirubin and identify the interfering substance(s) in sera of patients with hepatobiliary disease. As previously stated, what makes this study possible is that for the first time the exact composition of preparations

VIII METHODS

Chemicals: Bilirubin (ε_{452} in chloroform 61.0×10^3 liter mol^{-1} cm^{-1}) was from Koch-Light Lab. (U.K.). Chloroform (containing 1% ethanol) and methanol, used for HPLC, were from Burdick and Jackson Lab. (Muskegon, MI). Crystalline methyl ester of xanthobilirubic acid (ε_{410} in methanol 34.0×10^3 liter $mol^{-1}cm^{-1}$) used as internal standard, was a gift of D. Lightner, University of Nevada, Reno, NV. Chloroform (containing 0.75% ethanol as stabilizer) and methanol (absolute, acetone free) for all other purposes except HPLC were from Mallinckrodt (Paris, KY). Absolute ethanol (200 proof) was from Pharmco, Publiker Ind. Co. (Linfield, PA). Sephadex LH-20 was from Pharmacia (Uppsalla, Sweden). Bovine serum albumin (fraction V), β-glucuronidase (Type VIII, optimum pH 6.8-7.0, E. coli), TEAE cellulose (0.5 meq/L), EDTA disodium salt, dyphylline, sodium nitrite, ascorbic acid (acid form and sodium salt), Tris HCl, Tris base (Trizma^R), and glycine (NH_3 free and anhydrous) were from Sigma Chemical Co. (St. Louis, MO). Celite was from Johns-Manville Products (Denver, CO). Phenobarbital, sodium salt, was from the University of California pharmacy (San Francisco, CA). Cholestyramine resin was a gift from Merck, Sharp and Dome (West Point, PA). β-napthol (99%) was from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous disodium phosphate was from J.T. Baker (Phillipsburg, NJ). Sulphanilic acid monohydrate, sodium potassium tartrate·$4H_2O$, monosodium phosphate·H_2O, citric acid·H_2O, disodium citrate·$2H_2O$, concentrated HCl, glacial acetic acid, n-hexane, sodium chloride glucose,

anhydrous sodium carbonate, ethyl acetate, sodium acetate·H_2O, and sodium hydroxide (pellets) all analytical reagent grade were from Mallinckrodt (Paris, KY).

Materials and Methods

All work with bilirubinoids is done under dim light. Solvents are purged with argon and centrifugations are performed at 20-25°C at 3000 rev/min (1000g). Pre-coated silica gel G plates (Merck A.-G., Darmstadt, Germany of EM Laboratories, USA, 60G254/0025) are employed for T.L.C. Liquid chromatography is performed on a Perkin Elmer (Norwalk, CT) series 3 liquid chromatograph equipped with a LC 100 column oven, a LC 75 UV-Vis detector, a Rheodyne (Berkeley, CA) model 7105 injector, and a Hewlett-Packard (Palo Alto, CA) model 3380 A electronic integrator. Five micron LiChrosorb Si 60 columns (250mmX4.6mm) from Merck A.-G., EM Laboratories (Elmsford, NY) are used and kept at 45°C. A Cary (Varian, Palo Alto, CA) model 118 spectrophotometer is used for recording absorption spectra. A Durrum Stopped Flow apparatus (Dionex, Sunnyvale, CA) equipped with Beckman (Palo Alto, CA) DU optics is employed for the measurement of rapid reactions. Kinetic data is analyzed on a PDP-11/70 computer.

Solutions

Sulphanilic Acid 5 GM/L Dissolve 5 grams sulphanilic acid monohydrate in 500 ml of distilled water with heating, add 15 ml concentrate HCl and when cool dilute to one liter.

Sulphanilic Acid 10 Gm/L Same as 5 Gm/L except use 10 grams.

Dyphylline I Dissolved 50 grams dyphylline and 125 grams sodium acetate 3 H_2O in water with warming (40°C); add 1 gram EDTA, disodium salt, and dilute to 1 liter when cool. If necessary, the reagent should be filtered before use.

Dyphylline II Dissolved 64.52 grams dyphylline and 161.3 grams sodium acetate 3 H_2O in water with warming (40°C); add 1.31 grams EDTA, disodium salt, and dilute to 1 liter when cool. If necessary, filter before use.

Sodium Nitrite 5 Gm/L Dissolve 500 milligrams sodium nitrite in water and dilute to 100 ml (made fresh daily).

Diazo Reagent Mix 10 ml sulphanilic acid 5 Gm/L with 0.25 ml sodium nitrite. The solution is stable for approximately 3 hours.

1/4 Diazo Reagent Dilute diazo reagent 1:4 with sulphanilic acid 5 Gm/L.

4X Diazo Reagent Mix 4.7 ml sulphanilic acid 10 Gm/L, 4.3 ml sulphanilic acid 5 Gm/L and 1 ml sodium nitrite.

Alkaline Tartrate Dissolve 100 grams sodium hydroxide and 350 grams sodium potassium tartrate 4 H_2O in water and dilute to 1 liter.

Ascorbic Acid Dissolve approximately 200 mg ascorbic acid in 5 ml water (made fresh daily).

Glycine-HCl buffer pH 2.7 To a 0.4 M HCl solution enough solid glycine is added to bring the pH to 2.7.

Glycine-HCl buffer pH 1.8 Same as above except the final pH is 1.8.

Citric acid-Phosphate buffer pH 6.0 Thirty six ml of 0.5 M citric acid and 63.9 ml of 1 M disodium phosphate are mixed.

β-Naphthol Dissolve 3.42 grams sodium acetate 3 H_2O in 30 ml water, add 180 mg of β-Napthol and dilute to 500 ml with ethanol.

3 Gm% Albumin Three grams of albumin (Bovine, fraction V) is dissolved in 100 ml of a 10 mM phosphate buffer pH 7.4 containing 1 mM EDTA. The solution is stored at -20°C.

Stock Tris Buffer pH 7.2 Dissolved 7.02 grams of Tris HCl and 0.67 grams Trizma in water and dilute to 500 ml.

Working Tris Buffer Dilute Tris buffer by 10 with water.

Preparation of Bilirubin Free Serum

A serum pool is prepared by combining patients samples having a total bilirubin less than 0.6 mg/dl (10 μMole/L) and bleaching for two hours under a bank of fluorescent lights 30 cm above the serum. The serum is contained in a covered glass petri dish and placed on aluminum foil.

Collection of Bile Samples and Treatment of Animals (97)

Male Sprague-Dawley or Wistar R/A rats (300-400 gms body weight) are used. They are pretreated for three days with phenobarbital (sodium salt, 10 mg/ml in 0.15 M NaCl, 10 mg/100 grams body weight per day intraperitoneally). During the same period they are fed normal rat chow cantaining 5% (w/w) cholestyramine resin. On the fourth day the common bile duct and the jugular vein are cannulated with respectively a PE 50 and

PE 10 polyethylene catheter (Clay adams, Parsippany, NJ); the animals are then placed in restraining cages where they have access to drinking water and laboratory chow and are continuously infused with saline containing 5% (w/w) glucose. Normal body temperature is maintained by placing the animals under a heating lamp. After biliary drainage for 15-20 hours intravenous infusion of a bilirubin solution is started. The bilirubin solution is prepared by dissolving UCB in 0.1 M NaOH, diluting with a solution of bovine serum albumin (20 grams/L in 0.15 M NaCl) to a final concentration of 15.38 μMole/ml (9 mg/ml), and finally adjusting the pH to 8.0 with 0.15 M HCl. Bilirubin is infused at a rate of 5.13 μMoles/hr per 100 grams body weight. To minimize dipyrrole exchange, solutions are prepared every hour and bolus injections given. To avoid formation of non-1-0-acyl glucuronides bile was collected in two ml fractions,on ice, in tubes containing 0.5 ml citric acid-phospate buffer pH 6.0 and 50 mg of ascorbic acid. Each bile sample is then overlaid with 35 ml n-hexane, flushed with argon and stored at -20°C.

Purification and Extraction of Bilirubin Glucuronides (97)

Isolation of glucuronides is carried out as follows:

1. Separate hexane layer from enriched bile and extract again with fresh hexane (20 ml/ml bile). Discard hexane extracts.
2. Add bile to sephadex LH-20 (375 mg/ml bile), mix, and lyophilize over night.
3. Add the sephadex LH-20 from step 2 (equilibrated with

chloroform/ethanol (1:1; v/v)) to a 2 cm ID glass column containing a bed of sephadex LH-20 (200 mg/ml bile). All column work is performed at 4°C.

4. Wash column with $CHCl_3$ / EtOH (1:1;v/v) (200 ml/ml bile). Adjust the flow rate to 5 ml/min by applying pressure to the column with nitrogen.

5. Wash column with absolute ethanol (100 ml/ml bile). Maintain the flow rate at 5 ml/min.

6. Elute the bilirubin glucuronides with a minimum volume of ethanol/water (1:1;v/v) in an evaporation flask placed on dry ice and containing 35 mg ascorbic acid and 5 mg EDTA (disodium salt). Collect the eluate under argon. Adjust the flow rate to 1.5 - 2 ml/min.

7. Concentrate in vacuo the ethanol water eluate to about 2.5 ml per ml starting bile (water bath 40°C).

8. For each 2.5 ml concentrate add sequentially 2.5 ml ethanol and 1 ml glycine HCl buffer pH 1.8. Extract with 100 ml ethyl acetate (for each 2.5 ml concentrate). Centriguation is necessary to separate the phases. For quantitative recovery the aqueous phase can be extracted again with three volumes chloroform/ethanol (1:1;v/v, equilibrated with an equal volume of glycine HCl buffer pH 1.8). Discard the aqueous phase.

9. Concentrate in vacuo the ethyl acetate extract (or combined extracts) to approximately 30% of the amount of ethyl acetate used. Store at -20°C in sealed tubes purged with argon.

10. Apply 3 - 6 ml (approximately 1 micromole of bilirubin conjugates) of the concentrated ethyl acetate extract to a preparative thin layer plate. This step must be carried out quickly to avoid hydrolysis. Use a hot air stream to speed application. The conjugates are separated by development in chloroform/methanol/water (60:32:6; v/v).

11. While still wet, scrape the BMG and BDG bands from the plate and elute with methanol. Remove silica by decantation and filtration through a 2-3 mm celite column. Wash the silica several times with methanol.

12. Immediately evaporate in vacuo the methanol to dryness.

13. The bilirubin glucuronides may be stored dry under argon at -20°C or dissolved in bleached serum or 3 gm% albumin and stored frozen (-20°C).

Total and Direct Diazo Determination of Bilirubin (62)

Three tubes are set up in the following order, mixing after the addition of each reagent:

Total:	0.8 ml water
	0.2 ml serum (albumin solution)
	0.5 ml diazo reagent
	2.0 ml dyphylline I
	wait for 10 minutes
	0.1 ml ascorbic acid
	immediately
	1.5 ml alkaline tartrate
Direct:	0.8 ml water
	0.2 ml serum (albumin solution)
	0.5 ml diazo reagent
	wait for 10 minutes
	0.1 ml ascorbic acid
	immediately
	2.0 ml dyphylline I
	1.5 ml alkaline tartrate

Blank: 0.8 ml water
0.2 ml serum (albumin solution)
0.1 ml ascorbic acid
0.5 ml diazo reagent
2.0 ml dyphylline I
1.5 ml alkaline tartrate

The optical densities are read as soon as possilbe at 600 nm.

