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# A study of neonatal hyperbilirubinemia: the contribution of the enterohepatic circulation of bilirubin

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# A STUDY OF NEONATAL HYPERBILIRUBINEMIA: THE CONTRIBUTION OF THE ENTEROHEPATIC CIRCULATION OF BILIRUBIN

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and motivated the author from day to day. And lastly, I owe a great deal to my wife, Pat, for all her help through many hard times.

F. Sessions Cole, III
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New Haven, Connecticut

DEDICATION

To the three people who have influenced me the most in my last four years:

To Michael S. Kramer, who convinced me that learning and studying are not synonomous;

To G. Morris Dillard, M. D., a model for my own development as a physician, whose great sensitivity to patients and students is paralleled only by his awesome clinical knowledge;

To Pat, my wife, who has helped and continues to help me through many hard times.

## Neither Out Far Nor In Deep

The people along the sand
All turn and look one way.
They turn their back on the land.
They look at the sea all day.

As long as it takes to pass
A ship keeps raising its hull;
The wetter ground like glass
Reflects a standing gull.

The land may vary more;
But whatever the truth may be--
The water comes ashore,
And the people look at the sea.

They cannot look out far.
They cannot look in deep.
But when was that ever a bar
To any watch they keep?

Robert Frost

TABLE OF CONTENTS

# I. INTRODUCTION

## 1. Definition of problem

In the neonate, within the first five to seven days of life, the serum bilirubin may increase from less than one mg.% in cord blood to values as high as 25 mg.%, and then spontaneously remit. In an adult, such a rapid rise would indicate the need for investigation of hepatic function. In the neonatal age group, this phenomenon is viewed as a normal variation. Despite the prevalence of neonatal jaundice, both "physiologic" and as a complication of other medical and maternal problems, the exact causes and long-term effects of hyperbilirubinemia are incompletely understood. This investigation has examined one cause of neonatal jaundice, the enterohepatic circulation of bilirubin. To show the importance of this factor, current concepts of neonatal bilirubin metabolism will be reviewed. On the basis of this review, the rationale for and effectiveness of the various treatment modalities for hyperbilirubinemia will be examined. Finally, a brief survey of the developmental consequences of neonatal jaundice will be made. By defining factors in the neonatal period which lead to significant jaundice, developmental studies should help focus further research into the causes of hyperbilirubinemia.

2. Neonatal bilirubin metabolism

While liver enzyme immaturity and increased hemolysis are the usual explanations for neonatal hyperbilirubinemia, these concepts must be better defined, and other factors included to account for the rapid rise and fall of serum bilirubin values in some infants. As outlined in diagrams 1 and 2, each step in fetal and neonatal bilirubin metabolism will be reviewed, and the importance of its contribution to neonatal jaundice assessed.

a) Bilirubin metabolism *in utero*

The two primary sites of excretion of bilirubin for the fetus are the amniotic fluid and the placenta. While some studies have suggested that the fetal liver's ability to handle bilirubin may be induced by high circulating levels of unconjugated bilirubin, it plays a small role in normal fetal bilirubin excretion. (1,2)

The route along which bile pigments pass from the fetus to the amniotic fluid is not known. (3) Possible routes which have been investigated include the gastrointestinal tract, skin, tracheobronchial tree, and kidney. Cherry *et al.* collected data concerning the concentration of protein in amniotic fluid versus that in the umbilical cord. (4) They suggested on the basis of a gradient between these two compartments that a bilirubin gradient between fetal plasma and amniotic fluid might be established which favored the transfer of unconjugated

bilirubin across partitioning membranes without its albumin carrier. Such a gradient might occur across the fetal surface of the placenta, cord tissue, or fetal skin. Such an equilibrium would also explain the observation that in normal, non-immunized pregnancies, there is a drop in both bilirubin and protein concentrations in the amniotic fluid late in pregnancy. (3) In order to test this gradient hypothesis, 1500 mg. of albumin was injected into the amniotic fluid of three females. Subsequent amniocentesis revealed a significant passage of free bilirubin from the fetus into the amniotic fluid, a finding which supported their theory.

Another suggestion made by some authors was that the tracheobronchial tree contributes to the excretion of bilirubin into the amniotic fluid. (5) Recent animal studies by Goodlin et al. have shown convincingly, at least in the goat and sheep, that tracheal fluid has no significant excretory functions. (6)

A third possible excretory route of bilirubin into the amniotic fluid is the fetal kidney. (7) While fetal urine definitely contributes to the amniotic fluid, as indicated by the oligohydramnios which accompanies renal agenesis, it probably represents only a conduit system for the renal filtrate. (7,8) Although unconjugated bilirubin has been demonstrated in the urine of newborns (7),

neonatal renal excretory ability is limited by the low fetal glomerular filtration rate. (8)

The major excretory route for fetal bilirubin is the placenta. (2) The equilibrium across the placenta is similar to that between amniotic fluid and fetus in that the bilirubin crosses into the mother's circulation without its albumin carrier. (4) It appears to be conjugation, not protein binding, which impedes bilirubin clearance by the placenta. Experiments involving bilirubin clearance in animals, primarily monkeys, suggest that there is no significant transfer of conjugated bilirubin across the placenta. (5,9) However, because of the marked degree of deconjugation of fetal bilirubin, the placenta can usually handle the fetal bilirubin load. $H^3$-labelled bilirubin studies of dogs and monkeys have shown that while there is marked species variation, the combined rate of placental and fetal hepatic excretion of label was only slightly lower than normal adult hepatic values. (10) These data are supported in the human by the finding that cord bilirubin levels have little predictive value in neonatal jaundice unless there has been increased hemolysis <u>in utero</u>. (11) Thus, as long as the major part of the bilirubin load <u>in utero</u> is unconjugated, and there is no marked increase in bilirubin load (e.g., hemolysis), there should be no build-up of bilirubin in the fetus.

b) Neonatal bilirubin load

Many authors have noted that the bilirubin load generated secondary to hemolysis of neonatal red blood cells cannot adequately account for the magnitude nor the speed of the rise in serum bilirubin in the neonatal period. (12-16) First investigators of this problem examined the relationship between the hematocrit or total RBC mass and the degree of hyperbilirubinemia which develops in neonates. As measured by these parameters, the average amount of blood destruction in groups of jaundiced and nonjaundiced infants was essentially the same. (17,18) In an effort to examine the phenomenon of RBC turnover with a more dynamic tool, RBC survival times were measured. While the methods for measuring RBC survival varied, the results were all quite similar. In comparing normal newborns with adults, most investigators found a decrease of up to 33% in RBC survival time in the normal neonate. (1,2, 15,19-21) A reduced RBC survival time was found in premature infants when compared with normal newborns. (9,14) These sources differed, however, concerning the ability of the shortened RBC survival time to account for the phenomenon of neonatal jaundice.

Researchers then turned to an investigation of the molecular catabolism of hemoglobin to bilirubin. The primary area of concern has been the heme oxygenase system. This set of exzymes catalyzes the reduction of hemoglobin

to bilirubin. In the adult this system is present in highest concentration in the spleen, with activity also being located in the liver and bone marrow. (9,22) However, total hepatic activity predominates over splenic activity during intrauterine and neonatal life. (23) Recent work by Tenhunen et al. has pointed to a two step process whereby heme is first transformed to biliverdin by microsomal heme oxygenase. (22) The second reduction is then coupled with a soluble NADPH-dependent biliverdin reductase to form bilirubin. Although experimentation on this newly discovered system is incomplete, preliminary work indicates that some of the factors correlated with hyperbilirubinemia (e.g., hypoglycemia) also stimulate the heme oxygenase. (24) As Thaler points out, current evidence suggests that the hormones epinephrine and glucogon, secreted in response to a falling peripheral glucose level in the first 12 hours of life, stimulate a steep rise in the activity of many hepatic enzymes during the first post-partum day. (23) Among them is the heme oxygenase system. Moreover, in adult rats, bilirubin production is increased when the activity of heme oxygenase is stimulated without a corresponding increase in RBC destruction. (23,25) Thus, infants of diabetic mothers may form bilirubin at faster rates than normal newborns. (26) The activity of this enzyme system relative to the activity of the more slowly developing glucuronyl

transferase system may offer a reasonable molecular explanation for neonatal jaundice. A transient increase in bilirubin formation secondary to an increase in heme oxygenase activity rather than hemolysis is thus an attractive concept now gaining more attention.

Administration of labelled hemoglobin precursors has also increased understanding of the way in which hemoglobin catabolism affects the bilirubin load. When $N^{15}$-labelled glycine is administered to rats, three peaks of labelled bilirubin excretion in stool are found. (27) There are two peaks in the first three to five days, the so-called "early-labelled bilirubin." These peaks account for about 15% of the total label and are thought to be from a variety of sources, including by-products of heme synthesis in the liver, continuous low-grade production from cytochromes, myoglobin, and catalase, and, most importantly, ineffective erythropoiesis. The third peak at 40 to 80 days or "late labelled bilirubin" coincides with expected red cell survival, and accounts for approximately 65% of the label. When this experiment is done on human adults, the same pattern is obtained. (9) However, when Vest et al. injected labelled glycine into two normal infants, they found the first peak of fecal bilirubin pigment excretion to be approximately twice as high in the newborn as in the adult. (1) To investigate the non-hemoglobin contribution to this early labelled peak, studies using simultaneously

administered glycine and aminolevulinic acid (ALA) were performed. (27) ALA is a preferential precursor of non-hemoglobin sources of bile pigment. It was found that the newborn appears to be an overproducer of bilirubin from both hemoglobin and non-hemoglobin sources. (26) Thus, although the bilirubin load from both erythropoietic and non-erythropoietic sources is increased in the neonatal period, the increase is not sufficient to account for the rapid rise and fall seen in neonatal serum bilirubin levels.

c) Bilirubin transport in blood

Bilirubin is transported in the blood tightly bound to albumin. (28-30) In vitro studies have shown that one g. of albumin can bind approximately 16 mg. of bilirubin. (28) While the newborn's albumin can bind bilirubin (9), there is evidence that the binding capacity is not as great as in the adult. (31) As little as 1.5 g. of albumin may be available for binding bilirubin in some newborns. (31) Odell has pointed out that this decreased binding capacity may increase the infant's susceptibility to bilirubin toxicity, and that small changes in pH may alter the binding ability of albumin for bilirubin. (30) While the possibility of increasing the albumin concentration in the blood is one means of treatment of neonatal jaundice which has been tried in animals (32) and humans (33), its effectiveness is clearly limited by the amount that can be

administered before the protein's osmotic effect disrupts the infant's fluid balance. Moreover, Wong found no beneficial results from albumin therapy. (33) While the ability of the infant's albumin to bind bilirubin is thus a factor in his susceptibility to bilirubin toxicity, it is not a factor in the rapid rise and fall in serum bilirubin.

d) Neonatal liver in bilirubin metabolism

1) Uptake of bilirubin: In 1948, using a clearance measurement independent of the original serum bilirubin concentration, Fashena calculated that in every instance of neonatal hyperbilirubinemia, the velocity constant for bilirubin clearance was less than the velocity constant for neonates with a normal serum bilirubin. (17) In 1952, Obrinsky et al. demonstrated impaired clearance of BSP in newborn monkeys and humans. (34,35) Since the early 1960's, investigators have searched for the molecular means by which the hepatocyte can clear bilirubin from the blood. In 1966, Odell et al. demonstrated the ability of the liver to concentrate bilirubin by infusing adult rats with labelled bilirubin and then analyzing various organs for their content of label. (36) He found that despite the fact that the kidneys receive twice the blood flow of the liver, there was 19 times more bilirubin in the liver than the kidney. He hypothesized that there must therefore be a receptor carrier mechanism along the sinusoidal border of the hepatocyte to remove the albumin-bound bilirubin from the extra-cellular

fluid. Studies over the last four years on animals, using subfractionates of the liver, have revealed that infected labelled bilirubin was bound by proteins and/or lipids contained in liver cytoplasm. Specifically, investigators found that once inside the liver, the amount of bilirubin in cell sap exceeded the carrying capacity of intracellular albumin at least thirty fold. (23) In 1969, Levi et al. isolated two proteins, called Y and Z, from the liver cytoplasm. (37) They showed that these two proteins account for most of the intracellular bilirubin and BSP found after infection of these anions in vivo or their addition to liver supernatant in vitro. (38) Y was characterized as a basic protein which binds bilirubin, BSP, and other anions in vitro or in vivo. Z binds bilirubin or BSP when their concentrations on Y exceed a critical level. (38) Both proteins were found in liver of many mammalian species. Attention was then turned to the rhesus monkey, a species which manifests physiologic jaundice. It was found that Y was virtually absent at birth and attained adult levels by approximately the second week of life. Z developed prior to birth. (38) Maturation of the Y protein thus coincided with maturation of the hepatic uptake of BSP as well as amelioration of physiologic jaundice. (35,39) These data suggested that the Y and Z proteins represented the rate-determining steps in hepatic transport. Moreover, phenobarbitone has been shown to increase the concentration

of the Y protein. (40) It has therefore been suggested that this effect might account partially or completely for phenobarbitone's beneficial effect on neonatal jaundice (vide infra). However, this thesis has not been demonstrated in humans.

More recent investigation, however, has raised new questions about the roles of these proteins. Competition studies have shown that neither protein can dislodge bilirubin from its attachment to albumin. (23) These findings suggest that the membrane itself may play an active role in the uptake process. Selective receptors on the plasma membrane of the hepatocyte may mediate the uptake of organic anions, while the Y and Z proteins may simply distribute non-polar compounds to sites on the endoplasmic reticulum where conjugation is accomplished. (23) Further study of the hepatocyte's plasma membrane is required before our understanding of the uptake of bilirubin can be complete.

2) Conjugation: Along with increased hemolysis, inability of the hepatocyte to conjugate bilirubin has been the factor traditionally singled out as the cause of neonatal jaundice. This idea first became popular in 1958 from the work of Brown and Zuelzer. (41) They found that homogenized adult guinea pig livers could conjugate bilirubin to bilirubin diglucuronide. However, they were unable to demonstrate any conjugating ability in a fetal

or newborn liver. Their results also indicated a "gradual increase" in activity of glucuronyl transferase during the neonatal period. They felt that two enzymes were deficient, glucuronyl transferase (UDPGT), and uridine-diphospho-glucuronide dehydrogenase (UDPGD). In the same year, Lathe and Walker examined three premature human neonatal livers (ages 98 hours, 5 minutes, and 65 minutes) all within 1.5 to 2.5 hours of autopsy. (42) Their results also suggested deficient glucuronide forming enzyme. Other histological studies showed that UDPGT was located in the smooth endoplasmic reticulum, that the smooth ER was virtually absent at birth, and that phenobarbitone caused an increase in the smooth ER coincident with a decrease in unconjugated bilirubin. (38) Moreover, by showing impaired excretion of substances conjugated by liver in the same way as bilirubin (p-aminobenzoic acid, N-acetyl-p-aminophenol), investigators again indicated the inadequacy of the glucuronidating enzymes of the liver. (38)

However, as more experience was gained by various investigators with different species of laboratory animals and with the availability of an assay of UDPGT, it became apparent that the deficiency in transferase activity varied with both species and investigator. Gartner et al. working with liver slices from adult and fetal Wistar rats found that both adult and fetal livers had an equal ability for

phenol-glucuronide conjugation. (43) They noted that since the pattern differed from that observed earlier in the rat and guinea pig livers, the pattern was more dependent on the species and method than on true transferase activity. These findings were corroborated by Dutton et al. who worked with Wistar rat liver homogenates. (44) They observed that "though low in early fetuses, glucuronyl transferase activity in newborn preparations was as high as in adult males, rising to even twice that value in the first four days before falling below it." They also pointed to insufficient concentration of uridine-di-phospho-glucuronic acid in the assay for UDPGT as an explanation for the anomalously low results of other investigators. As these facts became known, more data accumulated showing that a decrease in transferase activity could not account for the serum bilirubin accumulation in the neonatal period. (12,16,35,38,45)

Thus, although the "functional immaturity" of fetal and neonatal liver for conjugation of bile pigment has been "amply documented" according to Schmid (46), recent evidence has led investigators away from the traditional concept of decreased transferase activity as a sole cause of neonatal jaundice. Researchers are now looking at steps in bilirubin excretion after conjugation for possible explanations of the rapid rise and fall in serum bilirubin.

3) Excretion: Excretion is one of the least understood steps in bilirubin metabolism. It is generally

accepted that in order for bilirubin to be excreted in the bile, it must be conjugated with a glucuronide. (16,47) This fact was first reported by Schmid in 1956. (48) Using chromatographic purification and beta-glucuronidase of animal or bacterial origin, he showed that hydrolysis of azo-pigment B, always found in bile and urine, resulted in its complete conversion to azo-pigment A. While occasional investigators have reported some unconjugated bilirubin in bile (49), most researchers feel that these small quantities of unconjugated bilirubin are probably produced by hydrolysis of the conjugated form during analysis. (47) In terms of its overall role in bilirubin metabolism, excretion has been shown in animal models to be more efficient in eliminating conjugated than unconjugated bilirubin, a fact which suggests that, at least in guinea pigs, conjugation and not excretion is the rate-limiting step in overall pigment metabolism. (50) However, as Thaler suggests, if hepatic transport, conjugation, and excretion of bilirubin are viewed as a single ongoing process, the excretory function which is known to require expenditure of metabolic energy is likely to be the final rate-limiting step. (23) More recent evidence has suggested that, at least in newborn monkeys, the clearance of bilirubin appears limited by conjugation in the first day of life and by excretion thereafter. (51) As in the previously discussed hepatic factors in neonatal bilirubin metabolism,

an adequate understanding of the excretory function of the hepatocyte has not yet been attained.

4) Vascular changes in the neonatal liver at birth: Several authors have pointed out that the profound circulatory changes in the liver at birth offer a reasonable explanation for the time curve of neonatal jaundice. (52, 53) At birth, the pressure in the umbilical vein drops from over ten ml. of mercury to zero, and the pressure gradient and oxygenation of the portal blood fall rapidly. (52) The blood reaching the relatively large left lobe of the liver thus switches from highly oxygenated umbilical vein blood to portal venous blood. While this adjustment is taking place, liver function presumably will be markedly impaired. Histologic studies have shown shrinkage of liver cells as well as decreased mass in the left lobe during the first week of life. The marked change in vascular supply has even caused infarcts to occur. (54) Moreover, these same authors have also observed that the ductus venosus remains patent for a variable length of time and thus provides a shunt around the liver. (52-54) Such a shunt and/or the previously described hypoxemic stress might lead to decreased liver function over the first week to ten days of life. While these changes undoubtedly do play a role in producing neonatal jaundice, their exact contribution remains difficult to evaluate.

e) Enterohepatic circulation of bilirubin

The final step in bilirubin metabolism is pigment excretion via the gut. While not a significant factor in adult metabolism, the unique situation of a sterile gut and high concentrations of beta-glucuronidase make this final stage more important in the neonate. The first evidence that bilirubin was reabsorbed from the gut came in the early 1960's with experiments on rats. In 1961 Lester et al. showed that when free and conjugated $C^{14}$-labelled bilirubin were placed in the gut of a rat, the label reappeared quickly in bile. (55) Their data also showed that the initial phase of absorption from the gut was more rapid for unconjugated bilirubin, and its total absorption appeared greater than conjugated bilirubin. These findings pointed up a "substantial" enterohepatic circulation of bilirubin. Two years later, Lester and Schmid, working with jj Gunn rats, found that conjugated bilirubin was not absorbed from the intestine, but had to be deconjugated before absorption. (56) They pointed out that "conjugation may be said to provide a barrier against intestinal absorption."

Data concerning the absorption of bilirubin in the human soon began to accumulate. In 1962, Gilbertson et al. infused both conjugated and unconjugated labelled bilirubin into a 79 year old male who had a T-tube in his common bile duct. (57) They found that when free bilirubin was

administered intraduodenally, it was recovered from the T-tube within the next 48 hours. None was found as either urobilinogen or bilirubin in the feces. With similar administration of conjugated bilirubin, highly significant increases of labelled fecal urobilinogen were recovered. Lester repeated this experiment in three patients in 1963 with similar results. (49) Brodersen and Hermann in 1963 first drew attention to the possibility that recirculation of bilirubin from the gut might be a contributing factor to neonatal jaundice. (58) They examined one or more stools from 13 infants from age zero to nine days for conjugated and unconjugated bilirubin and beta-glucuronidase activity. They found measurable enzyme activity as well as predominantly unconjugated bilirubin. Their conclusions were that the low degree of conjugation was due primarily to the combined absence of bacterial reduction and presence of large amounts of beta-glucuronidase. Based on the evidence cited above, they theorized that this lack of conjugation would promote re-circulation of bilirubin out of the gut. In 1964, Ulstrom et al. fed charcoal, which had the capacity in vitro to bind bilirubin, to newborn infants beginning at four hours of age or twelve hours of age in an effort to interrupt this enterohepatic circulation. (59) They found that those infants given charcoal at four hours had significant decreases in the levels of serum bilirubin while those given charcoal at 12 hours did not show

significant decreases. They suggested that the enterohepatic circulation was a significant factor in neonatal bilirubin retention, and that this enterohepatic shunt was most critical in determining the level of serum bilirubin during the first few hours of life. Moreover, such a shunt, they pointed out, would constitute the only normal prenatal route of exit for the slow luminal accumulation of bilirubin known to be present in the fetal bile. In 1968, Rosta et al. reported a significant relation between delayed meconium passage and the incidence of hyperbilirubinemia in 699 mature neonates, all delivered vaginally. (60) In 1971, Poland and Odell fed a formula which contained agar to nine normal neonates delivered by cesarean section and the same formula without agar to ten matched controls. (12) Both groups were fed within the first 20 hours of life. (The authors had already shown that agar could bind bilirubin in vitro.) They found that the agar fed infants had significantly lower levels of serum bilirubin and also significantly increased levels of fecal bilirubin excretion. Takimoto and Matsuda correlated increased amounts of fecal beta-glucuronidase with neonatal hyperbilirubinemia in a series of 54 newborns. (61) Uncertainty still exists, however, over the contribution of the enterohepatic circulation to neonatal jaundice in premature versus normal newborns.

f) Summary

Thus, neonatal jaundice is clearly a multi-factorial phenomenon. Certain factors which influence the serum bilirubin levels in one group of infants may be less important in other groups. No one factor can be singled out as most important. Increased hemolysis and hepatic immaturity, while important contributing factors, must be defined and understood in the context of a wide range of factors which play varying roles in different groups of newborn infants. While still concerned with the size of the bilirubin load and the hepatocyte's ability to excrete it, recent investigators have begun to focus on individual enzyme systems and the hepatocyte membrane, both sinusoidal and canalicular. Thaler's concept that neonatal jaundice may be due to the different rates of maturation of the heme oxygenase system and the glucuronyl transferase system exemplifies the new emphasis. (23) Unfortunately, greater understanding of these more sophisticated mechanisms for neonatal jaundice has not yet generated new therapeutic regimens. In general, therapeutic concepts are still based on the idea that hepatic immaturity is the primary cause of neonatal jaundice.

3. Treatment modalities

The three most commonly used treatment modalities, exchange transfusion, phototherapy, and phenobarbitone therapy, are based on the concept that liver immaturity

is the primary cause of neonatal jaundice. In exchange transfusion and phototherapy, iatrogenic elimination of bilirubin will avert bilirubin encephalopathy until the neonate can excrete his own bilirubin load. Phenobarbitone treatment is aimed at accelerating maturity of the liver enzymes. Some more recent therapeutic approaches have focused on more specific areas of bilirubin metabolism, e.g., the enterohepatic circulation.

a) Exchange transfusion

While exchange transfusion can lower the serum bilirubin dramatically, there is a significant morbidity and mortality associated with the procedure. This fact has generated disagreement about the indications for exchange transfusion. (62) According to some authors, the risk of mortality from the procedure exceeds the risk of bilirubin toxicity. Jablonski has put the risk at 1.5% for full-term infants. (63) Trolle, on the basis of a 6% incidence of severe neonatal jaundice (greater than 20 mg.%) in full-term infants and 11% in premature newborns derived from a series of 1000 livebirths, calculated that 2140 full-term babies would have to be transfused in order to prevent athetosis in one. (64) As a result, 21 infants would die as a complication of the exchange transfusion. His figures for premature neonates show that 92 infants would have to be exchanged to prevent one case of athetosis, with the risk of four dying. He put the

mortality for exchange transfusion for premature infants at four per cent, and that for full-term infants at one to two per cent. Crosse has reported a two-year experience in which 6.6 premature babies were transfused to save each expected case of kernicterus. (65) There were 92 babies transfused with one death resulting from transfusion and five infants with spastic kernicterus, all associated with serum bilirubin levels exceeding 22 mg.%. Fourteen cases of kernicterus with ten deaths had been predicted on the basis of the hospital's previous experience. As experience with exchange transfusion increased, the mortality from the procedure fell to zero over the following year. However, the possibility of developmental abnormalities at low serum bilirubin levels combined with significant mortality figures of exchange transfusion and the incidence of neonatal jaundice have motivated investigators to look for safer means of treating neonatal jaundice. (46, 66)

b) Phototherapy

Early in 1958, the head nurse in charge of the premature unit of the General Hospital, Rockford, Essex, England, reportedly noticed the apparent fading of the jaundiced color of babies' skin when they had been left for a short time in sunlight. (67) She also noted that only those areas of skin exposed to the light showed fading of yellow color. Cremer et al. studied 22 infants by

placing them in direct sunlight and artificial light for short periods of time and found that serum bilirubin fell markedly following light exposure. (67) *In vitro* studies of the effect of light on bilirubin by the same workers showed the mechanism to be photooxidation of bilirubin. Investigators quickly became concerned about two possible complications of therapy. First, some were concerned by the possibility that the photooxidation products might be more toxic than bilirubin itself. Others were worried that the rapid fall in bilirubin could be due to the displacement of bilirubin from its albumin carrier with its consequent deposition in tissues. However, no evidence of the formation of the possibly more toxic oxidation product, biliverdin, during phototherapy has been found. (35,68) Other investigators have shown that the toxic effects of bilirubin are abolished both *in vitro* and *in vivo* if bilirubin is previously degraded by illumination *in vitro*. (69) The photooxidation products have also been shown not to interfere with cell growth in culture. (68) More recent studies have shown that the breakdown of bilirubin by light is oxygen dependent, and probably involves formation of a singlet oxygen molecule, i.e., molecular oxygen in its first excited state. (46) This process results in eventual cleavage of the chain of four pyrrole rings that constitute the bilirubin molecule into fragments which contain two pyrrole rings linked by a methane bridge. (46)

Investigating both the concerns cited above, Callahan et al. have recently pointed out that light converts bilirubin to more polar, predominantly diazonegative derivatives which are excreted rapidly in bile and urine without detectable retention in plasma. (70) He proved this statement by infusing $C^{14}$-labelled bilirubin over two to twenty-four hours into two infants, five and seven months old, with the Crigler-Najjar syndrome. Both had previously responded to light therapy. Plasma, urine, and bile were collected to explore the disposition of the labelled pigment. Approximately three-fourths of the eliminated isotope was recovered from the feces with the remaining fraction appearing in urine. At the conclusion of the study, virtually all the isotope had been recovered. Callahan pointed out that this complete recovery makes it improbable that phototherapy reduces serum bilirubin levels by means of displacement of pigment from its albumin carrier or through significant redistribution of pigment between plasma and tissues.