HPLC Analysis of UCB and the Methyl Esters of BMG and BDG (34)

Approximately 20 mg of ascorbic acid (sodium salt), 5 mg EDTA (disodium salt), and 2 ml of methanol containing the internal standard is added to 0.2 ml serum in a 40 ml conical tube. The mixture is treated with 2 ml of 2% (w/v) KOH in methanol and immediately vortex mixed. After reaction for 60-90 seconds at 20 - 25°C, 2 ml of chloroform and 4 ml of glycine HCl buffer, pH 2.7, are added sequentially. The organic and aqueous phase are separated by centrifugation for ten minutes. The aqueous phase is aspirated off and the cholorform phase transferred to a 12 ml conical tube and evaporated to dryness under nitrogen at 30°C. The residue (methyl esters and UCB) can be stored for a week under argon at -20°C.

For HPLC analysis the pigment residue is redissolved in a small volume of chloroform (100-200 µl) and 10-20 µl of the solution injected into the liquid chromatograph. The pigments are separated by eluting the column at 1.5 ml/min with a convex gradient (curvature 0.2 according to the Perkin-Elmer liquid chromatograph model series 3 instruction manual) of mobile phase, starting with chloroform/acetic acid (199:1; v/v) (Solvent A) and ending with chloroform/methanol/acetic acid (197:2:1; v/v) (Solvent B) after ten minutes. Elution is continued for ten minutes with Solvent B, and the column

is then reequilibrated for ten minutes with Solvent A. The pigment in the effluent is detected at 430 nm, and the area under each peak in the chromatogram measured with an electronic integrator.

Kinetics

The kinetics of the diazo reaction is studied by monitoring the transmittance at 530 nm as a function of time using a stopped flow system. The data is digitalized into the computer from polaroid prints of oscilloscope tracings of the reaction. The data is then analyzed using a Marquardt-Levenberg type nonlinear least squares regression analysis of multiexponential functions (98,99). The stopped flow apparatus dispenses and mixes equal volumes of two solutions; consequently, the direct and total reactions had to be modified in the following manner:

Direct Reaction (volume ratios)

Syringe A	Syringe B
0.5 ml Diazo Reagent	0.2 ml Bilirubin Solution
0.25 ml water	0.55 ml water

Total Reaction (volume ratios)

Syringe A	Syringe B
0.5 ml Diazo Reagent	0.2 ml Bilirubin solution
1.25 ml water	1.55 ml Dyphylline II

All reactions are carried out at 22° ± 1°C. Bilirubin solutions in 3 gm% albumin are used.

Determination of Diazo Concentration

The basic method of Landis and Pardue (96) was used. In the analysis procedure, dilute and mix exactly 1.00 ml of the

p-diazobenesenesulfonic acid solution to be measured with 4.00 ml of water. Add 2.00 ml of β-Napthol to 180 µl of the diluted diazo reagent, mix, wait five minutes, and then measure the absorbance in a 1.0 cm cell at 486 nm versus a blank without β-Napthol. Compute the concentration as C, µmole/liter= $2.32X10^{3}XA_{486}$, which takes into account the dilution factors and the molar absorptivity of the coupling product.

Isolation of P-Diazobenezenesulfonic Acid (100)

Dissolve 1.1 grams of anhydrous Na_2CO_3 in 50 ml of water, add 4.0 grams of sulphanilic acid monohydrate and heat until it dissolves (if necessary add a little Norit and filter). After the solution cools to room temperature add 1.5 grams sodium nitrite with stirring until it dissolves. Pour the resultant solution into a 400 ml beaker containing 25 ml ice water and 5 ml concentrated HCl. After several minutes cooling on ice the diazonium salt precipitates. Filter the precipitate and wash five times with 50 ml cold 0.5 M HCl. The p-diazobenezenesulfonic acid is stored as a suspension in 20 ml of 0.5 M HCl at -20°C. Care must be taken not to let the salt dry out since dry diazonium salts are explosive.

Alkaline Hydrolysis of Bilirubin Conjugates (101)

To 0.2 ml of sample in a 12 ml conical tube add approximately 5 mg ascorbic acid (sodium salt), 1 mg EDTA (disodium salt), 1 ml methanol and 0.5 ml 1N NaOH. Flush with argon, seal, and incubate at 40°C for thirty minutes. After incubation add sequentially with mixing 0.06 ml glacial acetic acid,

1 ml chloroform, 2 ml glycine HCl buffer pH 2.7, and 1 ml methanol. Separate the two phases by centrifugation for ten minutes. The lower chloroform layer contains all the hydrolyzed (unconjugated) bilirubin.

Determination of the IIIα, IXα, and XIIIα Isomer Composition of Unconjugated Bilirubin (102)

Apply two hundred microliters of an UCB chloroform solution to be analyzed to a pre-coated silica gel plate and develop with a chloroform/acetic acid solvent system (99:1; v/v). Elute the pigments in chloroform and calucate their relative amounts from the A_{452} values.

Hydrolysis of Conjugated Bilirubins with β-Glucuronidase

Add 1.1 ml of a conjugated bilirubin solution (17 to 85 μmolar) in 3 gm% albumin to solid β-glucuronidase (25,000 units), and incubate for thirty minutes at 37° C. For comparison solutions without added β-glucuronidase were also incubated for thirty minutes at 37° C.

Isolation of Reaction Intermediate from Diazo Reaction with UCB

Dissolve two milligrams of UCB in 0.4 ml of 0.1 N NaOH and dilute to 10 ml with 3 gm% albumin in which 0.38 ml of 0.1 N HCl has been added. Diazo reagent containing no excess sulphanilic acid is prepared by adding enough diazonium salt suspension to 0.5 N HCl to make an approximately 2 μMole/L solution.

Add reagents as follows:

3.5 ml UCB solution
10.5 ml methanol
3.5 ml water
8.75 ml diazo reagent
wait twenty minutes
70 mg ascorbic acid (sodium salt)
10 ml chloroform

After the addition of chloroform the reaction mixture is shaken, centrifuged and separated. The lower chloroform layer contains the yellow reaction intermediate and the upper layer the azopigment.

Isolation of Reaction Intermediate from Diazo Reaction with Diconjugated Bilirubin

Precycle two grams of TEAE cellulose by adding 30 ml of 0.15 N HCl; letting it stand for thirty minutes; filtering; washing with water until the pH is 4.0; adding 30 ml 0.5 N NaOH; letting stand for thirty minutes; filtering; and finally washing with water until the pH is nearly neutral. Degass the TEAE cellulose and equilibrate with working Tris buffer. Remove fines by decantation and transfer to a 2 cm inner diameter column. Approximately 170 μMole/L (10 mg/dl) BDG in 3 gm% albumin is used. Diazo reagent containing no excess sulphanilic acid is prepared by dissolving enough solid diazonium salt suspension in 0.5 N HCl to make a solution of approximately 2 μMole/L.

Add reagents as follows:

2 ml BDG solution
8 ml water
5 ml diazo reagent
wait ten minutes

40 mg ascorbic acid (sodium salt)
1.6 ml 10X Tris buffer
adjust pH to 8 with 5 N NaOH

Apply the resulting reaction mixture to the TEAE cellulose column. The yellow reaction intermediate is eluted with working Tris buffer, and the azopigment remains on the column.

IX RESULTS

Reference Chromatographs

A chromatograph of reference pigments is shown in Figure 4. Peak assignments and their related structures are shown in Figure 2. Under the chromatographic conditions used the three isomers of unconjugated bilirubin, bilirubin IIIα, IXα, and XIIIα, appears as one peak or rather three overlapping, unresolved peaks (Figure 4, peak 2). The methyl esters of the two naturally occuring monoglucuronides, C-8 (endovinyl) and C-12 (exovinyl), are peaks 4 and 5, respectively, in Figure 4. The LC technique also separates the methyl ester derivatives of the IIIα (Figure 4, peak 3) and XIIIα (Figure 4, peak 6) isomers of BMG, which arise through dipyrrole exchange. The dimethyl ester of the naturally occuring IXα diglucuronide appears as peak 9 in Figure 4, and is well separated from peaks 8 and 10 which are given by the dimethyl ester derivatives of the IIIα and XIIIα isomers, respectively.

Figure 5 presents a chromatograph of bleached serum. It shows only a small UCB peak and several carotenoid peaks that do not interfere with the assay. One carotenoid peak has a retention time similar to bilirubin monoglucuronide IIIα, but is present in only a small amount and the two peaks can usually be resolved.

Purification and Extraction of Bilirubin Glucuronides

The pretreatment procedure performed before infusion of UCB into the rats was designed to produce bile containing

a high concentration of bilirubin conjugates and low levels of other normal bile constituents. Phenobarbital, a known stimulant of liver conjugating enzymes, was administered to increase the capacity of the liver to clear the infused unconjugated bilirubin. Phospholipid and bile acid content of the enriched bile was minimized according to the procedure of Billing et al. (103), in which the rats were given cholestyramine and the bile drained overnight before beginning bilirubin infusion and bile collection.

Using this collection technique and the purification described in the methods section it is possible to produce BMG and BDG preparations in which the specified conjugate is 90% of the total bilirubin. BMG preparations (a typical chromatograph is presented in Figure 6) contained less than 1% BDG and 8-10% UCB. BDG preparations (a typical chromatograph is presented in Figure 7) contained less than 1% UCB and 8-10% BMG. The relative proportions were calculated by area per cent. The instrument response of BDG is less than that of equimolar amounts of BMG and UCB; therefore, the amount of BDG is slightly underestimated.

The isomer composition of the infused unconjugated bilirubin was approximately 93% IXα, 3% IIIα, and 4% XIIIα as determined by TLC. Analysis of the eluate (Methods, step 6) by TLC immediately after collection showed the isomer composition to be identical to the starting material proving that dipyrrole exchange (see chemistry discussion of iso-

merization) is insignificant in the first six steps. After elution from the Sephadex column work must be performed as quickly as possible, since some dipyrrole exchange is unavoidable. The final preparations have varied from 55 to 75% IXα. Dipyrrole exchange is a problem particularly for BMG since it leads to the formation of a mixture of UCB, BMG and BDG. The extent of dipyrrole exchange in the steps following elution (Methods, steps 7-12) must be determined and ways to minimize it developed.

If spots from the preparative thin layer plates are not removed before the organic solvents have evaporated (Methods, step 11) significant hydrolysis occurs resulting in greater contamination of BMG preparations with UCB and BDG preparations with BMG and UCB. Another problem is oxidation of bilirubin to biliverdin in the ethyl acetate extracts (Methods, step 9). This oxidation limits the time the ethyl acetate extracts may be stored. Biliverdin is easily separated from the conjugated bilirubins on preparative thin layer plates, but its formation means a reduction in yield. Exclusion of oxygen may help reduce biliverdin formation.

Both BMG and BDG can be stored for at least a month in 3 gm% albumin or dry under argon at -20° C.

Kinetics

The object of these studies was to characterize the kinetics of the diazo reaction used in the clinical labor-

atory. Most of the studies utilized conditions for measuring conjugates (direct reacting). The total reaction is expected to be two consecutive second order reactions (see chemistry section) which in simplified terms can be written as follows:

$$B + D \xrightarrow{k_1} A_1 + I$$

$$I + D \xrightarrow{k_2} A_2 + CH_2O$$

B = Bilirubin
D = Diazonium ion
A_1 and A_2 = azodipyrroles
I = reaction intermediate
k_1 = rate constant first step
k_2 = rate constant second step

The diazonium ion is present in large excess; therefore, the reaction simplifies to two consecutive pseudo first order processes. The three possible combinations of k_1' and k_2' (pseudo first order rate constants) give the semi-log plots of absorbance verse time shown in Figure 8. When k_1' is much greater than k_2' (Figure 8a) the two steps will be well separated, and the semi-log plot will be two straight lines with slopes of k_1' and k_2' respectively. When k_2' is much greater than k_1' (Figure 8b) the second step follows immediately after the first step occurs, and the semi-log plot will be a straight line with a slope equal to $2k_1'$ (because both A_1 and A_2 are being measured). Finally k_1' and k_2' can be of the same order of magnitude (Figure 8c) in which case the two steps are not well separated, and the semi-log plot is

curved.