While these reports suggested that light therapy was a technically easy, effective, and safe way to reduce neonatal hyperbilirubinemia, other investigators were less convinced. Light found most effective in treatment was that in the 420 to 475 nanometer region of the spectrum. (69) Odell has pointed out that other compounds are disrupted by light in this range. (71) For example, albumin,

the primary carrier protein of bilirubin in blood, has several histidine molecules which contain imidazole rings. When these imidazole rings are exposed to bright light, they are cleaved with consequent reduction in bilirubin binding capacity in the photooxidized albumin. (71) An incidental finding in a recent study of the effectiveness of phototherapy and albumin infusion again raised the question of light's effect on albumin. (33) In comparing albumin levels in eight control infants with eight infants who had received both phototherapy and an intravenous infusion of 1.5 g. of albumin per kg. body weight, it was noted that the plasma albumin levels were not significantly different. Moreover, other enzymes that contain histidine, e.g., phosphoglucomutase, are completely and rapidly inactivated by photooxidation. (71) In addition, Hakani et al. have pointed out that while later products of photooxidation are non-toxic and do not bind with albumin, early products are toxic and do bind to the carrier protein. (72) Besides this biochemical evidence, it has also been shown that phototherapy is not universally effective. It is relatively ineffective for rapidly rising serum bilirubin, and the degree of reduction of serum bilirubin varies among subjects. (66,69,73)

The systemic effects of phototherapy have been underlined by recent evidence concerning its effect on the rate of hepatic excretion of bile. Ostrow, working

with jj Gunn rats found that most bilirubin derivatives excreted during phototherapy are identical with those found under dim lighting conditions. However, there is a markedly enhanced hepatic excretion of diazoreactive material. He concludes, therefore, that the primary effect of phototherapy is to stimulate alternate pathways of bilirubin catabolism that exist normally in both humans and animals. (69)

Thus, while phototherapy may be somewhat easier and safer to administer than exchange transfusion or phenobarbitone and by itself is beneficial in mild cases, long-term controlled studies are still needed. Its systemic effects on enzyme and organ systems as well as on the child's overall developmental status need to be assessed. (66)

c) Phenobarbitone therapy

Catz and Yaffe in 1968 noted that administration of phenobarbitone to newborn mice caused a significant increase in the activity of the liver glucuronide conjugating system for bilirubin. (74) They also found that mice whose mothers had been treated with phenobarbitone while pregnant had a significant increase in activity in the same enzyme system to values which approximated those of a normal adult. Yaffe's results with animals led him to study newborns to determine, firstly, if phenobarbitone could prevent a rise in bilirubin in the first five days of life, and secondly, if phenobarbitone could lower already elevated levels of bilirubin between the fifth and tenth days. (74) He found that in 40 full-term

infants (20 treated infants and 20 controls), he could alter the peak of the bilirubin curve from day four to day two. Moreover, he found that in five to ten day old infants, phenobarbitone therapy caused a significantly more rapid decline in serum bilirubin values. Trolle, in a study of 808 newborns with birth weights greater than 2500 g., found that administration of phenobarbitone to both mother prenatally and baby was more effective than just to mother or just to baby. (75) Stern et al. in 1970 confirmed the effect of phenobarbitone on the glucuronidating capacity of treated and control infants by administration of salicylamide. (2) He found that phenobarbitone treated infants had an increased glucuronide conjugating capacity. Yeung et al. more recently have shown by BSP tests that phenobarbitone treatment increased both uptake and excretion in a series of 20 jaundiced Chinese infants when matched with 20 jaundiced controls. (76)

However, despite these encouraging data, Behrman and Fisher have pointed out both disadvantages and uncertainties concerning phenobarbitone treatment. (77) First, they point out there is no good evaluation of mortality and morbidity from the therapy. Complications of sedation, with slow feeding and aspiration have been observed. Moreover, neonatal rats from phenobarbitone treated mothers have decreased growth rates, altered rates of gonadal steroid metabolism, and increased mortality rates. They also

raise the interesting question that drug-induced sedation in the neonatal period could interfere with neurobehavioral development, e.g., imprinting. In the light of these unexplored complications, and the estimates that 77 to 95% of term infants have peak bilirubin levels of under ten mg.%, they feel there is little justification for pre-natal treatment of a term baby. In examining the current small amount of data concerning treatment of premature infants, they point out that phenobarbitone appears less effective in lowering serum bilirubin in this more susceptible group of infants.

Recently, Yeung et al. have responded to these questions. (78) Working in Hong Kong with a population in which up to 40% of newborns have bilirubin levels >15 mg.%, they have treated 2500 jaundiced newborns with phenobarbitone over the last three years. By using phototherapy, they have been able to limit the number of newborns who required exchange transfusion. Sedation was the commonest side effect. There were also six drug rashes and three cases of apnea and cyanosis. In defending their mode of therapy, they point out that in the animal studies cited by Behrman and Fisher, the total dose (>45 mg./kg.) and the length of treatment were both far greater than those employed on human newborns.

Without adequate follow-up of a large series of phenobarbitone treated infants, however, the arguments of

Behrman and Fisher legimately question the indiscriminate treatment of neonatal jaundice with phenobarbitone.

d) Other treatment modalities

Other treatment modalities have been aimed primarily at either the maturity of the glucuronyl transferase system or at the enterohepatic circulation of bilirubin. Serein et al. reported a series of 12 sets of twins treated with diethylnicotinamide, a substance which induces liver glucuronidating enzymes. (79) They found that 96 hours after starting drug administration, the treated infants had statistically lower serum bilirubin values. Waltman et al. infused ethanol (118 g.) slowly into ten pregnant women and found a significant lowering of their infants' serum bilirubin values over the first five days, when compared to ten controls, without maternal or neonatal complications. (80) No Apgar scores were reported to be <8 at one minute. Uridine-di-phospho-glucose has also been used to decrease neonatal serum bilirubin. (81) No large series have been reported, however, for any of these methods.

Others have attempted to lower serum bilirubin by interrupting the enterohepatic circulation of bilirubin. Charcoal (60), agar (12), and 1,4-disaccharolactone (82) (a beta-glucuronidase inhibitor) have all been tried with varying degrees of success. The disaccharolactone study was poorly designed and needs re-evaluation. The major

complication of the other two treatments has been poor weight gain during the first week of life. Again, because of insufficient numbers studied, definite effects, either beneficial or hazardous, on different groups of neonates cannot be adequately assessed.

More recent studies have examined the effectiveness of combination therapies, specifically phenobarbitone and light (83,84), or light plus albumin (33). Neither combination was found to be more successful than one or the other individual component alone.

As the means of treatment of hyperbilirubinemia are being repeatedly redefined, the need for treatment is likewise being re-examined. Historically, proof of the toxicity of bilirubin in newborn infants has long been established. (85) More recently, factors which increase the infant's susceptibility to toxicity have been identified. An at-risk population is currently becoming recognized on the basis of developmental studies which control for a variety of neonatal complications. An outline of these developmentally based concepts in the context of neonatal bilirubin metabolism will now be presented.

4. Developmental studies

a) Toxicity of bilirubin

The toxicity of bilirubin is influenced by a wide variety of factors. These factors can be divided into two

categories: 1) levels of indirect serum bilirubin; and, 2) increased susceptibility of certain infants.

1) The level of indirect serum bilirubin: The most dramatic cause of hyperbilirubinemia in the neonatal period is erythroblastosis fetalis. In 1952, Hsia et al. reported 229 infants with serologically proven erythroblastosis fetalis. (86) He showed data which compared serum bilirubin levels in infants with erythroblastosis fetalis, premature newborns, and normal neonates. The values for the infants with erythroblastosis fetalis rose much more dramatically than those for either premature or normal newborns. He also showed that the height of the serum bilirubin, which was predominantly unconjugated, correlated well with the incidence of kernicterus. No infants with erythroblastosis fetalis who had total serum bilirubin values <5 mg.% showed signs of kernicterus; three per cent of those with six to 15 mg.% developed kernicterus; while 18% of those between 16 and 30 mg.%, and 50% in whom serum bilirubin levels were >30 mg.% developed kernicterus. Unfortunately, Hsia's data did not reflect maximum serum bilirubin values, because the serum bilirubin was not measured immediately prior to exchange transfusion, and duration of high bilirubin was not considered. Nor were the results controlled for gestational age of the infants. Moreover, the incidence of kernicterus without treatment was probably underestimated

because almost all of the babies had exchange transfusions. These data suggested, however, that the liability of bilirubin to cause brain damage was correlated with the serum level, and other studies have confirmed this. (65) Researchers have also looked at the ability of bilirubin to gain access to the brain. Nasralla et al. to assess the relationship between spinal fluid bilirubin and serum bilirubin in 100 newborns, including 34 normal newborns, 49 normal premature newborns (4.5 pounds or less), and 17 infants with erythroblastosis fetalis. (87) They found a positive correlation coefficient between serum and spinal fluid bilirubin levels. Because they felt that the greater fraction of neonatal serum bilirubin was unconjugated, no attempt was made to measure direct reacting bilirubin. These data suggested that the higher the level of unconjugated serum bilirubin, the greater the level of CSF bilirubin to which the brain is exposed. The danger of such exposure was suggested by in vitro experiments. Using a system which parallels the solubility properties of the intra- and extra-cellular spaces, Brodersen and Vind have shown that it is possible for unconjugated bilirubin to penetrate brain cells. (88) Claireaux et al. confirmed these findings clinically by isolating unconjugated bilirubin from the brains of four infants, two of whom were premature by dates and two of whom had hemolytic disease. Their identification was based on diazo

reactivity, chromatographic behavior, and absorption spectra from the affected infants' brains. Two of the four infants had seizures, but the other two had no clinical history to suggest kernicterus. All four had relatively deep jaundice, but no serum bilirubin levels were reported. These data thus suggested that free bilirubin can be deposited in neonatal brain, and its ability to do damage may be a reflection of the indirect or unconjugated serum levels. (133)

Factors besides hemolysis have been correlated with increased levels of unconjugated bilirubin. Prematurity has long been considered to predispose the neonate to increased serum bilirubin levels. In a study of 383 infants, Trolle divided infants of approximately equal weight (2000-2500 g.) into mature and premature by gestational age. (89) He excluded blood group incompatibilities, maternal diabetes mellitus, and heart disease, but not sepsis, hypothermia, and various drugs. He found that premature infants have a higher incidence of jaundice of unknown etiology than under-weight full-term infants. Under-weight full-term infants had an incidence and degree of severity of jaundice similar to mature infants who weighed approximately 3000 g. Unfortunately, since the authors did not control for certain medical complications of the neonatal period, (known to be correlated with hyperbilirubinemia), it is difficult to isolate prematurity by dates as a specific cause. Taylor et al. in an

earlier study of 142 full-term newborns and 173 premature infants (also controlled for maternal diabetes mellitus and blood group incompatibilities) did not mention the incidence of medical complications in either group. (20) Their data showed that the degree of hyperbilirubinemia correlated with either birth weight or length of gestation. Harris also observed in a study of 114 premature newborns a reciprocal correlation between birth weight and peak bilirubin levels. (90) Controlled for infection and hemolysis, she found in addition that 27% of her subjects had bilirubin levels >15 mg.%, and ten per cent had values >20 mg.%. On the basis of a review of 800 cases in the literature, Lucey roughly corroborated these percentages and called hyperbilirubinemia of prematurity "a common occurrence." (91) The incidence of jaundice in premature infants is greater than that in normal newborns, although the greater incidence of complications in the former group often makes the data difficult to interpret. Parenthetically, it may be noted that this kind of data gave credence to the belief that "liver immaturity" was one of the primary causes of neonatal jaundice.

Maternal factors have also been implicated in influencing higher neonatal serum bilirubin levels. Taylor et al. showed that when third day bilirubin values of 48 infants of diabetic mothers were compared with controls matched for gestational age (33 to 40 weeks), the

infants of diabetic mothers had significantly greater hyperbilirubinemia ($p < 0.005$). (11) While no labelling studies were done, no evidence for increased hemolysis could be demonstrated in these infants. Earlier series have also noted this increased incidence of hyperbilirubinemia without evidence of hemolysis in infants of diabetic mothers. (92,93) Recent data concerning the effects of epinephrine and glucagon on the heme oxygenase system make these results somewhat more intelligible. (23)

Another maternal factor thought to predispose to increased serum bilirubin levels is breast-feeding. Substances isolated from breast milk (pregnanediol) and maternal serum (also thought to be a progestational steroid) have both been reported to be associated with unconjugated neonatal jaundice. Although controversial, some investigators feel that these substances are responsible for hyperbilirubinemia by inhibiting glucuronyl transferase in the neonate's liver. (23,94,95) Other maternal factors including age, medical complications, duration of labor and means of delivery have been reported as having no effect. (20,65) The influence of race and sex on neonatal serum bilirubin is evident primarily in premature newborns. (20,96)

The factors most convincingly correlated with an increased incidence of neonatal jaundice are thus

erythroblastosis fetalis, prematurity, and maternal diabetes. Circulating maternal steroids (transient familial neonatal hyperbilirubinemia) and prenanediol in maternal milk are most controversial in their correlations. Any factor that predisposes to elevated unconjugated serum bilirubin levels also puts an infant at risk for bilirubin encephalopathy. Several other factors, however, may increase the infant's susceptibility to bilirubin toxicity.

2) Increased susceptibility to bilirubin: Factors which may lead to an increased risk of bilirubin toxicity include a wide spectrum of neonatal problems. These problems can be considered in two groups: 1) those complications which affect the permeability of neurons and other tissues to bilirubin; and, 2) those which decrease the bilirubin binding capacity of serum, thus increasing the amount of freely diffusible bilirubin.

The first category includes asphyxia, acidosis, hypothermia, hypoglycemia, and sepsis. (65,97) Although the exact pathophysiologic mechanisms of these complications have not been elucidated, they are probably related to injury of cells, especially neurons, with consequent increased permeability to bilirubin. (98) The second category includes oxygen treatment in premature infants which may increase hemoglobin degradation (99), large doses of intramuscular vitamin K (65), any drugs which are protein bound in competition with bilirubin (e.g.,

sulfa drugs and salicylates), hypoproteinemia, and acidosis. (30) Any one or a combination of these factors might lead to marked increases in permeability to bilirubin of brain tissue and/or diffusibility of bilirubin. These complications can thus increase the risk of bilirubin toxicity in both premature and full-term infants. Boon has reported 26 full-term infants, none of whom had hemolytic disease, who developed kernicterus. (100) At necropsy, all had bilirubin staining of basal ganglia, as well as diffuse fatty change in the liver. A large percentage (62%) had a history of dehydration and sepsis. Some combination of the diffusibility of free bilirubin and increased cell permeability is probably required for bilirubin to exert its toxic effects on neonatal brain.

From this examination of factors which influence neonatal bilirubin levels and susceptibility to bilirubin, several categories of infants emerge which must be considered at special risk from bilirubin toxicity. It is also clear that many complications can affect either the maximum serum bilirubin level or the infant's susceptibility to bilirubin. The usual explanations for neonatal jaundice are difficult to apply to these observations. Attention should be directed to the effects which these various risk factors have on the balance between the heme oxygenase and glucuronyl transferase systems, or the

ability of the hepatocyte to clear bilirubin and its ability to excrete it, or the possible change in the rate-limiting step from conjugation to excretion.

The implications of these factors are only beginning to be understood through studies which define the risk to the infant's subsequent growth and development of various levels of serum bilirubin.

b) Developmental risks of hyperbilirubinemia

Kernicterus is the extreme example of neonatal bilirubin toxicity. Its classical features include mental retardation, athetoid palsy, high frequency deafness or neonatal death. (101) While some researchers feel that physiologic hyperbilirubinemia can never cause kernicterus in a full-term infant (102), there are case reports of such occurrences. (100) Claireaux et al. examined necropsies of liveborn infants over a four-year period and found that 33 of 376 had "brain jaundice," and of those 33, nine had been premature infants without increased hemolysis (2.4%). (133) Only two had had infection or other medical complications. Hsia et al. found the incidence of kernicterus in babies with erythroblastosis fetalis to be 18% if the serum bilirubin rose to a level between 16 and 30 mg.%, and 50% if the serum bilirubin was >30 mg.%. (86) Because there is poor understanding of the factors which define the at-risk population for kernicterus, it is difficult to find data which can be compared concerning the

long-term developmental effects of neonatal hyperbilirubinemia, especially in the full-term age group. Through the mid-1960's, there were two basic viewpoints concerning the effects of neonatal hyperbilirubinemia on the growth and development of the child.

A typical earlier investigation was that of Gerver et al. in 1950. (103) They matched 68 children who had been diagnosed as having erythroblastosis fetalis, but who had had neither neonatal symptoms of kernicterus nor gross motor difficulties, with older brothers and sisters to achieve a close hereditary and environmental match. Using the Stamford Binet intelligence test, they examined the children at an average age of 4.5 years and found a variable, widespread, and usually moderate lowering (from 114.5 to 102.7) of intelligence in affected children. The authors were quick to point out, however, that any severely ill newborn might suffer some degree of permanent functional impairment of intellect, and that lowering of IQ could not be singled out as a specific hallmark of this disease. Other authors were similarly impressed by the variability in the neurological effects of hyperbilirubinemia. In 1948, after reviewing eight patients with kernicterus, Lande pointed out that "there is no correlation between the degree of jaundice or anemia during the neonatal period and the occurrence or degree of cerebral damage." (104) Evans, in 1950, observed that while most of the survivors of kernicterus developed severe, symmetrical muscular incoordination,

usually associated with athetosis, some patients have severe mental defects while many others have "impaired" intelligence. (105) Gerrard noted that "mental retardation is by no means invariable or inevitable." (106)

Against this background of confusing evidence concerning the effect of hyperbilirubinemia on growth and development, Hsia and others established the all-or-none concept. Central to this idea was the hypothesis that a cerebral threshold of toxicity existed which could be assessed by the level of serum bilirubin. Both his own data (86) and that of other investigators tended to support 20 mg.% as the threshold for development of neurological abnormalities. Shiller and Silverman examined a group of 110 three-year-olds who had all been premature (less than 2000 g.). (107) In this sample, approximately one fourth had uncomplicated hyperbilirubinemia (maximum concentrations 18 to 22 mg.%) in the neonatal period. Approximately one fifth exhibited signs which were interpreted at three years of age as either suspicious or definite evidence of brain damage. They were unable to demonstrate a significant correlation between uncomplicated hyperbilirubinemia in these prematurely born children and neurological deficit at age three years. Ose et al. showed that in a questionnaire follow-up of 157 patients with neonatal hyperbilirubinemia, 90% with serum bilirubin values >35 mg.% died or had cerebral palsy, while there were no

instances of developmental retardation when the maximum serum bilirubin was <20 mg.%. (108) Wishingrad et al. found no difference between two groups of 50 premature infants each with bilirubin values >18 mg.%, when only one group had been treated by exchange transfusion. (102) Their follow-up at one year showed neurologic abnormalities in seven infants from the transfused group compared with six infants in the untreated group. The frequency of neurologic deficits was similar to that found in any series of low birth weight babies. They suggested that exchange transfusion was necessary in uncomplicated non-hemolytic hyperbilirubinemia of the premature infant only when unconjugated bilirubin values were >24 mg.%. Jablonski studied 204 full-term infants with neonatal jaundice of undefined etiology. (63) Forty-five had serum bilirubin levels >20 mg.%, and nine had levels >25 mg.%. There were no proven cases of kernicterus. At six month follow-up, he found no greater incidence of neurologic deficit in 19 infants whose bilirubin levels rose to 20 to 25 mg.% than in 58 whose levels never exceeded 15 to 20 mg.%. He therefore suggested that there was no indication for exchange transfusion in full-term infants with jaundice of undetermined etiology until the serum bilirubin reached 25 mg.%.

However, over the last two to three years, the idea of a threshold for bilirubin toxicity has been questioned.

The largest series was published by Boggs et al. in 1967. (109) It included 23,000 single, live-born infants unselected for any medical reason. Both Coombs positive and negative babies were included as well as those who had been treated by exchange transfusion. The maximum serum bilirubin concentration was the single most important variable. They noted correlations between the maximum recorded neonatal serum bilirubin concentration and the incidence of low total motor and mental scores at an eight month examination. The series was controlled for the infants' birth weights and five minute Apgar scores but not for gestational age. The developmental test used was a standardized modification of the Bayley Scale of Infant Development. This examination included test items arranged in a scale to permit classification of a given infant in terms of percentiles of expected achievement up to fifteen months. The results showed that the correlation between neonatal hyperbilirubinemia and low mental and motor score did not begin abruptly at 20 mg.%, but could be seen to rise progressively and to become substantial at 15 to 19 mg.%. Moreover, this relationship was found in all birth weight groups, independent of, although superimposed upon the effects of low birth weight and/or distress at birth as measured by a low Apgar score. Bilirubin thus seemed to have definite neurological effects below 20 mg.% at least in these short-term follow-ups. Other investigators have not confirmed these findings.

More reports subsequently appeared of both low birth weight and full-term infants who either developed kernicterus or showed subsequent neurological abnormalities with low serum bilirubin values, and without clinical indications of kernicterus in the neonatal period. (110-112) Gartner et al. recently examined the autopsy findings in a series of 14 low birth weight infants who expired during the third to the sixth day of life. (113) Nine of the 14 had pathological evidence of kernicterus, i.e., yellow staining of one or more areas of brain with necrosis of brain cells in stained areas. None had clinical signs of kernicterus nor hemolytic disease. Moreover, the peak total serum bilirubin values in the group with kernicterus was 9.4 to 15.6 mg.%, while in those without kernicterus, the values were similar, 8.8 to 17.2 mg.%. In a follow-up study of low birth weight infants with jaundice, Crichton et al. examined three groups of 30 low birth weight infants. (114) Each group had a different maximum serum bilirubin level (>20 mg.% , 11 to 19.9 mg.%, and <11 mg.%). After four to 11 years, they found that the mean IQ scores were slightly lower in the most severely jaundiced group, and that this same group had a significant excess of mentally retarded (IQ <70). The mean IQ scores of the three groups did not, however, significantly differ. Neither was there a significant difference in verbal and performance scores, as had also been suggested by earlier studies. (115)

Ackerman has suggested that two new categories be established for infants who have had increased bilirubin values: 1) "neurological damage, definitely associated with hyperbilirubinemia," specifically athetosis or deafness or other clear-cut neurological signs in the newborn period; 2) "neurological damage, possibly associated with hyperbilirubinemia," specifically more subtle brain damage, in which bilirubin may have been a factor. (116)

Odell has recently proposed a new method to assess an infant's susceptibility by testing the ability of his serum to bind bilirubin and thus keep it from diffusing into cells. (117) A saturation index is established on the basis of the amount of salicylate bound by the serum albumin of the infant. In a study of 32 children between the ages of four and seven, all of whom had been jaundiced, 14 were considered normal by psychometric testing, and 18 were classified as having brain damage. (117) There was no significant correlation between the presence or absence of brain damage and maximum bilirubin concentration, birth weight, sex, presence or absence of hemolytic disease, or use of exchange transfusion. There was, however, a significant correlation between the presence or absence of brain damage and the saturation index of serum protein with bilirubin during the neonatal period. While large scale studies have not yet been reported, the saturation

index may be a reliable method to select infants at particular risk from bilirubin toxicity.

Thus, recent developmental studies seem to indicate that the neurological deficits secondary to hyperbilirubinemia in the neonatal period may be observed over a wide range of serum bilirubin values. The infant least likely to be affected is the healthy term baby. The most likely affected is the sick premature infant. There are no definite conclusions about the level of serum bilirubin at which risk occurs and when treatment is indicated. Differing susceptibilities of different groups of infants suggest that different steps in neonatal bilirubin metabolism play varying roles in determining the height of the serum bilirubin as well as the infant's susceptibility to it. Studies must therefore be designed to examine the interaction of this wide variety of neonatal factors. In this way, more reliable identification of and treatment for at-risk infants will be found.

## II. MATERIALS

### 1. Patient selection

Infants included in this study were ten full-term neonates by weight, gestational age, and Dubowitz score, delivered by cesarean section without complicating maternal factors of diabetes mellitus or toxemia. (See Table 1) No mother was on any pre-natal medications except vitamins and diuretics. None had a prior history of Rhesus sensitization or jaundiced babies. The indications for section were either cephalo-pelvic disproportion or repeat section. Babies delivered by cesarean section were chosen, because they could be followed in the hospital for five days. The Apgar scores at five minutes were all eight or greater. No abnormalities were detected on examination, and there were no medical complications in the newborn period. All were Coombs negative. All infants were cared for in the well-baby nurseries or in the Newborn Special Care Unit, Yale-New Haven Hospital. All were given intramuscular vitamin K as Aquamephyton in a dose of 0.5 mg. Iron fortified cow's milk formula was introduced at approximately 12 hours of life, and all infants were fed on the same schedule. None was breast-fed. Informed consent was obtained from each mother. The mother's medical and obstetrical history was derived from an interview with her and from her chart.

2. Collection of data

Blood samples (in heparinized capillary tubes) for bilirubin determinations and hematocrit were obtained for five successive mornings. All were analyzed within 15 minutes of sampling. Blood grouping and Coombs test, daily weights, feeding and stool times were all obtained from normal records kept on each infant and mother. Stools left in diapers were collected by the nurses and wrapped quickly in Saran Wrap. The exposure to light was minimized. The time of stooling and baby's name were noted on each stool. All stools were picked up within six hours, weighed, and were analyzed within 12 hours.

3. Preparation of reagents

a) Serum bilirubin: Diazo reagent -- ten ml. of a solution of 2.5 g. of sulfanilic acid in 15 ml. of concentrated hydrochloric acid diluted to 1000 ml. were added to 1 ml. of 0.5% sodium nitrite.

b) Stool beta-glucuronidase assay: Acetate buffer (0.07 M acetic acid; pH 5.0) -- To 800 ml. of distilled water was added 4.0 ml. of acetic acid. The pH of the mixture was titrated to 5.0 with 10% sodium hydroxide. The mixture was then diluted to one liter with distilled water.

Glycine buffer (pH 10.4) -- 15 g. of glycine and 11.7 g. of sodium chloride were added to 700 ml. of distilled water. The pH of the mixture was adjusted to 10.4

with 10% sodium hydroxide, and diluted to one liter with distilled water.

Phenolphthalein standard (Sigma Chemical no. 105-1; 1 mg./ml. of ethanol) -- 2.5 ml. of phenolphthalein standard was added to 7.5 ml. acetate buffer to give a final concentration of phenolphthalein of 2.5 mg./10 ml. of solution.

Phenolphthalein glucuronic acid (Sigma Chemical no. 105-4) -- 5 ml. of phenolphthalein glucuronic acid were added to 28.3 ml. distilled water to give a final substrate concentration of 0.0015 M.

c) Stool bilirubin assay: Hydrogen peroxide reagent -- To 200 ml. of 94% ethanol was added 0.8 ml. of 30% hydrogen peroxide and 4 ml. concentrated hydrochloric acid.

Fifty per cent ethanol in water

Ascorbic acid (1 g.% in distilled water)

Pure Bilirubin (Fisher Scientific Co. no. B-315)

## III. METHODS

### 1. Serum bilirubin

Serum bilirubin values were run in duplicate on 0.03 ml. aliquots of serum obtained from centrifugation for three minutes of four full heparinized capillary tubes in a Model HN Centrifuge (International Equipment Company, Needham Heights, Mass.). Spectrophotometric determinations were carried out in a Bilirubinometer (Advanced Instruments, Inc., Newton Highlands, Mass.). The diazo reagent was made up as described by the manufacturer (Advanced Instruments bulleton no. Bl 2-2702). Two values each for total and direct bilirubin were obtained, and the average taken. Results obtained by this method are comparable to those obtained when the standard Evelyn and Malloy technique is used. (118)

### 2. Hematocrit

The hematocrit was obtained after centrifugation for three minutes by a Micro-capillary reader (International Equipment Co., Needham Heights, Mass.).

### 3. Stool beta-glucuronidase assay (119,120)

Six 25 ml. Erlenmeyer flasks were numbered one through six: one and two were unknowns, three and four were blanks, and five and six were standard points. Into flasks one, two, three, four, and six, 0.9 ml. aliquots of

acetate buffer were pipetted. Into flask five was pipetted 1.0 ml. of acetate buffer. Into flasks one and two were pipetted 0.2 ml. aliquots of 0.0015 M phenolphthalein glucuronic acid. One-tenth ml. of phenolphthalein standard solution was pipetted into flask five, and 0.2 ml. into flask six. The fresh, undried meconium was weighed out on a Mettler balance to the nearest hundredth of a g. and diluted one g. per 5 ml. of acetate buffer. It was homogenized for 15 minutes in a glass homogenizing tube with a Teflon mallet attached to a 5000 r.p.m., 1/18 horsepower electric motor. The meconium homogenate was then placed in a separate 25 ml. Erlenmeyer flask, and all seven flasks were corked and equilibrated in a water bath shaker at 37°C. for ten minutes. To each numbered flask was added 0.3 ml. of the meconium homogenate. The six flasks were then corked and incubated with shaking for an exact appropriate multiple of 30 minutes. The reaction was stopped with the addition of 5 ml. of glycine buffer to each flask. The flasks were allowed to shake for two minutes, then 0.2 ml. aliquots of 0.0015 M phenolphthalein glucuronic acid were added to flasks three and four. The flasks were then allowed to shake for five minutes. The contents of the flasks were next poured into appropriately labelled conical centrifuge tubes and spun in a Sorvall Superspeed RC2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 5000 r.p.m. for 15 minutes. The supernatant from each tube was separately decanted into matched

spectrophotometer (Model 6A, Coleman Instruments, Inc., Maywood, Illinois). Tubes three and four were blanks and were first adjusted to 100% transmission. The per cent transmission of the two standard points, tubes five and six (25 mg. and 50 mg. of phenolphthalein respectively), were then located on a graph and a line parallel with that of the standard curve drawn through these points. The values for tubes one and two were averaged and the amount of phenolphthalein released determined from the line drawn through the two standard points. The amount of phenolphthalein produced in the reaction was then converted into the amount of phenolphthalein released per hour per g. of meconium. This conversion was based on the linearity of the reaction rate and the uniformity of the meconium homogenate. Since one unit of beta-glucuronidase was defined as that amount of enzyme which releases one mg. of phenolphthalein in one hour, this number represents the number of units of beta-glucuronidase per g. of meconium.