Data collection was limited to a thirty second time interval with the Durrum stopped-flow instrument because data taken for longer periods of time is subject to drift. In all experiments the reaction was incomplete within this time period. A typical experiment is shown in Figure 9 which is derived from an oscilloscope trace of voltage (proportional to transmittance) versus time. Computer analysis (Figure 10) converts transmittance to absorbance and plots both absorbance and the log of the absorbance with the best fit exponential function versus time. The direct reaction data seemed to fit a single exponential function best. This suggests one of two possibilities. The first is that k_2' is much greater than k_1' as in Figure 8b. The second is that k_1' is much greater than k_2' (Figure 8a) and during the time of measurement only the first step has taken place. Earlier work (see chemistry section) points to the second possibility as being more likely. In either case there could be a large error in the infinity time measurement, in the first case because the reaction has not gone to completion, and in the second because there is no clear break point between the steps. To test the power of the computer analysis to handle such situations a hypothetical single exponential function (Figure 11), and the same function with a 30% error in the infinity time point (Figure 12) were generated. Figures 11b

and 12b are semi-log plots of the data, and it can be seen that errors in the infinity time measurement cause a spurious non-linearity. Figures 11c and 12c show the computer analysis of the data, and it can be seen that in spite of the large error, the a(1) and b(1) best fit parameters are the same; i.e., the computer corrected the error in the infinity time measurement by altering a(0). This demonstrated that the programs have the capability of correcting for the expected uncertainties in the infinity time measurements.

Although a single exponential form was the best fit of the data, at each level of bilirubin tested the rate constant varied. This is inconsistent with a first order reaction. Before any conclusions on the reaction mechanism can be drawn, data over the entire reaction course must be collected. The reaction can be slowed by reducing the diazo concentration to a level where the time course can be followed on a double beam instrument. Alternatively, the rate can be increased by employing a diazo concentration sufficient to drive the reaction to completion within thirty seconds. Lowering the diazo concentration was attempted and did not work because the reaction does not go to completion (see stiochiometry section).

Since sulphanilic acid catalyzes the second step, an experiment using a diazo reagent without excess sulphanilic acid was tried in an attempt to isolate the first step.

This failed because the diazo concentration was high enough that the reaction proceeded beyond the first step, and there was not a clear separation of steps (single exponential functions did not give the best fit).

In a preliminary experiment, the total reaction (see Methods for conditions) also did not reach completion within thirty seconds. Two exponential functions gave the best fit with the first step being about three times faster than the second. More experiments on the total reaction must be done before any firm conclusions can be drawn.

Stoichiometry

Tables 1 and 2 present results comparing total and direct reactions on paired samples of BMG and BDG. Both steps were assumed to go to completion in the total reaction, and the percent completion of the direct reaction was calculated using the total reaction as 100%. The amount of UCB in BDG preparations was less than 1% and no correction was made for it; however, four BMG preparations were used which contained 8, 9, 14 and 23% UCB, and corrections were made for its presence. BDG concentrations up to approximately 85 μMole/L (5 mg/dl) and BMG concentrations up to approximately 51 μMole/L (3 mg/dl) were used. The direct reaction went to virtual completion with both BMG and BDG, and there is no statistical evidence that the total and direct reactions were different. When the diazo concentra-

tion was reduced to one quarter of that normally used, while holding the sulphanilic acid concentration constant, the reaction did not go to completion even when the time was extended to an hour. In ten minutes (n=4) BMG reacted to an extent of 79-85% of the controls run under standard conditions. The extent of reaction of BDG (n=4) was 85-90%. Diazo reagent concentration was measured to establish consistent composition. The standard diazo concentration ranged from 1.4 to 1.6 μMole/L and the reduced diazo concentration ranged from 0.36-0.39 μMole/L. Raising the diazo concentration would only increase the amount of UCB reacting since the reaction goes to completion for the conjugates when the standard diazo concentration is used.

The results of a comparison between a one minute, two minute and a ten minute direct reaction are shown in Table 3. At one minute greater than 90% of the reaction has taken place, and at two minutes greater than 95% of the reaction has taken place. Table 4 shows the results of the direct and total reaction of mixtures of UCB, BMG and BDG. In all cases the observed absorbances were equal to the sum of the individual components, thus it appears that UCB, BMG and BDG react independently of each other.

To determine the validity of employing the standard curve for UCB to measure conjugated bilirubins, the diazo reactivity of conjugated samples was compared with paired samples which had been hydrolyzed with β-glucuronidase (Table 5). Hydrolysis of conjugates was verified by AMHPLC analysis of

specimens after incubation with glucuronidase. After hydrolysis BDG samples contained no detectable BDG, 15% BMG, the remainder being UCB. With BMG, 17% remained unhydrolyzed and BDG was not detected. No significant difference in absorbance between hydrolyzed and unhydrolyzed samples was observed, thus suggesting that the presence of glucuronide has little effecton the alkaline chromaphore.

Factors Affecting the Second Step of the Diazo Reaction

The catalytic affect of sulphanilic acid on the second step has been reported (see Chemistry section); however, its dependence on pH and diazo concentration has not been studied. In the standard procedure the diazo reagent is made with an excess of sulphanilic acid; if a diazo reagent is prepared which contains only diazonium ion and no sulphanilic acid and used in the direct reaction, the reaction does not go to completion. Spectra of starting bilirubin conjugates (A_{max}= 420nm), complete reaction (A_{max}= 530nm), and partial reaction in the absence of sulphanilic acid are shown in Figures 13 and 14. Since UCB (not shown) reacts very slowly in the direct reaction it was observed for 24 hours. However, the prolonged reaction time leads to the formation of a complex mixture of products. If methanol is added to the reaction it is accelerated, but in the absence of sulphanilic acid it does not go to completion. This is analogous to the reaction of conjugates in the direct reaction.

The amount of azopigment produced can be increased by

increasing the diazo concentration (from a 100 fold excess to a 1000 fold excess). Once the reaction has terminated, the addition of sulphanilic acid and/or more diazo reagent produces no further reaction. At pH values higher than 6 this phenomenon was no longer observed, which indicates that sulphanilic acid is no longer needed as a catalyst for the second step.

Visible Spectra of BMG and BDG in Serum

The spectra of UCB, BMG, and BDG in serum versus a serum blank are shown in Figure 15. The λ_{max} of 462 for UCB and 415 nm for BDG agree well with published values (104, 105, 106). Spectra of well characterized BMG preparations have not been published, and it has generally, but erroneously, been concluded that the spectrum of BMG is the same as BDG. In serum at neutral pH BMG has the same λ_{max} as UCB, and the spectra exhibits a marked shift to λ_{max}=420 nm at pH 2 (Figure 16). The two peaked spectra reported for BDG in protein solutions (106) may be due to contamination with BMG.

X CONCLUSIONS

The recent development of a definitive method for measuring bilirubin and bilirubin conjugates and of improved procedures for isolating conjugated bilirubins has made possible a more detailed study of the chemistry of the diazo reaction. Previously, quantitation of conjugated bilirubins by diazo methods was thought to be inadequate for two reasons. The first is that UCB interferes, and the second is that conjugates were thought to react incompletely (see Chemistry section). With well characterized preparations of BMG and BDG this study has established that the direct reaction goes to virtual completion in ten minutes for both BMG and BDG. Earlier conclusions that conjugates react incompletely can be attributed to contamination of the preparations with UCB. The analysis of purified conjugates (by AMHPLC) is required to insure that significant hydrolysis has not occurred and to correct results for the presence of UCB, if necessary. Two minutes was found to be a satisfactory time for the direct reaction. At this time greater than 95% of the reaction has occured, with a minimum of interference from UCB. The reactivity of UCB in the direct reaction (2-5%) was found to be similar to that observed in earlier work. The reaction of mixtures of bilirubins was equal to the sum of the individual components thus showing that they react independently and eliminating the possibility that the presence of conjugates increases the reactivity of UCB in the direct reaction.

The molar absorptivities of the diazodipyrrole products of conjugated bilirubin is not known. To determine them exactly would require purifying each conjugate substrate to nearly 100%, which we have as yet not been able to accomplish. However, by comparing the diazo reaction of conjugated preparations in the presence of dyphylline with samples subjected to hydrolysis with glucuronidase, it was established that little or no difference exists in the absorbance of the conjugated and unconjugated dipyrrole products. This means that the standard curve for UCB can be employed for conjugates; something that is always done though the validity has never been proven.

The formation of a non-reactive intermediate in the direct reaction, when a diazo reagent is used which contains no excess sulphanilic acid, is inconsistent with the commonly proposed mechanism in which a hydroxypyrrocarbinol is formed in the first step and then reacts with another diazonium molecule. One possibility (Figure 17) is that in the first step an intermediate is formed which can be converted to either a diazo reactive or non-reactive intermediate. Sulphanilic acid may catalyze the formation of the diazo reactive product, stabilize the reactive intermediate and/or catalyze its further reaction. This mechanism also explains the lower yield found when the diazo reagent concentration is reduced even in the presence of sulphanilic acid. Perhaps a lower reaction rate allows formation of some non-reactive side product. The

failure to see a "non-reactive" side product when the pH is raised, may be due to an increase in the rate of reaction, stabilization of the reactive produce, and/or increase in reactivity of the non-reactive side product. Isolation and characterization of the reaction intermediates, procedures for partial purification are given in the Methods section and should help resolve some of the questions regarding the reaction mechanism.

Formidable problems were encountered in the kinetic studies of the diazo reaction. The reaction rate was found to be too slow for the stopped flow instrument and too fast for use on a standard laboratory double beam spectrometer. Studies of the early part of the reaction on the stopped flow instrument did not yield any useable information. Slowing the reaction by reducing the diazo concentration and studying it on a double beam instrument will not work because at lower diazo concentrations the reaction does not go to completion. Assuming that the reaction is two consecutive second order reactions, then raising the diazo concentration so that the reaction is fast enough to be studied on a stopped flow instrument should yield information that is applicable to conditions used in the laboratory. These experiments have yet to be done. However, since the second step appears to be more complicated that previously thought it may turn out that the kinetics for the entire reaction is too complex to characterize with currently available laboratory and data handling techniques. Binding to proteins and the possibility of intramolecular

interactions (dimerization) may also complicate the kinetics.

This work established that conjugates react completely in the direct reaction and that bilirubins react independently of each other. However, diazo methods still suffer from two major liabilities. The first is that in patients with hepatobiliary disease there is a significant amount of non-bilirubinoid diazo positive material which interferes with the assay, and the second is that small amounts of conjugates in the presence of UCB cannot be detected because of the reactivity of UCB in the direct reaction. The AMHPLC method solves these problems but is too complex for routine use in the clinical laboratory. This work suggests several avenues of future investigation in the development of better total and conjugate bilirubin assays which are practical for the routine clinical laboratory. It might be possible to find conditions under which only the first step of the reaction takes place, such as lowering the pH or using another diazonium reagent. If the first step can be isolated its kinetic properties can be studied independently and could eventually lead to the development of a kinetic assay. The most promising possibility for the development of an assay which is specific and sensitive for small amounts of conjugates lies in the spectral properties of BMG, which has a λ_{max}= 460 nm at pH 7 and a λ_{max}=420 nm at pH 2. A difference spectra might be able to detect BMG in the presence of both UCB and BDG. Since serum from patients with hepatobiliary disease contains predominantly BMG such a test should be a sufficient measure of conjugates.