4. Stool bilirubin assay

Four conical centrifuge tubes of 10 ml. capacity were labelled one through four: tube two served as a blank for tube one, and tube four as a blank for tube three. The meconium was weighed out in a glass homogenizing tube on a Mettler balance to the nearest one-hundredth of a g., mixed with 50% ethanol in a concentration of one g. per 10 ml. of ethanol solution, and homogenized for 15 minutes. Before homogenization, the homogenizing tube was

wrapped in tin foil to protect the homogenate from light. Into tubes one and two were placed 0.5 ml. aliquots of meconium homogenate, into tubes three and four, 1.0 ml. aliquots of meconium homogenate. Tube one was then diluted with 1.5 ml. of 50% ethanol, tube three with 1.0 ml. of 50% ethanol; tube two receives 5.5 ml. of 50% ethanol, tube four 5.0 ml. of 50% ethanol. One ml. aliquots of 1% ascorbic acid were pipetted into tubes two and four. Five ml. aliquots of reagent were added to tubes one and three, and the time noted. After being tightly sealed with rubber caps, all four tubes were shaken in a Rotary Evapo-Mix shaker (Buchler Instruments, Fort Lee, New Jersey) at room temperature for ten minutes. They were then allowed to stand for exactly 80 minutes at room temperature. All four tubes were centrifuged immediately at the end of 80 minutes at 5000 r.p.m. for 15 minutes. The supernatant from each tube was decanted separately into matched spectrophotometer tubes. The per cent transmission was then read in a junior spectrophotometer at 660 mu. (Model 6A, Coleman Instruments, Inc., Maywood, Illinois). Tube two was set at 100% transmission, and the per cent transmission for tube one recorded; tube four was then set at 100% transmission, and the per cent transmission for tube three recorded. These per cent transmission values were converted to mg. of bilirubin from

a previously established standard curve. The completeness of the extraction was assessed by the fact that the amount of bilirubin in tube three should represent twice the amount in tube one. Knowing the original concentration of meconium (1g./ml. solution), the concentration of bilirubin in mg./g. of meconium could be calculated from tubes one and three. The average of the two values was taken as the final answer.

5. Preparation of bilirubin standard (121)

Ten mg. of bilirubin was weighed out in subdued light on a Mettler balance and placed in a 250 ml. Erlenmeyer flask. Ten ml. of 0.05 N sodium hydroxide was quickly added and swirled to dissolve the bilirubin. Eighty-nine ml. of degassed 50% ethanol was then added and swirled. One ml. of concentrated hydrochloric acid was added, and the mixture stored under nitrogen away from light under refrigeration. The final concentration of bilirubin was 10 mg./ml. of solution.

# IV. RESULTS

## 1. Stool beta-glucuronidase assay

A standard curve was first established by adding phenolphthalein to meconium and acetate buffer digests, incubating, adding glycine buffer, centrifuging, and plotting per cent transmission against known amount of phenolphthalein (see Graph 1). Each point in the standard curve was run in triplicate, standard deviation was derived from these three values.

To prove the accuracy and reproducibility required the ability to recover a known amount of enzyme. A known quantity (200 units) of beta-glucuronidase was therefore added to digests containing both meconium homogenate and substrate. The blank contained meconium homogenate and beta-glucuronidase with substrate added after incubation and alkalinization. Two hundred units of beta-glucuronidase was recovered from those digests to which enzyme was was added. This recovery corresponded exactly to the amount added. Thus, the inclusion of standard points in the assay allows accurate correction for interference created by stool pigments.

Other parameters of the reaction were also investigated. In order to establish the linearity of the reaction rate, the reaction was stopped at 30, 60, 90,

120, and 150 minutes. A straight line was obtained when per cent transmission or amount of phenolphthalein released were plotted versus time (see Graph 2). Authors working with beta-glucuronidase from other sources have shown linearity from 30 minutes to 20 hours. (122) In order to test the average uniformity of the meconium homogenate, increasing amounts of homogenate were placed in digests with substrate and incubated. (123) The straight line relationship obtained indicates the homogeneity of the meconium-acetate buffer mixture (Graph 3). Other authors have reported inhibition of the enzyme from both meconium and other sources by the sugar 1,4-disaccharolactone. (123-125) The enzyme was strikingly inhibited by this sugar in this assay system (see Table 2). Similar to the findings of other investigators, the enzyme was also found to be stable when the meconium homogenate was frozen immediately after homogenization (see Table 3). (120,123)

2. Stool bilirubin assay

A standard curve was established by adding known, increasing amounts of a standard bilirubin solution to the assay procedure described above (see Graph 4). To assure the reproducibility and accuracy of the assay, known increasing amounts of meconium were analysed with and without known added quantities of bilirubin (see Table 4 and Graph 5). An error of no greater than eight

per cent was found, a figure which compares favorably with that of Evelyn and Malloy. (126)

3. Clinical results

The neonatal and maternal blood groups, the Coombs test results, the birth weights, and the per cent weight loss of the infants are shown in Table 1. In Tables 5 and 6 are shown the daily total serum bilirubin levels on all ten infants, and the quantities of beta-glucuronidase and bilirubin in the first day's stool and first stool for all ten infants. In Table 7 are shown the maximum serum bilirubin of the neonate during the first five days of life, the change in the serum bilirubin during the first 24 hours of life, the maximum change in the bilirubin between the first day level and the highest value during the first five days, the ratio of the concentrations of beta-glucuronidase to bilirubin in the total 24 hours.

Analysis of these data was accomplished by linear regression correlation from a standard program written for a 9100 B Hewlett Packard Calculator. Both the ratio in the first stool and the ratio in the first day's total stool were compared individually to each of the cited bilirubin measurements, first on a scatter-graph, and then by calculation of the correlation coefficients (see Graphs 6-9). The ratios were always represented on the y axis, and the bilirubin values on the x axis. The point for each infant

is represented by his number from Table 1. Correlation coefficients are given on each scatter graph.

# V. DISCUSSION

## 1. Beta-glucuronidase assay

Assays for beta-glucuronidase have been applied to an almost endless number of tissues, body fluids, and experimental models. (123) These investigations have centered primarily around two themes. First, researchers have sought a correlation between increased enzyme activity in tissue or blood and neoplastic lesions. (123) Secondly, investigators have looked at the reciprocal effects of steroid hormones and beta-glucuronidase in both sex and non-sex organs. (123) While substrates for the assays used in these investigations have been as varied as the projects, the standard assay that has emerged utilized phenolphthalein glucuronic acid as a substrate. (120) This substrate has proven especially valuable in impurified tissue homogenate or body fluids. (127) The procedure involves incubation of two digests of the test substance with the substrate for a time that depends on the amount of activity anticipated in the digests. The reaction is run in an appropriate acetate buffer. (123) The control is made up of buffer plus the test digest without substrate. The blank contains only acetate buffer, glycine buffer, and substrate. Alkalinization of the digest with glycine buffer (pH 10.4) at the

end of the incubation not only stops the reaction but also causes the free phenolphthalein to assume its maxumum stable color intensity. (122) At this alkaline pH, unreacted phenolphthalein glucuronic acid interferes minimally with absorption at 540 mu. (123) A standard curve can thus be established. The difference between the spectrophotometric reading of the blank and that of the control is added to the average of the two test digests, and, by using the standard curve, the amount of phenolphthalein released during the incubation can be calculated.

The primary problem encountered in applying this procedure to meconium and neonatal stool is that different stools interfere with spectrophotometric determinations to widely varying degrees because of differing amounts of fecal pigment and proteins. This variation clearly restricts the ability of the assay to detect high enzyme activity in stools with high interference. Earlier efforts to overcome this problem in beta-glucuronidase assays on bile included addition of charcoal after incubation and extraction of phenolphthalein with alcohol. (128) In an effort to find a shorter, easier solution to this problem, standard curves were established for several different stools. Known amounts of phenolphthalein were added to meconium homogenates, incubated, centrifuged, and the per cent transmission recorded. A standard curve without meconium was also run. It was found that although two

different stools shifted the position of the standard curve slightly, the slopes of the curves were both identical and were also the same as the standard curve without meconium (see Graph 1). Knowing this fact, two standard points, i.e., known amounts of phenolphthalein plus meconium without substrate were included in each assay. In this way, correction could be made for the varying amount of interference in each stool.

2. Bilirubin assay

A wide variety of methodologies has also evolved for bilirubin assays in meconium and neonatal stool. (9) The assay that has won widest acceptance is that of Evelyn and Malloy. (126) After examining the differences in maximum color stability generated by using different reagents, they concluded that a hydrogen peroxide reagent gave maximum color intensity and stability in the most convenient time. They did not, however, explore differences in color development and disappearance with amounts of bilirubin greater than ten mcg. Based on previous studies (12,18), the amount of bilirubin that was to be measured in the assay described above necessitated standardization of values greater than ten mcg. In standardizing this higher range, it was noted that the stability of the color generated by the hydrogen peroxide reagent was not as great as that in the lower range. In an effort to observe the different rates of color development in varying amounts

of bilirubin, the reagent suggested by Evelyn and Malloy (126) was added to three digests with widely different amounts of bilirubin. Using a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) to read per cent transmission in each digest every 15 minutes over a four-hour period, it was found that the higher amounts of bilirubin took longer than an hour to develop maximum color intensity and stability (see Chart 1 and 2). In order to find a reagent that would allow maximum color intensity and stability to develop in a reasonably narrow time period over the range of 10-100 mcg. of bilirubin, the amounts of hydrochloric acid and hydrogen peroxide in the reagent were varied (see Chart 3). It was found that the Evelyn and Malloy reagent cited in the above gave maximum color intensity in 90 minutes for bilirubin from 10-150 mcg. (see Chart 3). However, unlike Evelyn and Malloy's findings for the lower range, the stability of the color is not long-lasting. Unless per cent transmission is measured at 90 minutes, the color begins to disappear. The importance of an exact time period for the reaction was thus established.

A single extraction procedure was employed in this assay for two reasons. First, it was felt that a constant substantial percentage of the bilirubin in the stool was obtained by a single extraction. (121) By

running 0.5 ml. and 1.0 ml. aliquots of stool homogenate simultaneously, this assumption was assessed with each run and found to be reliable. Secondly, a second extraction would make the assay prohibitively time-consuming for application on a large scale basis.

Two other problems which arose concerned the blanks used with each run. The first problem is that of auto-oxidation of bilirubin in the blank. Such a process limits the ability of the assay to detect small differences in amounts of bilirubin. As suggested by Odell, ascorbic acid was included in each blank to prevent autooxidation. (12) A second and more serious problem occurred when the blank, because of pigment and protein in the stool, became more opaque than the reagent tube. If such opacity developed in the blank tubes, values were obtained by using tube one as a blank for tube three. The difference in the amount of bilirubin in the two tubes represented the amount of bilirubin in 0.5 ml. of stool. The concentration of bilirubin in stool was then calculated on the basis of this value. This complication was infrequent and occurred primarily in stools after the third day.

Thus, by examining the problems of standardization of Evelyn and Malloy's bilirubin assay in neonatal stool, the method was adapted and applied more accurately and reproducibly.

3. Role of enterohepatic circulation in neonatal juandice

As indicated in the introduction, the contribution of the enterohepatic circulation of bilirutin to neonatal jaundice in the term infant has been recognized by a variety of investigators since 1961. The unique neonatal circumstance of a sterile gut and high concentrations of beta-glucuronidase make deconjugation physiologically necessary for bilirubin reabsorption. In the normal adult gut, the conjugated bilirubin is excreted into the gut lumen conjugated with glucuronide. Because most of the small intestine is relatively sterile in a normal adult down to the terminal ileum, the conjugated bilirubin reaches the large intestine before it is deconjugated and/or converted to urobilinogen. (129,130) The reduction of bilirubin probably is catalyzed by dehydrogenases of anaerobic organisms. The origin of the beta-glucuronidase in the neonatal gut seems to be mucosal rather than bacterial on the basis of its pH optimum. (58,61) While the enzyme has been isolated from almost every tissue in the body, its presence in the neonatal gut has not been satisfactorily explained. Preliminary results from an investigation in our laboratory of stool enzyme levels in babies whose mothers receive phenobarbitone show markedly increased levels of enzyme in first day stools. The enzyme might be originating in the liver, and be carried into the gut by increased bile flow caused

by the phenobarbitone. (131) Alternatively, phenobarbitone might be inducing increased secretion of the enzyme by jejunal or ileal mucosal cells. (132)

The exact site of bilirubin reabsorption is not clear, but the unconjugated bilirubin most probably gains access to the blood stream anywhere along the small or large bowel, not just in the terminal ileum where other portions of bile are also absorbed. Due to inconvenient and inadequate assay systems for stool bilirubin and beta-glucuronidase, no other available studies have correlated in a systematic manner enzyme to bilirubin ratios in the stool and the level of serum bilirubin.

With these assays at hand, a significant correlation has been demonstrated between the circumstances in the neonatal gut which might lead to increased bilirubin reabsorption, the ability of the neonate to excrete bilirubin in his stool, and the maximum serum bilirubin level. Expression of the ratio of the concentrations of enzyme to bilirubin was chosen rather than absolute amounts, because the stools were wrapped in diapers for significantly different amounts of time (15 minutes to six hours). The error introduced by the change in stool weight through absorption into the diaper was avoided by expressing the ratio of the concentrations. It was also felt that such a ratio would reflect more accurately the interaction of

the enzyme and bilirubin in the lumen of the gut where transit times, absorption of water, and other variables could not be controlled.

It was found that the ratio of the concentrations of enzyme to bilirubin in the first day's stool plotted against the maximum bilirubin level had the highest correlation coefficient of the combinations tried (correlation coefficient equal to 0.76). This correlation was significant ($p < 0.02$). In order to assure a suitably symmetrical distribution of points along both sets of axes, as assumed by this linear regression analysis, the log of the ratio was plotted against the maximum bilirubin levels (see Graph 10). This correlation was also found to be significant with a correlation coefficient of 0.75 ($p < 0.02$).

The correlation of the ratio of the concentrations of the enzyme, beta-glucuronidase, and of bilirubin in the stool over the first 24 hours of life with the maximum serum bilirubin during the first five days of life suggests that in the term infant, the initial circulation of bilirubin reabsorbed from the gut contributes significantly to the bilirubin load. These data also suggest that any treatment which would reduce the enterohepatic circulation of bilirubin during the first 24 hours might significantly reduce maximum serum bilirubin levels in full-term neonates. Odell et al. have devised such a treatment with

which they have been able to alter serum bilirubin levels in the first five days of life. (12) While phenobarbitone treatment both pre- and post-natally shifts the peak serum bilirubin from day four to day two, the same kind of rise and fall is observed as in controls. In agar fed infants, the rise is much flatter with no marked increased and decline in serum bilirubin values, but stool excretion of bilirubin is significantly increased. (12) These observations suggest that enterohepatic circulation plays a significant role in the full-term neonate.

The two major criticisms which might be made of the data presented in this study are, first, that the bilirubin load from both erythropoietic and non-erythropoietic sources might have varied in this population of infants. Red blood cell survival studies might have been done to prove that the increase in serum bilirubin was not in fact a result of increased hemolysis. An effort was made, however, to keep the test population as homogeneous as possible. Secondly, measurement of conjugated and unconjugated bilirubin in the stool was not attempted, primarily for technical reasons. It would have been interesting to see if a correlation existed between the amount of enzyme and the amounts of conjugated and unconjugated bilirubin excreted in the stool.

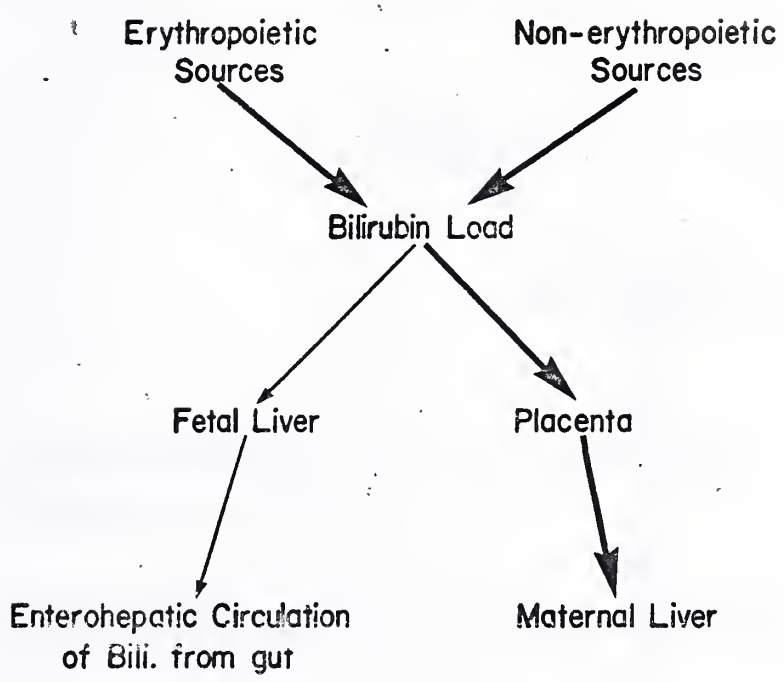
The next step in studying the significance of the enterohepatic shunt is to look at different categories of

infants. Although no large series of infants treated with agar has been reported, the possible efficacy of such therapy should be evaluated in premature infants and other at-risk populations. A prospective study might also be done whereby first day stools of normal newborns are analyzed, and those who appear to have a significant chance of high serum bilirubin levels secondary to a large enterohepatic shunt be treated with agar. The ability of phenobarbitone both pre- and post-natally to increase excretion of bilirubin might also be studied.

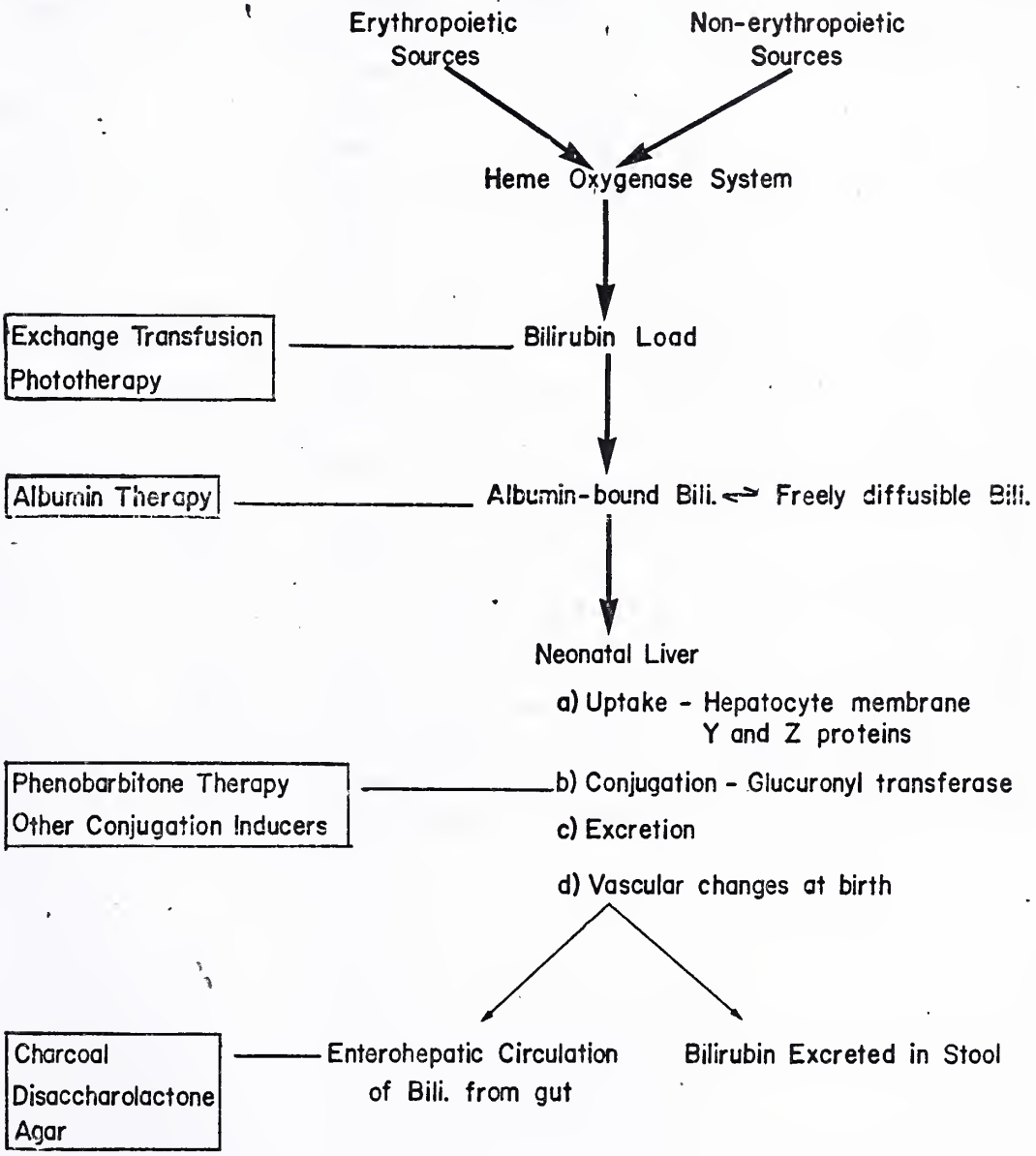
4. Summary

In summary, on the basis of the correlation between the ratio of the concentrations of beta-glucuronidase and bilirubin in the first day's stool and the maximum serum bilirubin during the first five days of life, the enterohepatic circulation of bilirubin has been shown to be a significant cause of hyperbilirubinemia among full-term normal neonates.

# Diagram I - Fetal Bilirubin Metabolism



## Diagram 2 - Neonatal Bilirubin Metabolism and Sites of Therapeutic Intervention



# TABLE I - Tabulation of Relevant Neonatal Parameters

| Infant's no. & sex | Infant's blood group | Mother's blood group | Coombs test | Birth weight | % weight loss | Apgars 1 min/5 min |
|---|---|---|---|---|---|---|
| 1 - M | A pos. | A pos. | neg. | 3295 | 1 | 9/9 |
| 2 - F | A pos. | AB pos. | neg. | 2820 | 2 | 9/9 |
| 3 - M | O pos. | O pos. | neg. | 3840 | 6 | 8/9 |
| 4 - M | O pos. | O pos. | neg. | 3635 | 1 | 7/8 |
| 5 - F | B pos. | O pos. | neg. | 3010 | 5 | 9/10 |
| 6 - F | O pos. | B pos. | neg. | 3310 | 7 | 9/9 |
| 7 - F | A pos. | A pos. | neg. | 2725 | 9 | 7/9 |
| 8 - F | O pos. | O pos. | neg. | 3840 | 5 | 9/9 |
| 9 - M | AB pos. | B pos. | neg. | 3080 | 4 | 8/8 |
| 10 - M | A pos. | A pos. | neg. | 3570 | 5 | 9/9 |

# TABLE 2 - Inhibition of Beta-Glucuronidase by 1,4-Saccharolactone

| | %T | mcg. of phenolphthalein released | % Inhibition |
|---|---|---|---|
| Stool without saccharolactone | 76.5 | 15 | 87 |
| Stool with saccharolactone | 96.5 | 2 | |

# TABLE 3 - Stability of Beta-Glucuronidase in Frozen Stool Homogenate

| | 0 hrs. | 24 hrs. | 48 hrs. | 72 hrs. | 168 hrs. |
|---|---|---|---|---|---|
| Units of Beta-Gluc. per gram of Stool | 450 | 420 | 425 | 425 | 420 |

S.D. = 12.6.
Avg. = 428.0

# TABLE 4 - Accuracy of the Bilirubin Assay

| ml. of stool homogenate | bili. in sample (mcg.) | bili. added (mcg.) | bili. calculated (mcg.) | bili. determined (mcg.) | % recovery |
|---|---|---|---|---|---|
| .5 | 37 | 25 | 62 | 62.5 | 100 |
| 1.0 | 74 | 25 | 99 | 107 | 108 |
| 1.5 | 111 | 25 | 136 | 142 | 104 |
| 1.75 | 129 | 25 | 154 | 162 | 104 |
| 2.0 | 148 | 0 | 148 | 142 | 95 |

## TABLE 5 - Daily Total Serum Bilirubin Levels

| Infant no. | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|---|---|---|---|---|---|
| 1 | 4.4 | 7.0 | 5.1 | 4.7 | 4.4 |
| 2 | 7.3 | 9.0 | 10.5 | 9.3 | 8.7 |
| 3 | 4.7 | 6.0 | 5.3 | 4.5 | 4.3 |
| 4 | 3.5 | 4.0 | 3.3 | 2.7 | 3.0 |
| 5 | 6.7 | 7.6 | 7.0 | 6.5 | 5.5 |
| 6 | 3.5 | 3.6 | 3.5 | 3.4 | 3.1 |
| 7 | 7.0 | 9.7 | 13.5 | 15.5 | 17.5 |
| 8 | 4.3 | 5.3 | 3.5 | 2.0 | 1.0 |
| 9 | 2.3 | 3.5 | 3.5 | 2.3 | 1.7 |
| 10 | 4.5 | 7.5 | 10.5 | 12.0 | 14.5 |

All bilirubin values expressed in mg %

# TABLE 6 - Excretion of Beta-Glucuronidase and Bilirubin

| Infant's no. | [B-G] in first stool | [B-G] in first day | [Bili] In first stool | [Bili] in first day |
|---|---|---|---|---|
| 1 | 657 | 610 | 168 | 218 |
| 2 | 2533 | 2533 | 127 | 127 |
| 3 | 866 | 866 | 352 | 352 |
| 4 | 316 | 316 | 250 | 250 |
| 5 | 1291 | 1291 | 610 | 610 |
| 6 | 266 | 266 | 165 | 165 |
| 7 | 1916 | 1916 | 45 | 45 |
| 8 | 583 | 421 | 235 | 629 |
| 9 | 1250 | 807 | 30 | 181 |
| 10 | 1150 | 921 | 320 | 270 |

[B-G] = concentration of beta-glucuronidase expressed as units of enzyme per gram of stool

[Bili] = concentration of bilirubin expressed as micrograms of bilirubin per gram of stool

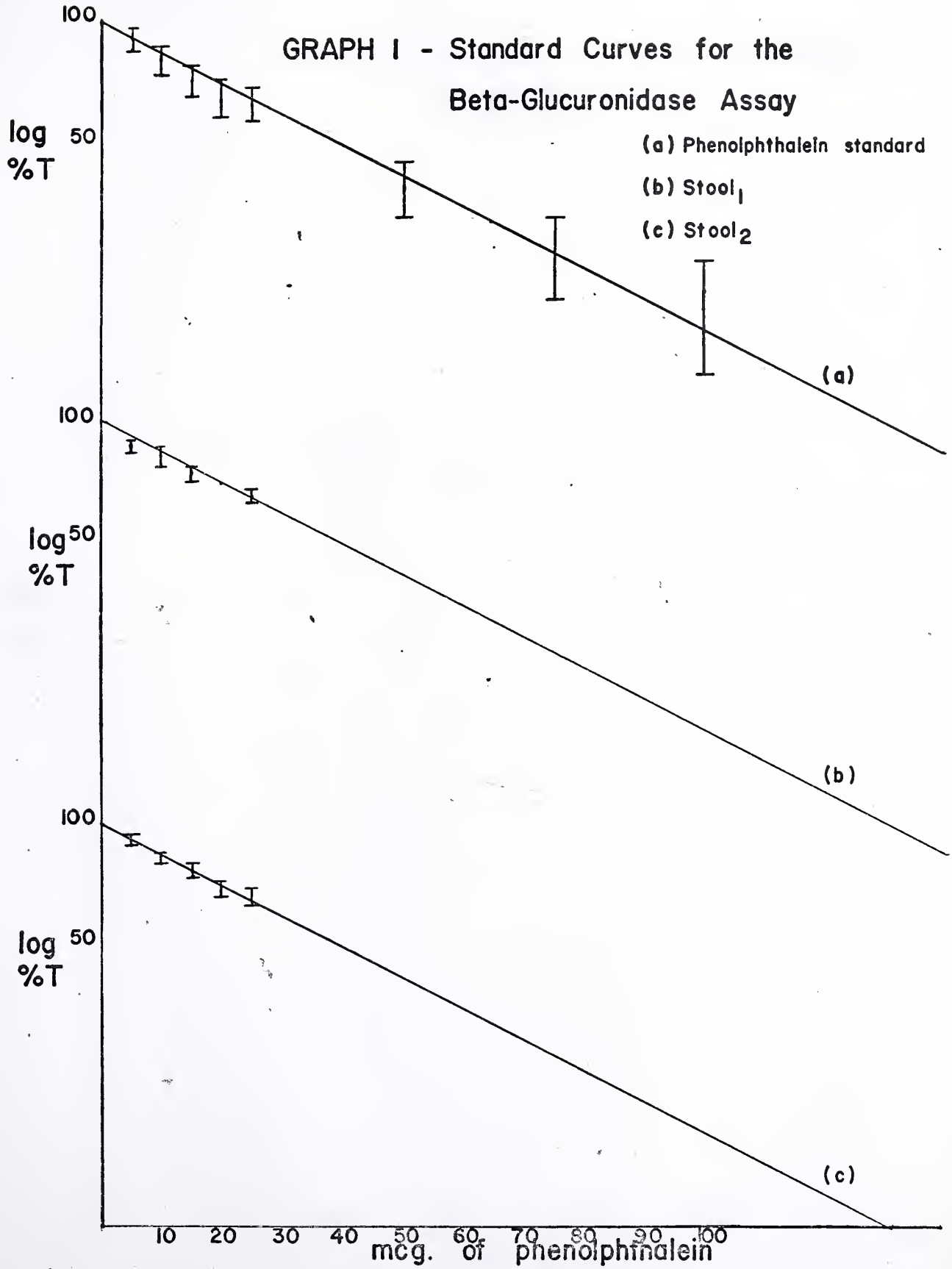
# TABLE 7 - Correlation of Serum Bilirubin Values $\frac{[B\text{-}G]}{[bili]}$ with [bili] in Stool

| Infant's no. | Max. bili. | Change in bili. 24 hrs. after first stool | Max. change in bili. | $\frac{[B\text{-}G]}{[bili]}$ in first stool | $\frac{[B\text{-}G]}{[bili]}$ in first day |
|---|---|---|---|---|---|
| 1 | 7.0 | 2.5 | 2.5 | 3.9 | 2.8 |
| 2 | 10.5 | 1.7 | 3.2 | 20.0 | 20.0 |
| 3 | 6.0 | 1.3 | 1.3 | 2.5 | 2.5 |
| 4 | 4.0 | 0.5 | 0.5 | 1.3 | 1.3 |
| 5 | 7.6 | 0.9 | 1.1 | 1.9 | 1.9 |
| 6 | 3.5 | 0.1 | 0.1 | 1.6 | 1.6 |
| 7 | 17.5 | 2.7 | 10.5 | 42.0 | 42.0 |
| 8 | 5.3 | 1.8 | 2.0 | 2.5 | 0.7 |
| 9 | 3.5 | 1.2 | 1.5 | 41.0 | 4.4 |
| 10 | 14.5 | 3.0 | 10.0 | 3.6 | 3.4 |