TABLE 1

Comparison of 10 Minute Direct and Total Reaction for Diconjugated Bilirubin

(Six Paired Samples)

Direct (OD)	Total (OD)	Difference
.153	.147	0.006
.154	.154	0
.167	.167	0
.167	.171	-0.002
.267	.264	-0.003
.274	.273	0.001

Average Difference = 0.0003

S = 2.88

t = 0.26

$t_{95\%} = 2.57$

TABLE 2

Comparison of 10 Minute Direct and Total Reaction for Monoconjugated Bilirubin

(8 Paired Samples)

Direct (OD)	Total* (OD)	Difference
.171	.172	0.001
.094	.098	0.004
.094	.098	0.004
.165	.169	0.004
.079	.079	0
.079	.079	0
.063	.062	-0.001
.060	.061	-0.001

Average Difference = 0.0016

S = 2.52

t = 1.68

$t_{95\%}$ = 2.36

*corrected for amount of unconjugated bilirubin

TABLE 3

Time Dependence of Direct Reaction*

	1 minute n=6		2 minute n=8	
	%	S	%	S
Monoconjugated Bilirubin	92	5.1	96	4.4
Diconjugated Bilirubin	94	3.3	96	2.3

*expressed as per cent 10 minute Direct Reaction

TABLE 4

Diazo Reactivities of Bilirubin Mixtures

	Direct reaction		Total reaction	
	A	sum of components	A	sum of components
UCB	.026	-	.310	-
BMG	.062	-	.068	-
UCB + BMG	.080	.088	.374	.378
BDG	.168	-	.169	-
UCB + BDG	.197	.194	.480	.479
UCB + $\frac{BMG}{2}$ + $\frac{BDG}{2}$	.134	.141	.435	.429

TABLE 5

Comparison of Optical Densities of Nine Paired Samples of Conjugates After One Has Been Hydrolyzed*

Unhydrolyzed	Hydrolyzed[F]	difference
.052	.053	0.001
.052	.052	0
.055	.055	-0.003
.140	.141	+0.001
.138	.143	+0.005
.139	.143	+0.004
.233	.232	-0.001
.231	.231	0
.227	.236	+.009

Average difference = 0.002

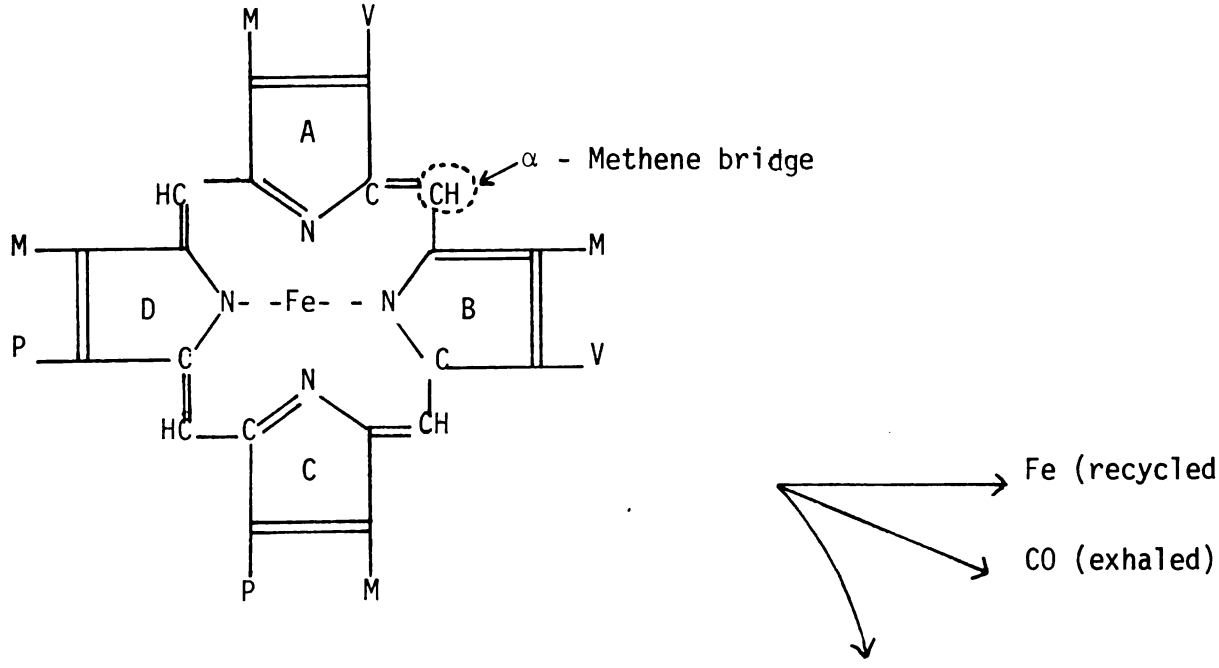
S = 4.6

t = 0.84

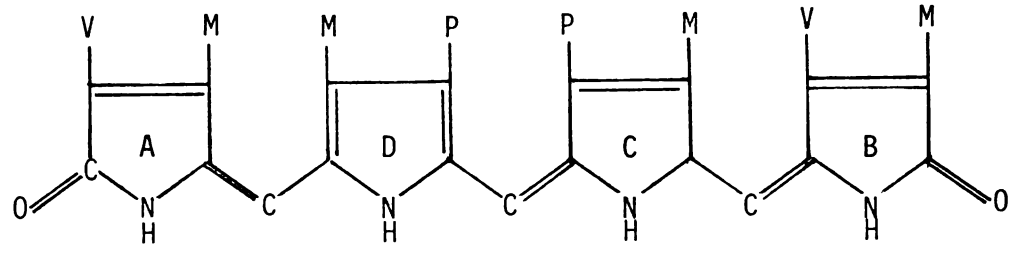
$t_{95\%}$ = 2.31

*hydrolyzed samples corrected for diazo positive material in β-glucuronidase (always less than 10% of total O.D.).

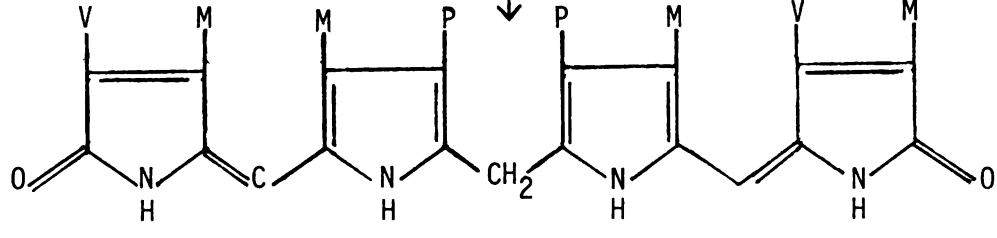
[F]after hydrolysis monoconjugated bilirubin contained 17% monoconjugated bilirubin with the rest being unconjugated bilirubin, and diconjugated bilirubin contained no detectable diconjugated bilirubin and only 15% monoconjugated bilirubin with the rest being unconjugated bilirubin.



Protoporphyrin IX



Biliverdin IX α



Bilirubin IX α

FIGURE 1. - Conversion of Protoporphyrin IX to Bilirubin IX α

V = vinyl group
M = methyl group
P = propyl group

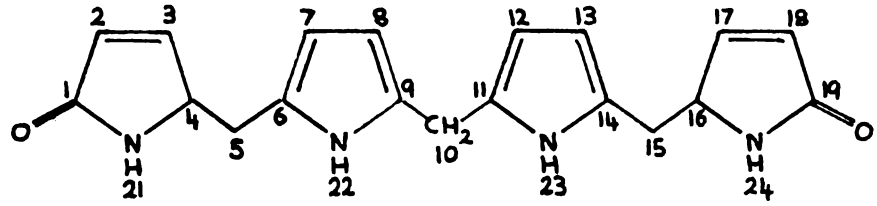


Figure 2. Bilirubin Skeletal Structure and Isomers.

*PG groups are PM esters in HPLC analysis.

Compound	Substituents in Position						Chromatography assignment in Figure 4.*
	2	3	8	12	17	18	
Bilirubin III α	V	Me	P	P	Me	V	2
Bilirubin IX α	V	Me	P	P	Me	V	2
Bilirubin XIII α	Me	V	P	P	Me	V	2
Bilirubin III α Monoglucuronide	V	Me	P	PG	Me	V	3
Bilirubin IX α C8 - Monoglucuronide	Me	V	PG	P	Me	V	4
Bilirubin IX α C12 - Monoglucuronide	Me	V	P	PG	Me	V	5
Bilirubin VIII α - Monoglucuronide	Me	V	P	PG	V	Me	6
Bilirubin III α - Diglucuronide	V	Me	PG	PG	Me	V	8
Bilirubin IX α - Diglucuronide	Me	V	PG	PG	Me	V	9
Bilirubin XIII α Diglucuronide	Me	V	PG	PG	V	Me	10

In all of the bilirubins listed, the substituent at position 7 and 13 is a methyl group. Me=Methyl; V=Vinyl, P=Propionic acid, PG=Glucuronide ester of propionic acid.

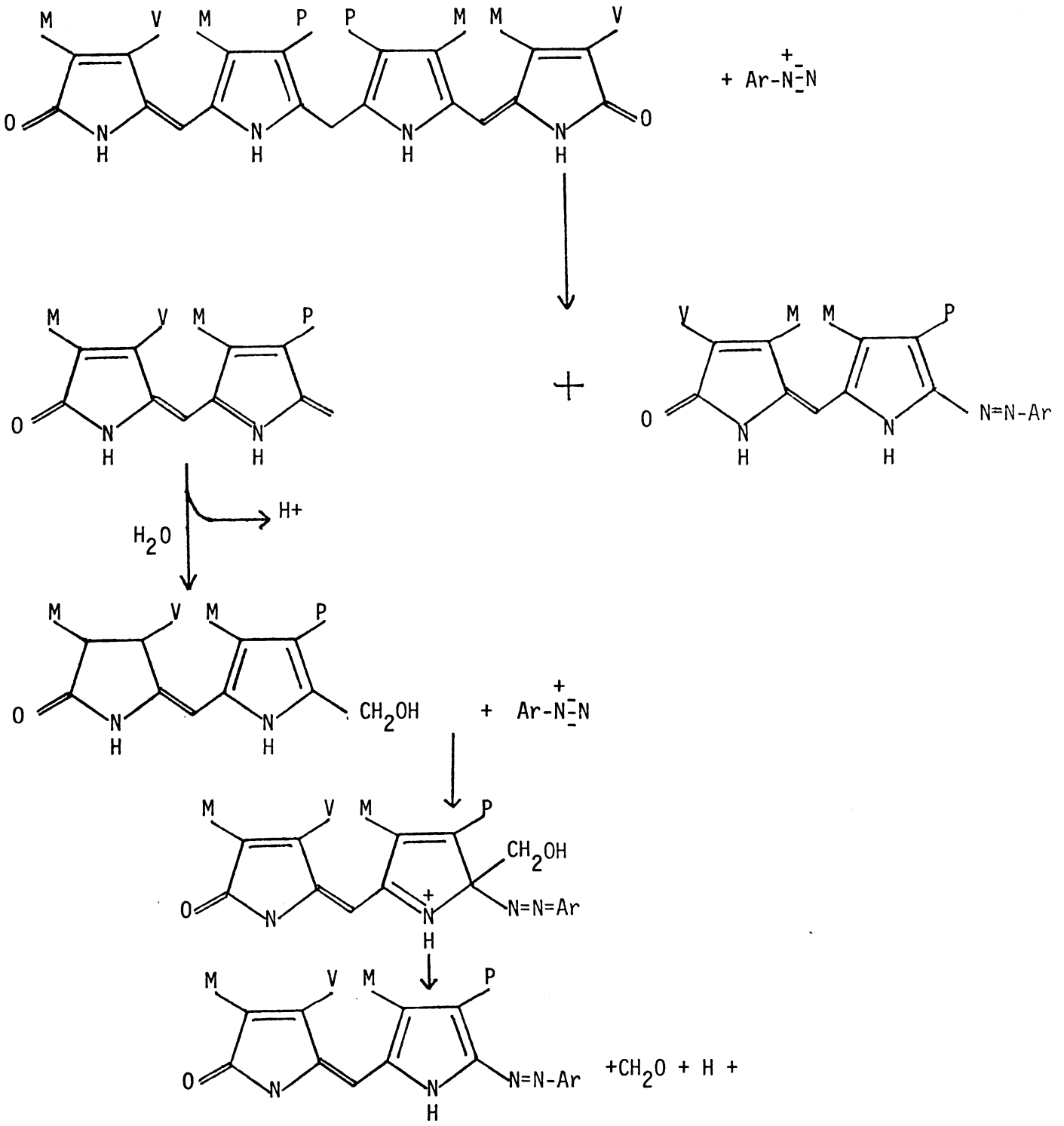


Figure 3. Mechanism of Reaction of Bilirubin with Aromatic Diazonium Ion.

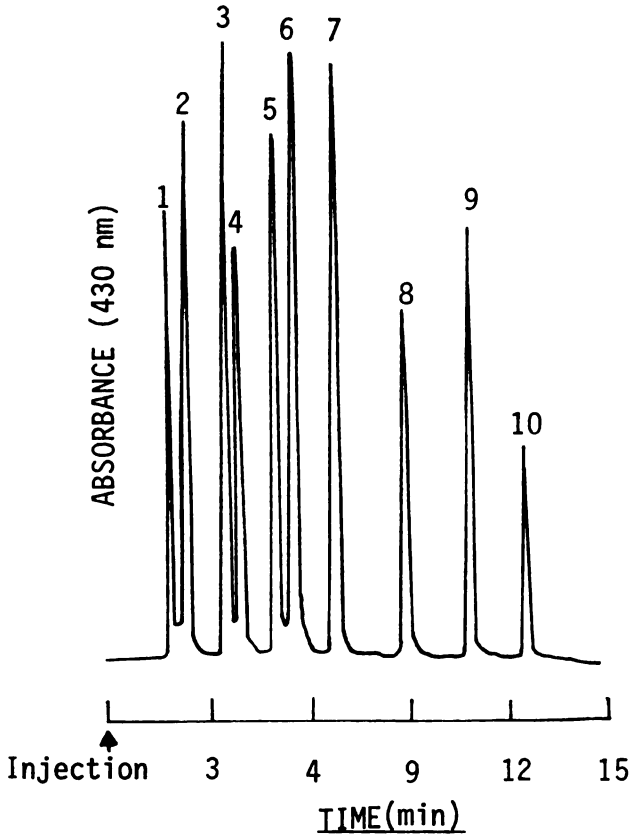


Figure 4. Chromatogram of the reference pigments.

A mixture of β-carotene, internal standard, and the reference bilirubins, dissolved in chloroform, was injected in the liquid chromatography at time 0. The structure of the pigment corresponding to each peak in the chromatogram is given in Figure 2. Peak 1 is β-carotene; Peak 7 is internal standard.

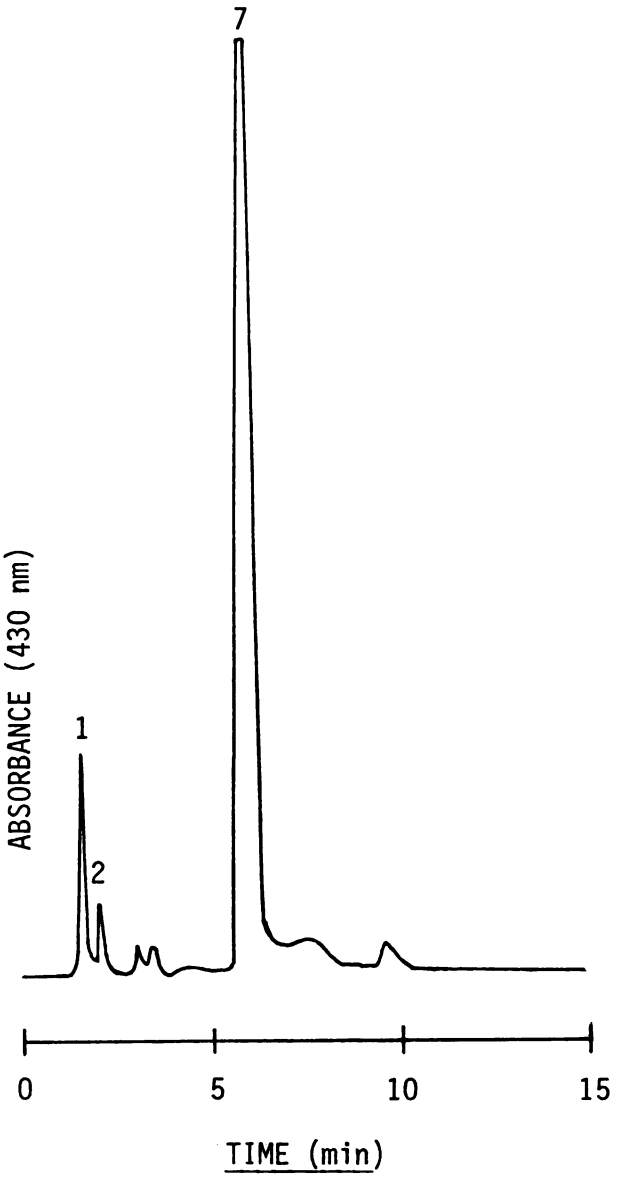


Figure 5. AMHPLC analysis of Bleached Serum.
Peaks identified in Figure 2.
Peak 7 internal standard.

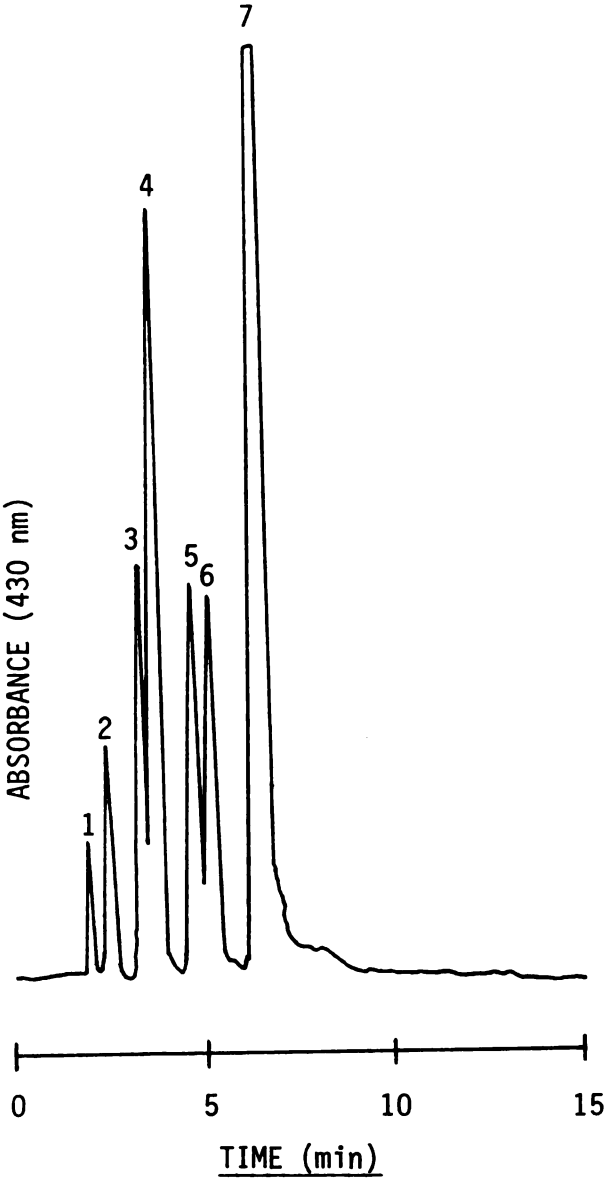


Figure 6. AMHPLC Analysis of BMG preparation.
Peaks identified in Figure 2.
Peak 7 internal standard.

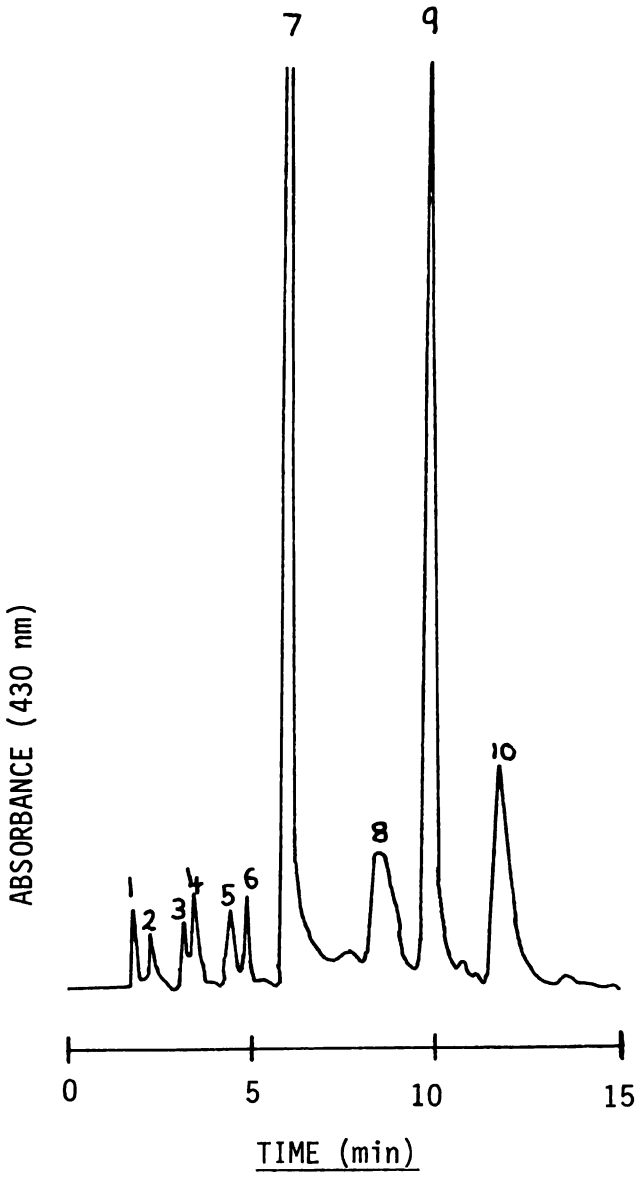
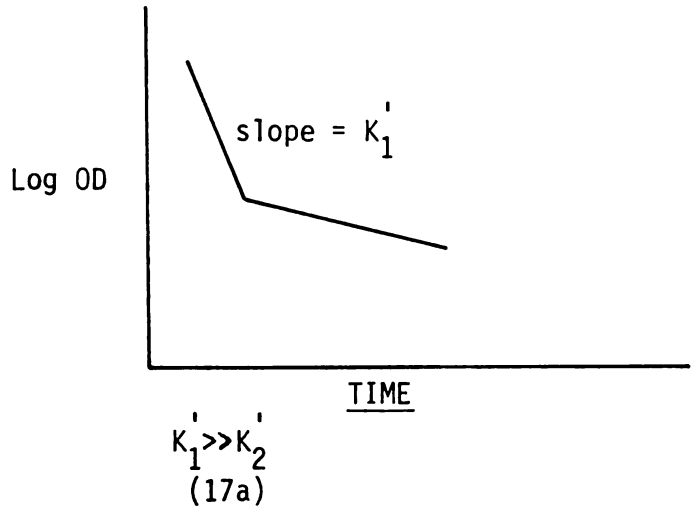
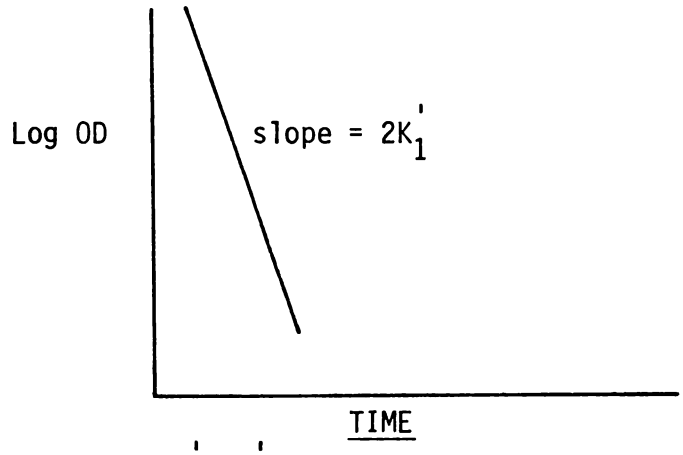


Figure 7. AMHPLC Analysis of BDG preparation.
Peaks identified in Figure 2.
Peak 7 internal standard.