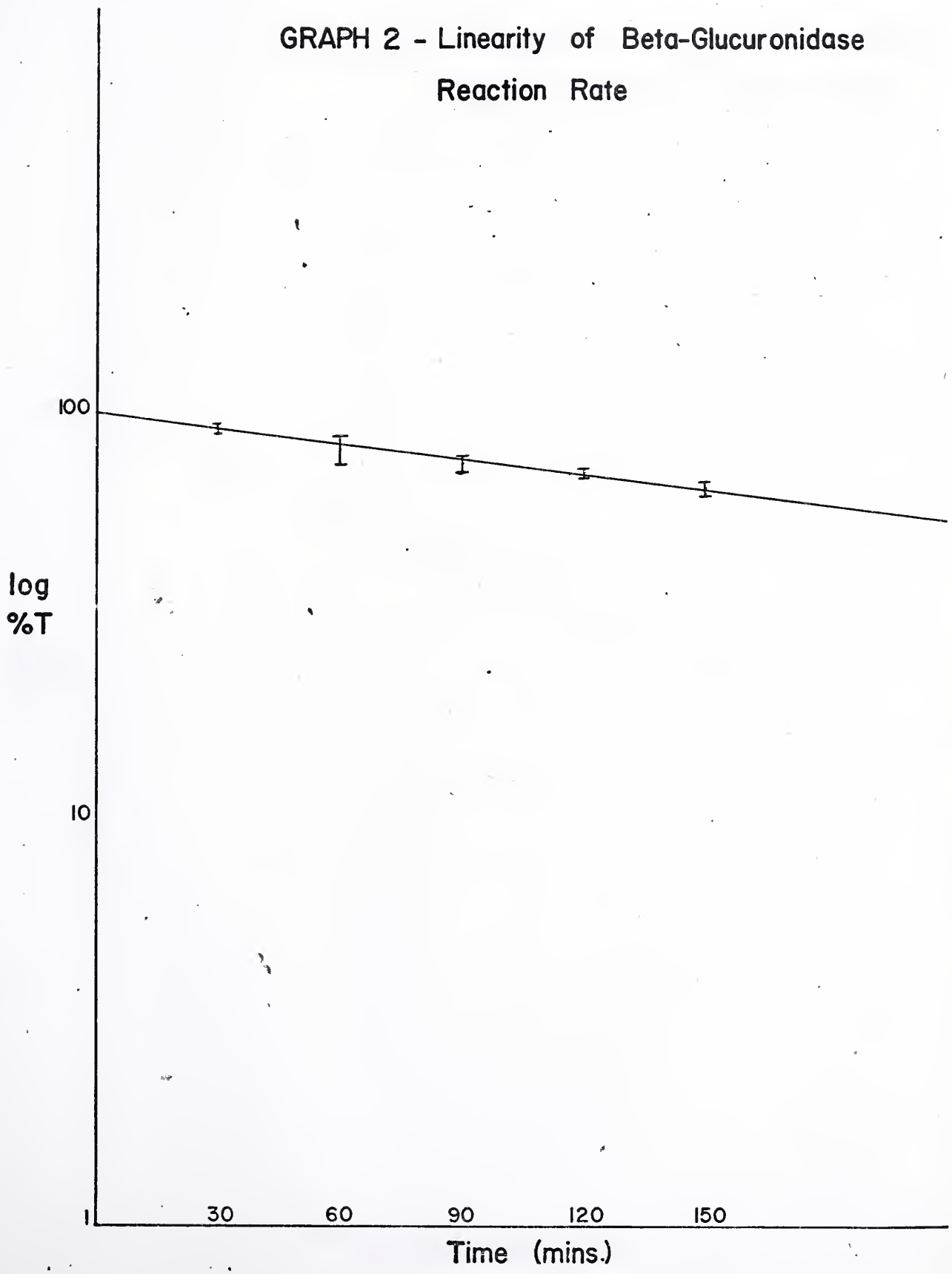
All serum bilirubins expressed in mg%

[B-G] = concentration of beta-glucuronidase expressed as units of enzyme per gram of stool

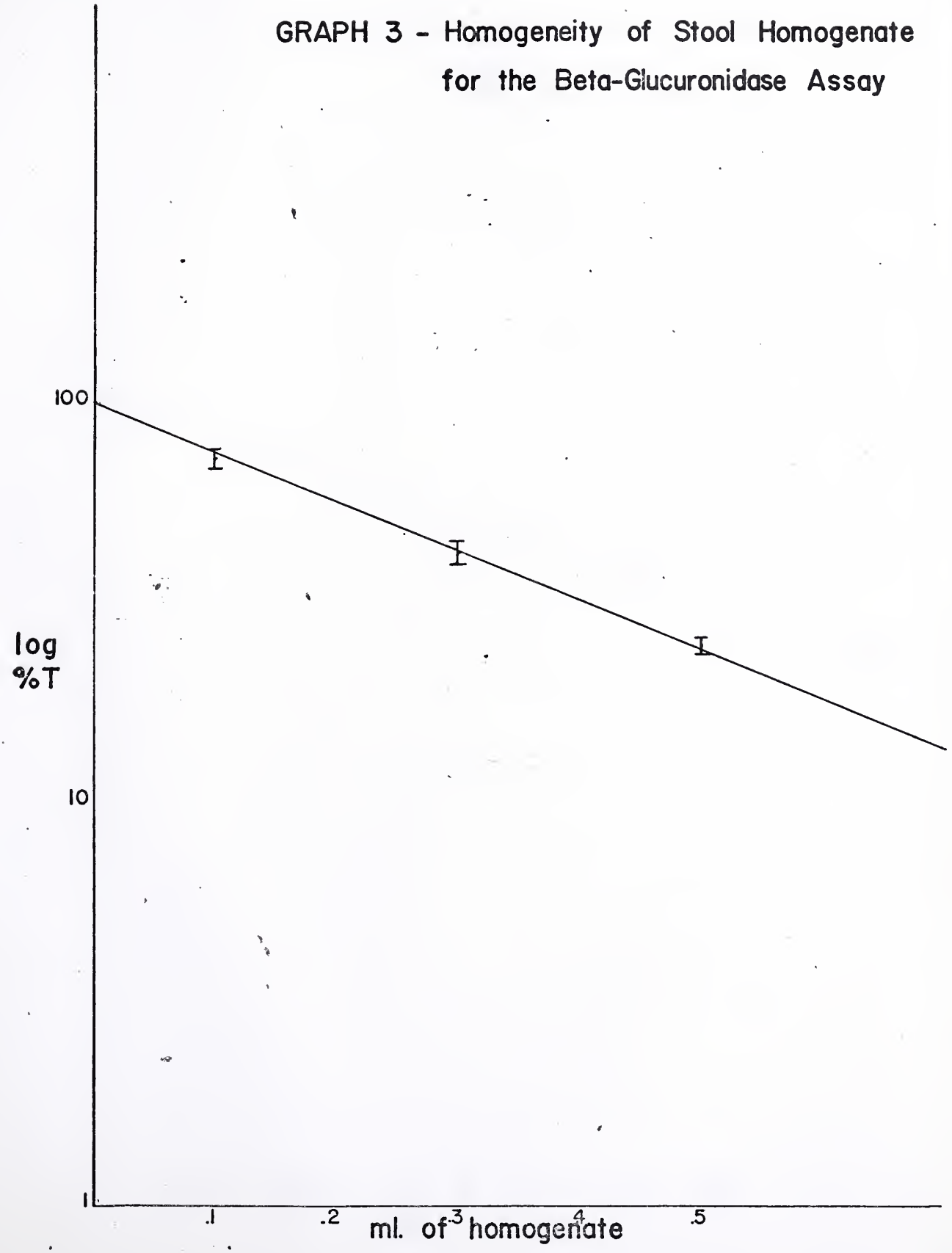
[bili] = concentration of bilirubin expressed as micrograms of bilirubin per gram of stool


GRAPH I - Standard Curves for the
Beta-Glucuronidase Assay
(a) Phenolphthalein standard
(b) Stool1
(c) Stool2
100
log %T
50
(a)
100
log %T
50
(b)
100
log %T
50
(c)
10 20 30 40 50 60 70 80 90 100
mcg. of phenolphthalein

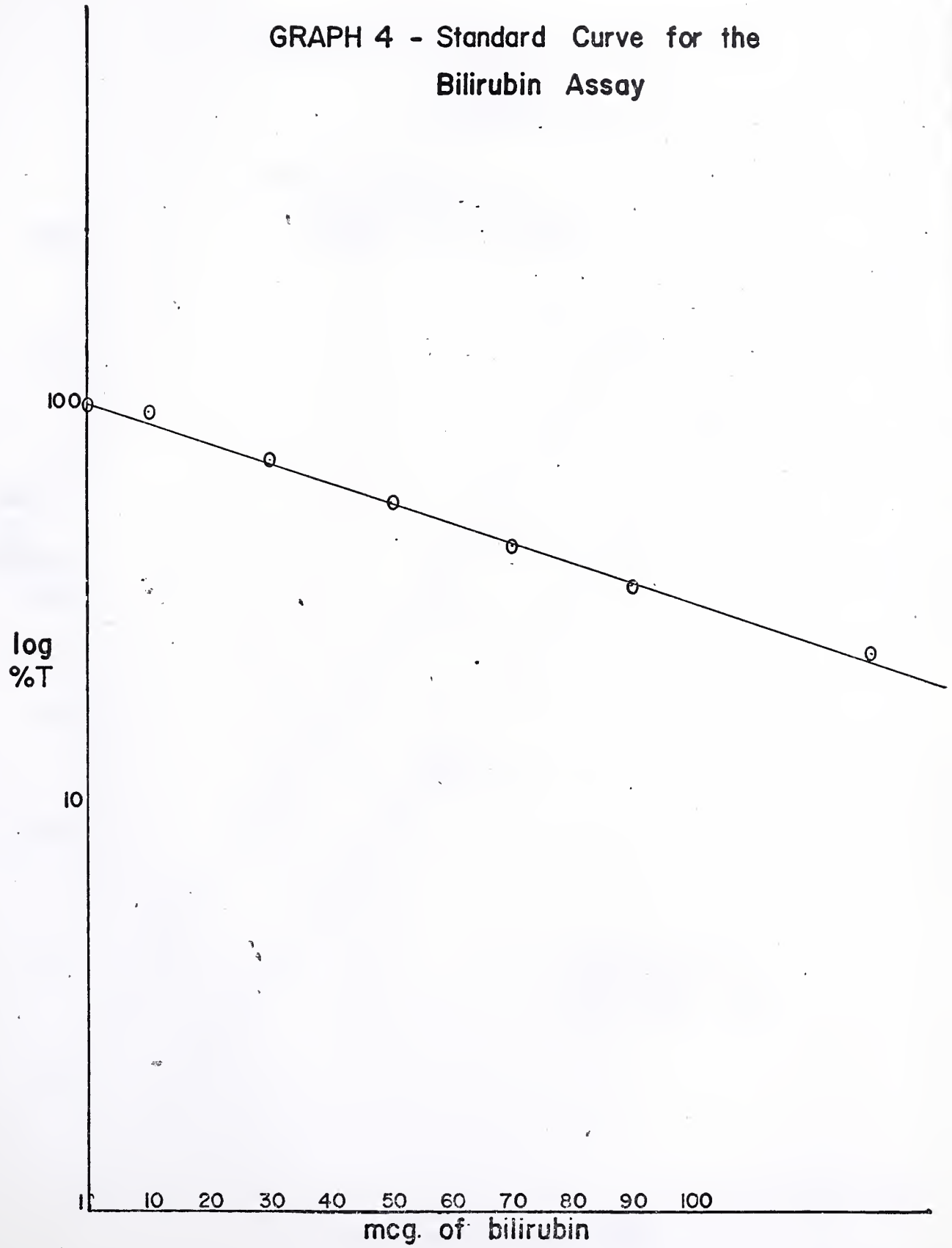
GRAPH 2 - Linearity of Beta-Glucuronidase Reaction Rate