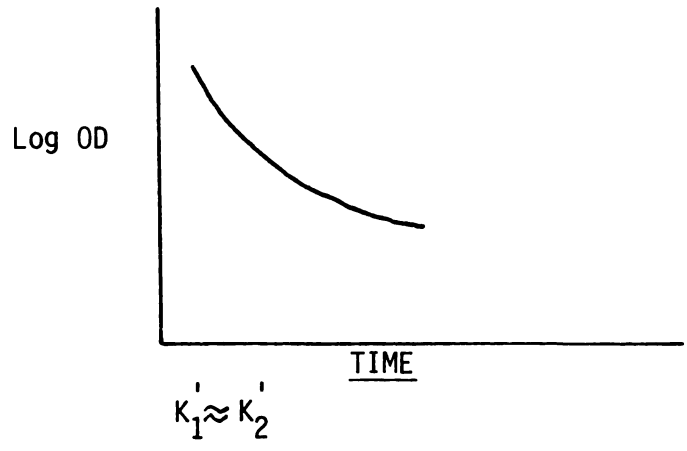
$K_1' >> K_2'$

(17a)



$K_2' >> K_1'$

(17b)



$K_1' \approx K_2'$

(17c)

Figure 8. Semi-Log Plots for Two Consecutive First Order Reactions.

K_1' = rate constant first step.

K_2' = rate constant second step.

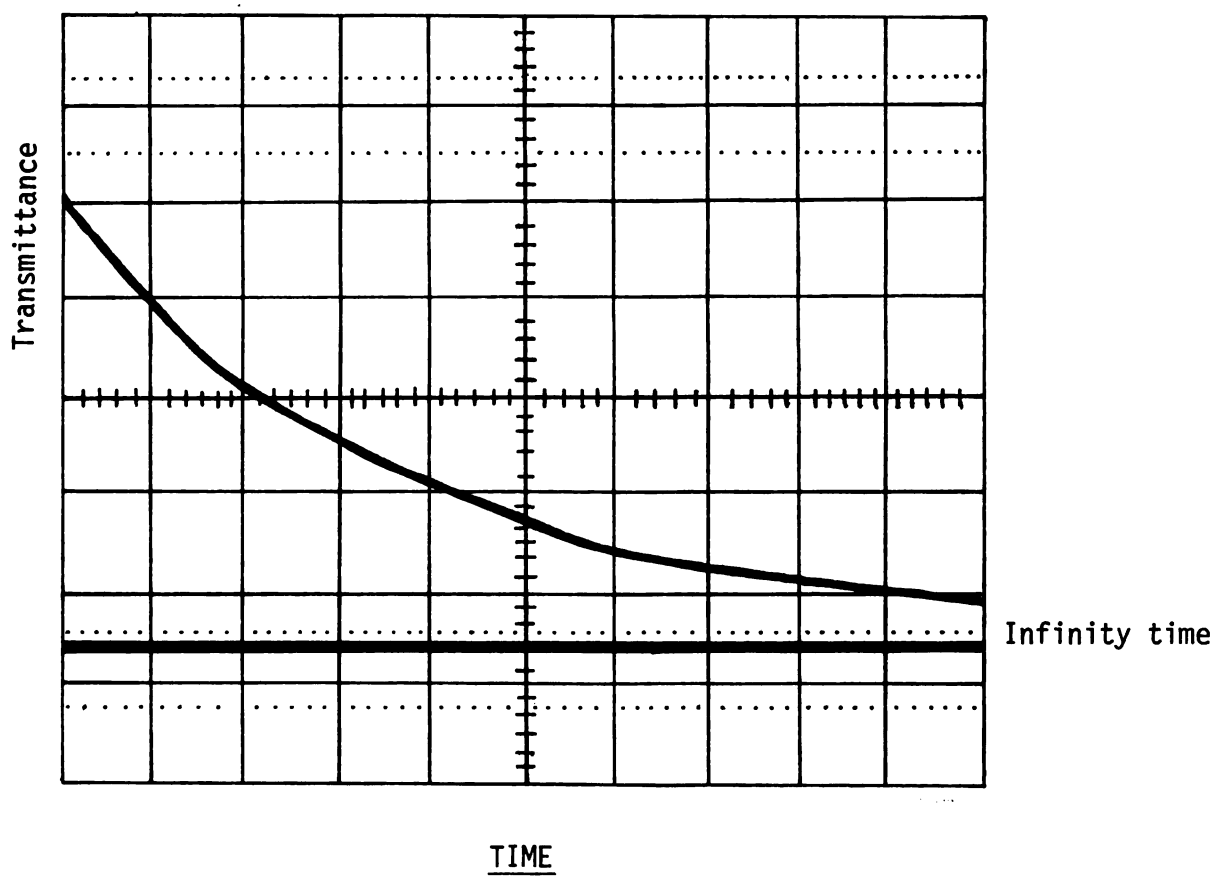


Figure 9. Kinetic Experiment of Direct Reaction (Oscilloscope trace).

0 - 10 volts 100% T

Y - axis 1 volt/div offset 4.13 volts
X - axis 3 sec/div

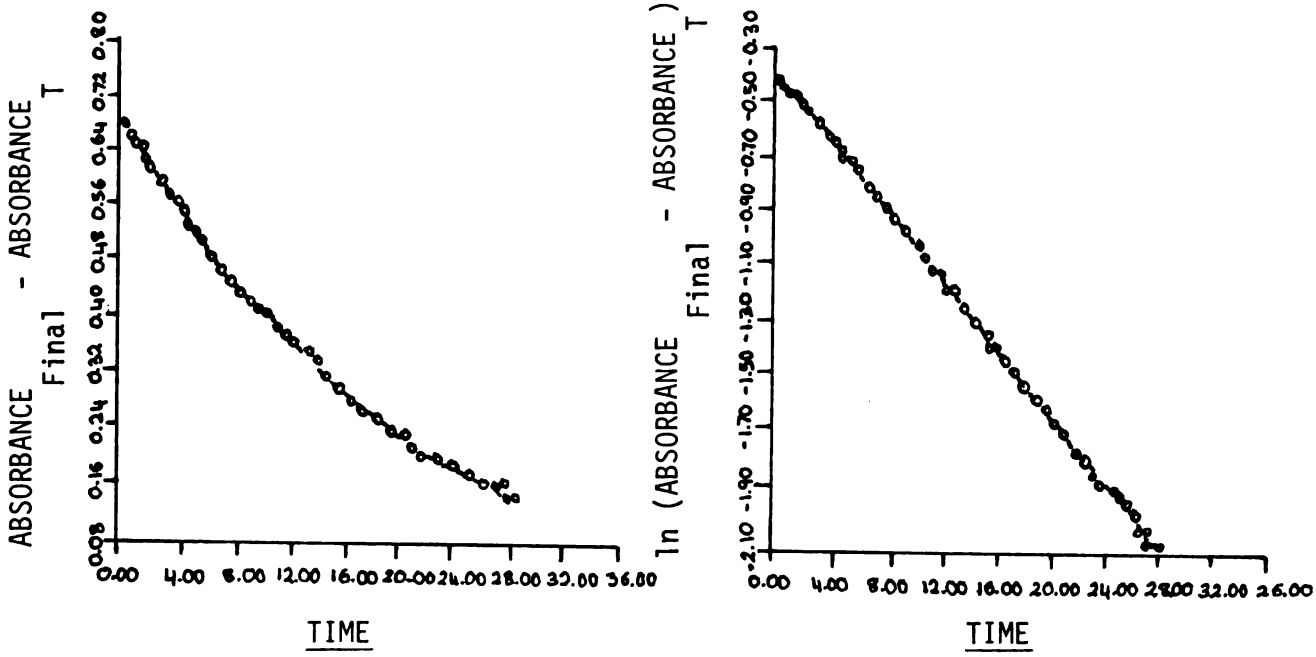
Reaction Conditions

Diazo reagent 600 µmolar diazonium ion, 8.3m molar Sulphanilic Acid.

pH = 2

BDG 0.6 µmolar

Albumin 0.4 gms/dl



10a 10b

Figure 10. Analysis of Experiment Shown in Figure 9.

------- best fit conputer generation

ooooooo actual data points.

fit is to the funcitonal form

1(0) + a(1)*exp(-b(1)*t) + a(2)*exp(-b(2)*t) +...

best fit parameters with standard errors are:

a(0) = 0.185d-01 s.e.=0.435d-02

a(1) = 0.690d+00 s.e.=0.376d-02

b(1) = 0.661d-01 s.e.=0.900d-03

10(c)

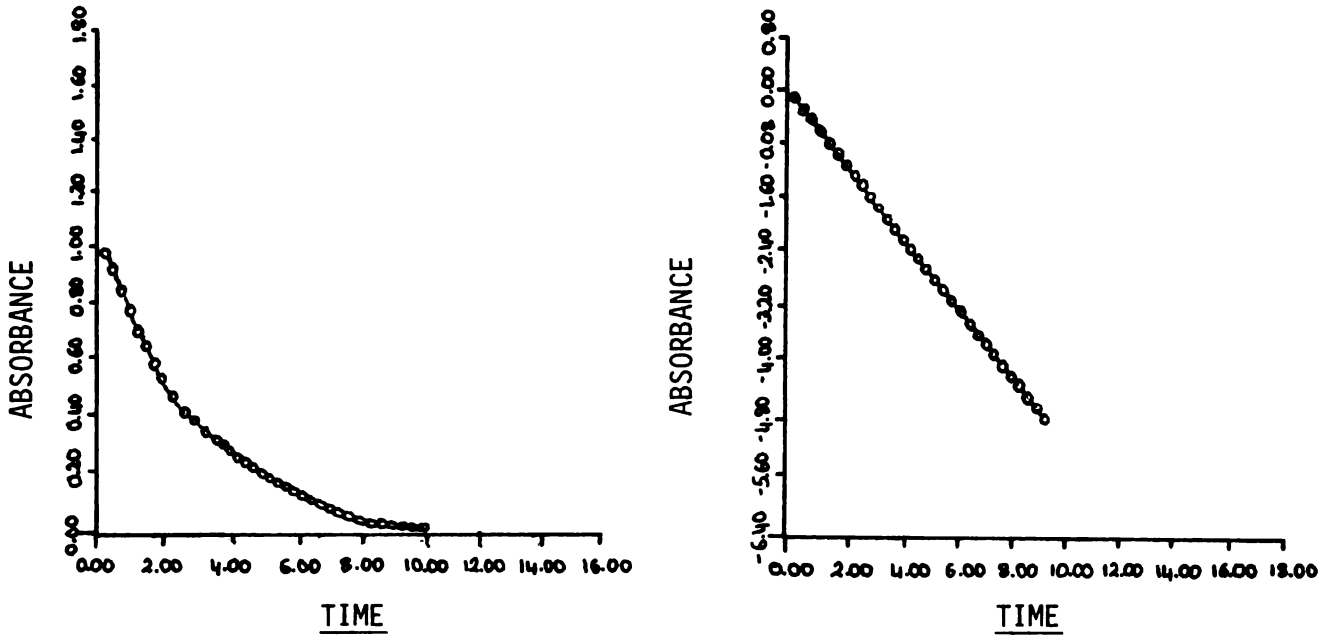


Figure 11. Analysis of Generated One Exponential Function.

------ best fit computer generation

oooooo actual data points.

fit is to the functional form.

a(0)=a(1)*exp(-b(1)*t) + a(2)*exp(-b(2)*t) +...

Best fit parameters with standard errors are:

a(0)=-0.562d-05 s.e. =0.739d-05

a(10= 0.999d+00 s.e. =0.187d-04

b(1)= 0.499d+00 s.e. =0.192d-04

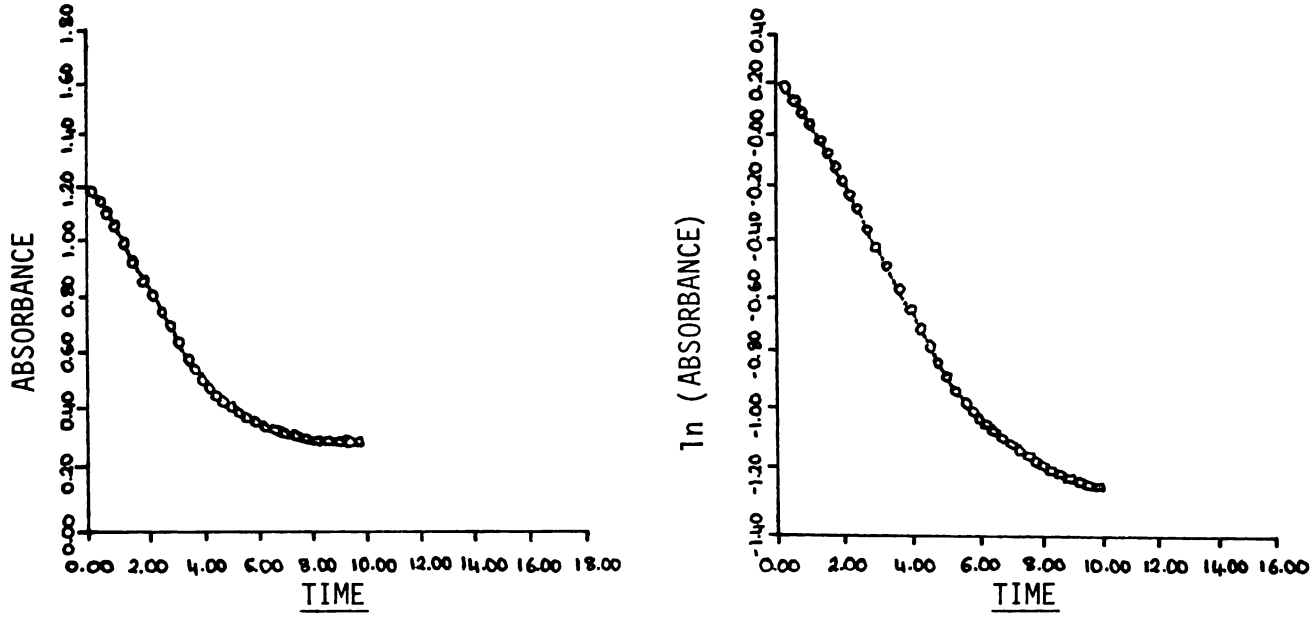


Figure 12. Analysis of Generated One Exponential Function with a 30% error in the base line.

------ best fit computer generation

oooooo actual data points.

fit is to the functional form.

a(0)=a(1)*exp(-b(1)*t) + a(2)*exp(-b(2)*t) +...

Best fit parameters with standard errors are:

a(0)=0.300d-00 s.e. =0.739d-05

a(1)=0.999d+00 s.e. =0.187d-04

b(1)=0.499d+00 s.e. =0.192d-04

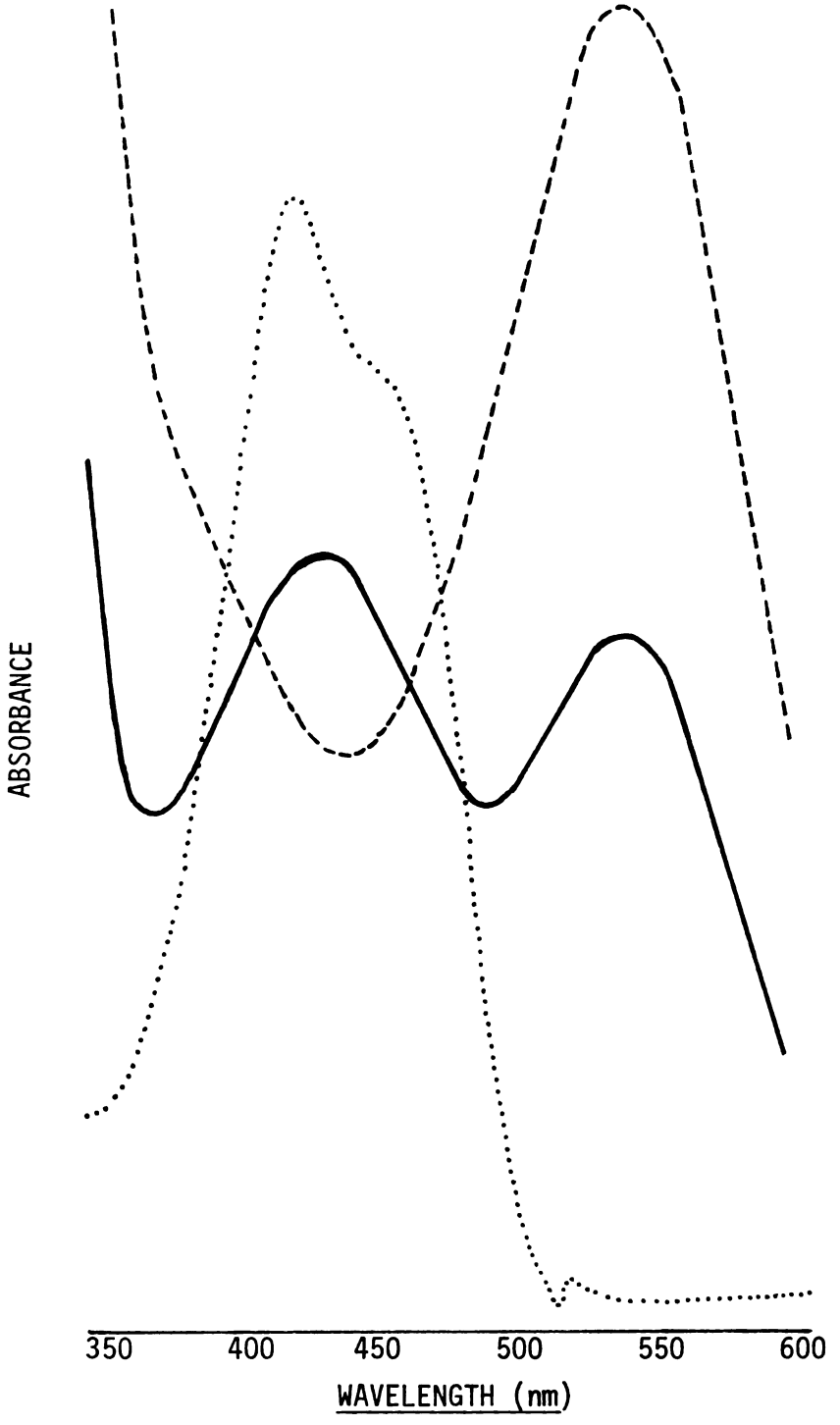


Figure 13. Reaction of BMG in direct reaction with and without sulphanilic acid.

..... BMG - sulphanilic acid added instead of diazo reargent.
-------- BMG - direct reaction.
_____ BMG - direct reaction, diazo reagent no excess sulphanilic acid.

Ascorbic acid, dyphylline, and alkaline tartrate were not added, spectra taken after 20 minutes reaction time.

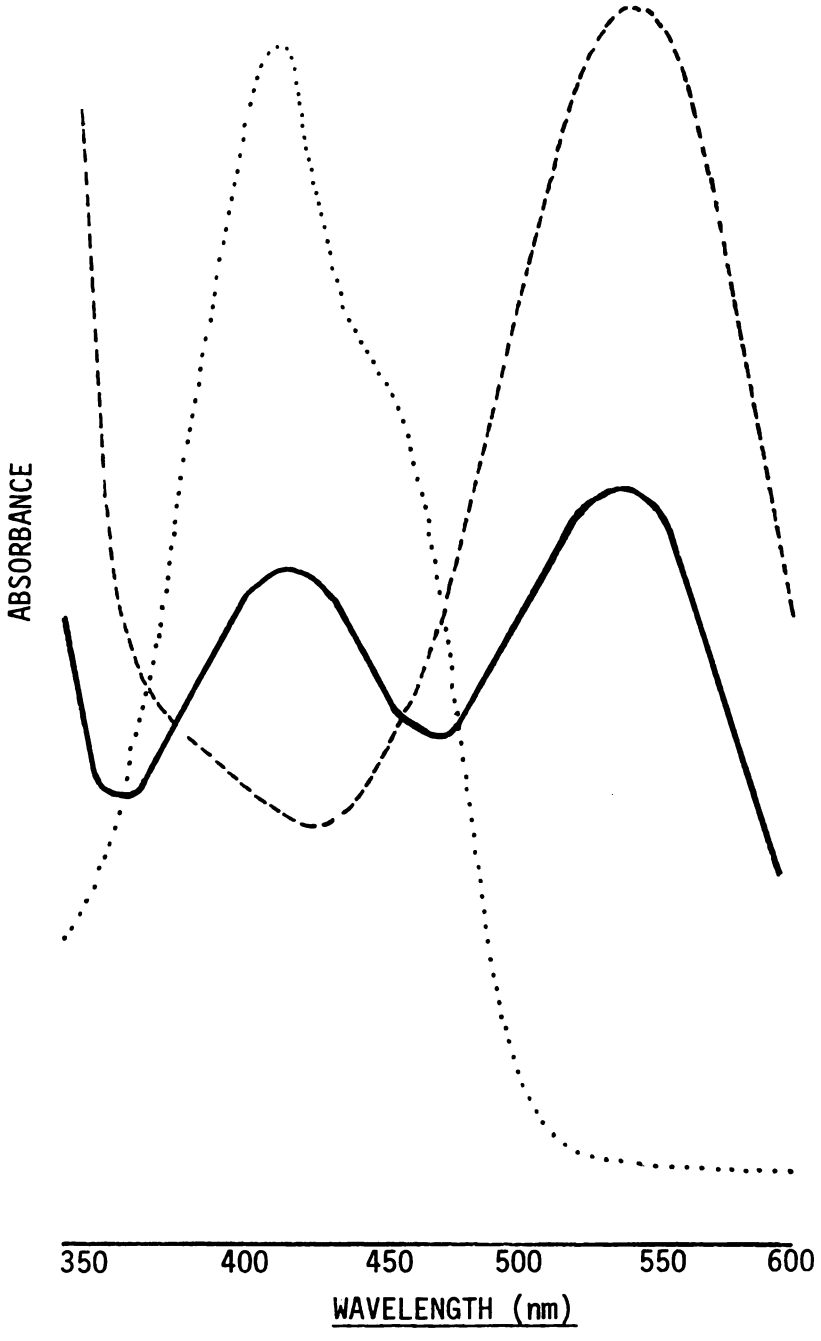


Figure 14. Reaction of BDG in direct reaction with and without sulphanilic acid.

..... BDG - sulphanilic acid added instead of diazo reargent.
-------- BDG - direct reaction.
_____ BDG - direct reaction, diazo reagent no excess sulphanilic acid.

Ascorbic acid, dyphylline, and alkaline tartrate were not added, spectra taken after 20 minutes reaction time.

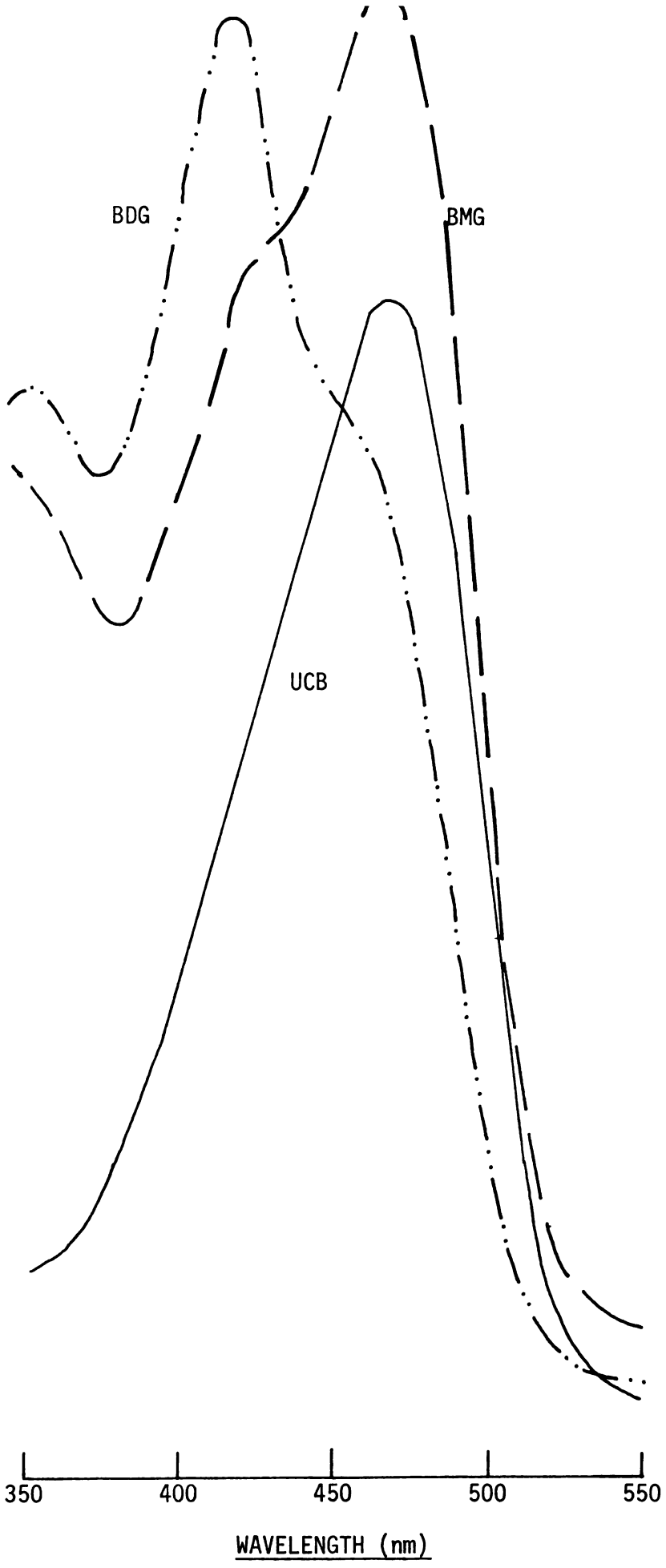


Figure 15. Spectra of UCB, BMG and BDG in Serum.

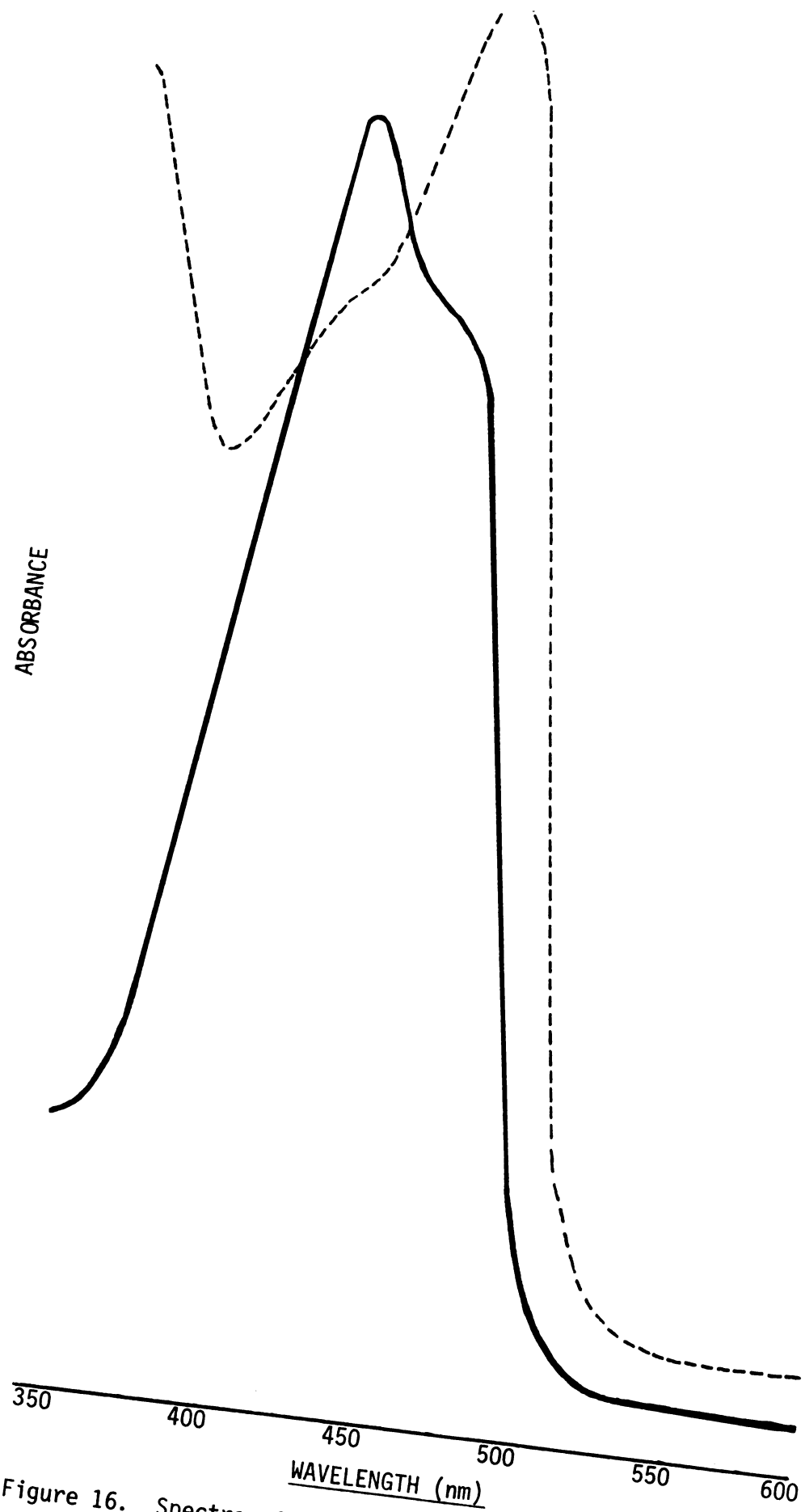
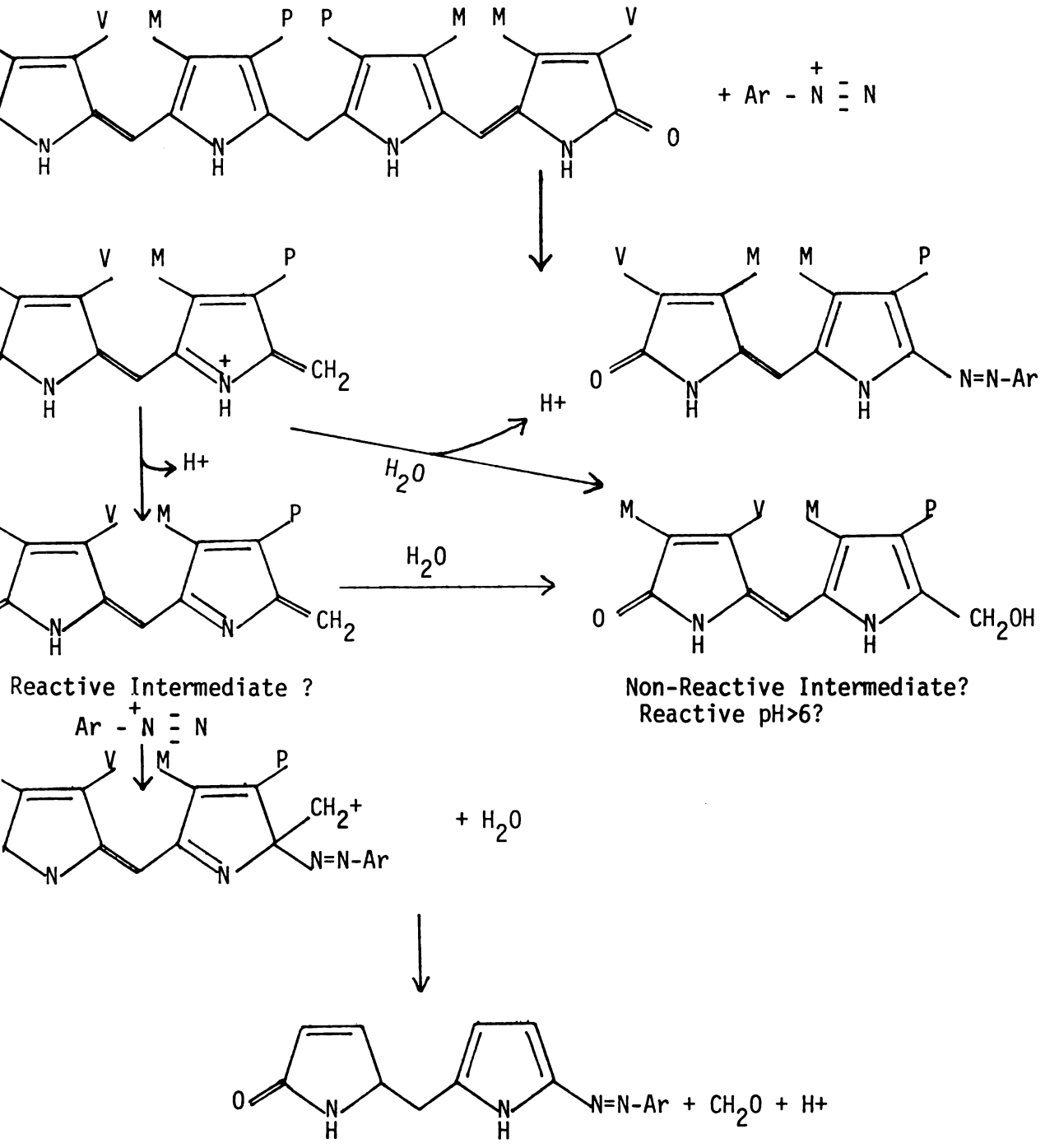


Figure 16. Spectra of BMG in sera at pH of 7 and 2.
——— pH= 2
- - - - - - pH= 7



re 17. Proposed Diazo Reaction Mechanism of Bilirubin with Aromatic Diazonium Ion.

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