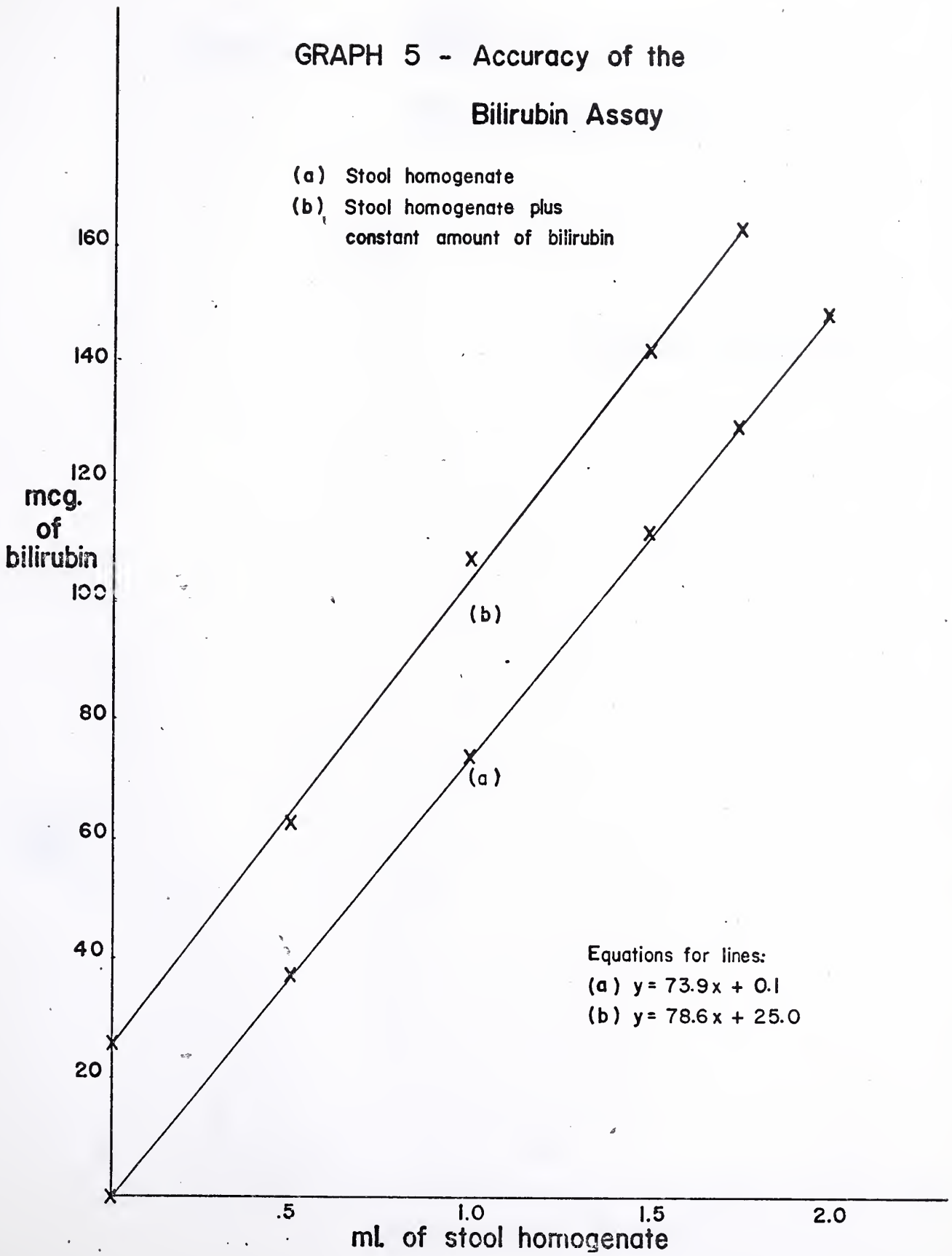


GRAPH 3 - Homogeneity of Stool Homogenate for the Beta-Glucuronidase Assay

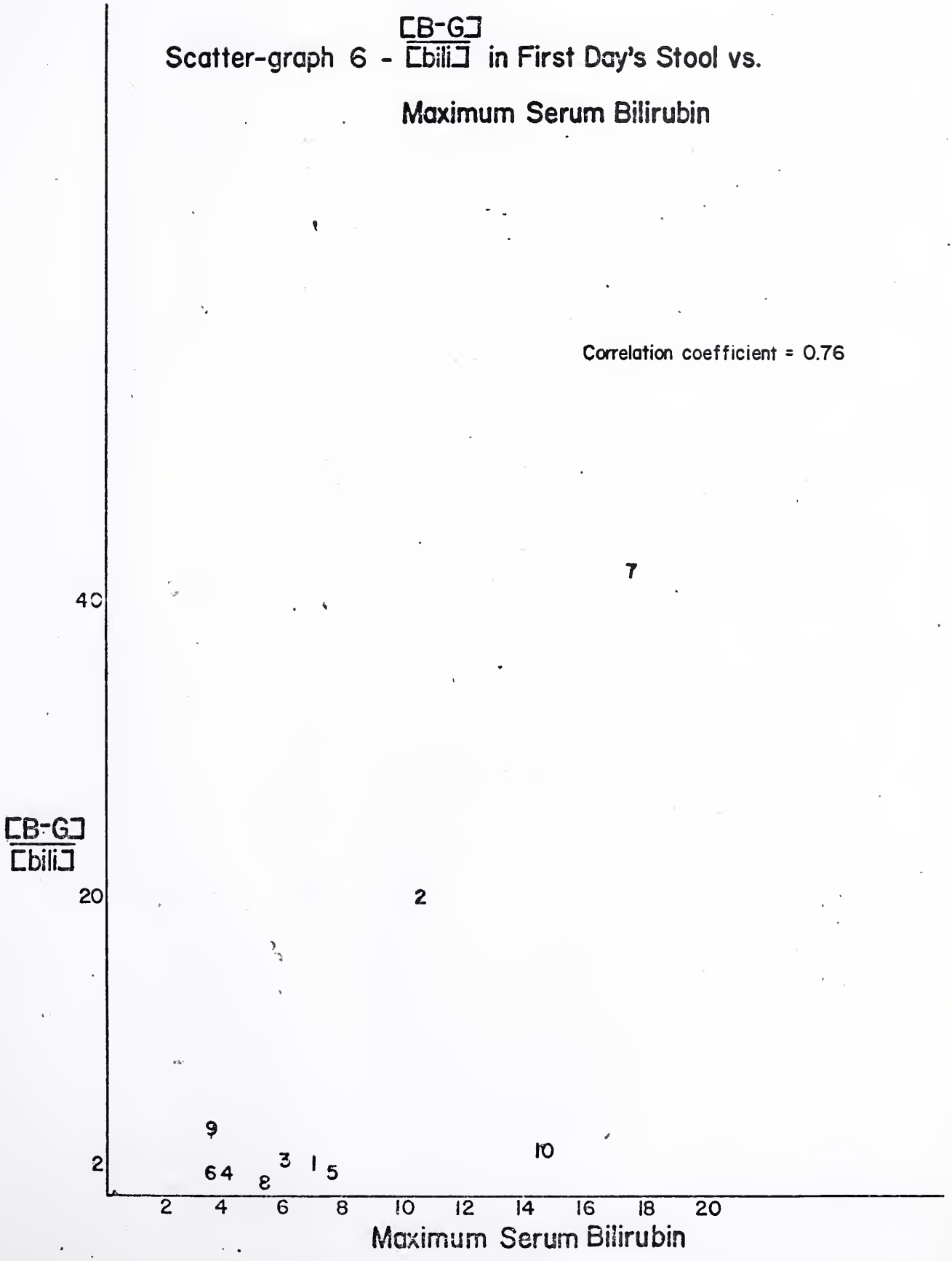


GRAPH 4 - Standard Curve for the Bilirubin Assay



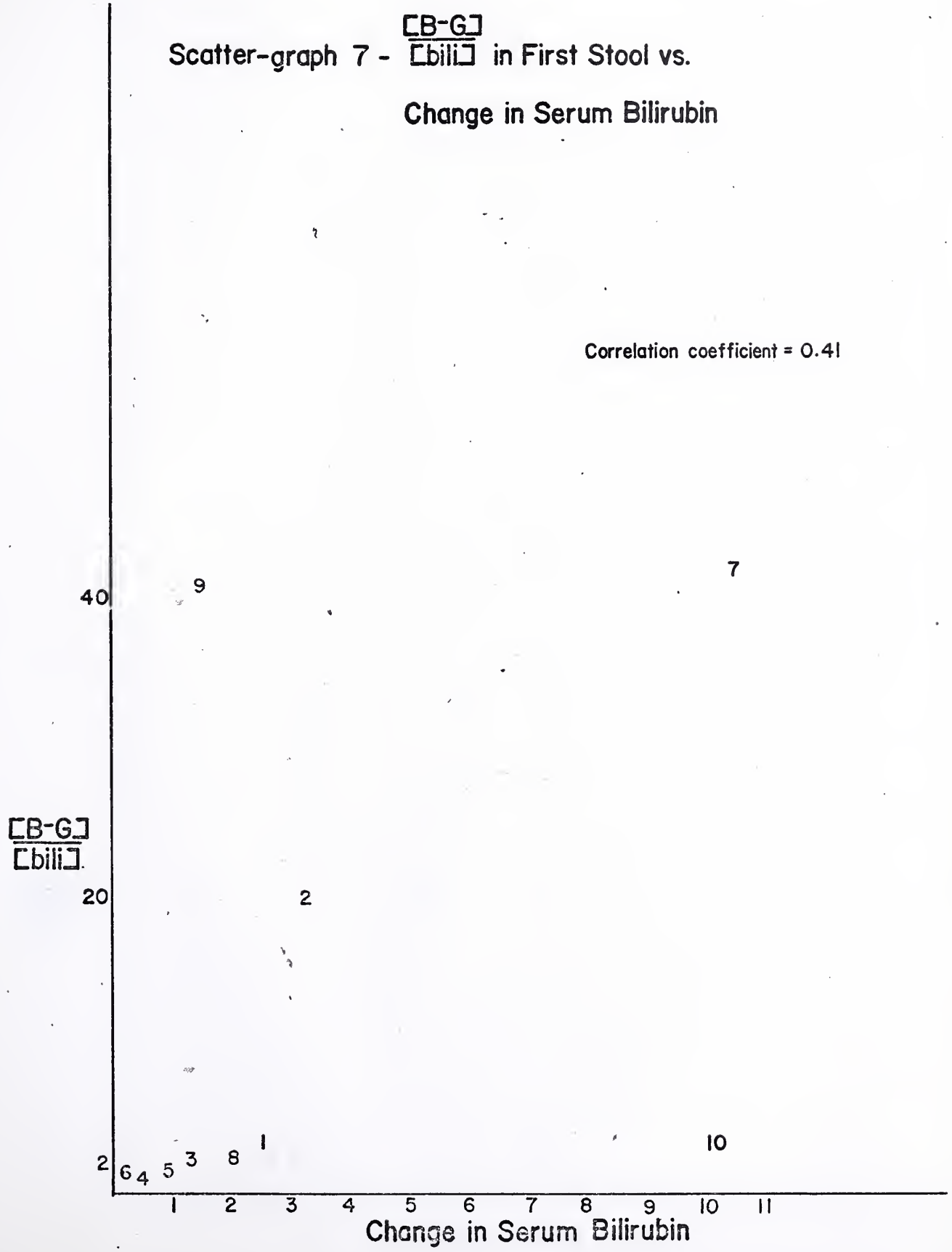

GRAPH 5 - Accuracy of the Bilirubin Assay
(a) Stool homogenate
(b) Stool homogenate plus constant amount of bilirubin
160
140
120
mcg. of bilirubin
100
80
60
40
20
(b)
(a)
Equations for lines:
(a) y = 73.9x + 0.1
(b) y = 78.6x + 25.0
.5
1.0
1.5
2.0
ml. of stool homogenate

Scatter-graph 6 - $\frac{[B-G]}{[bili]}$ in First Day's Stool vs. Maximum Serum Bilirubin



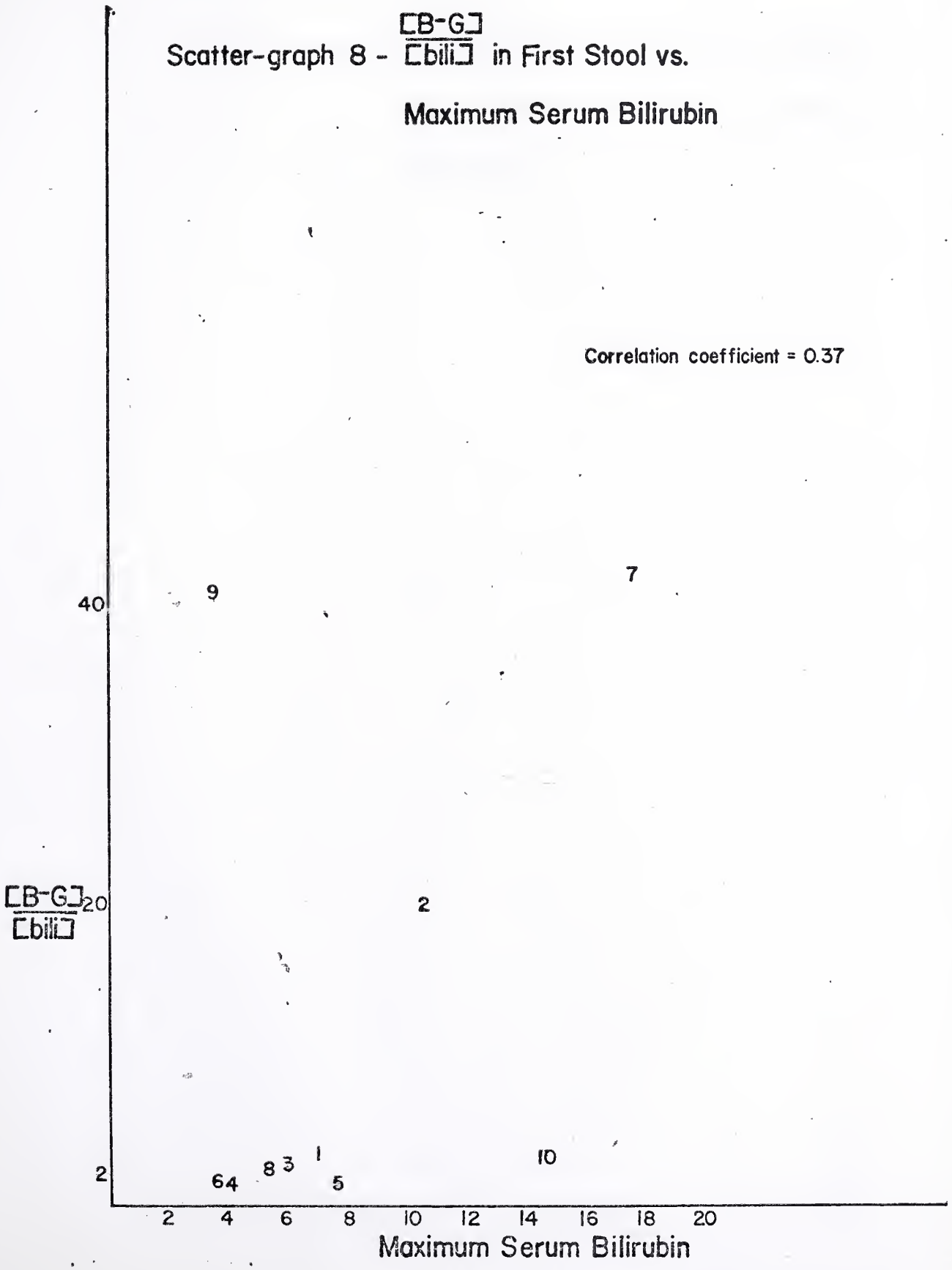
Scatter-graph 7 - $\frac{[B-G]}{[bili]}$ in First Stool vs.

Change in Serum Bilirubin

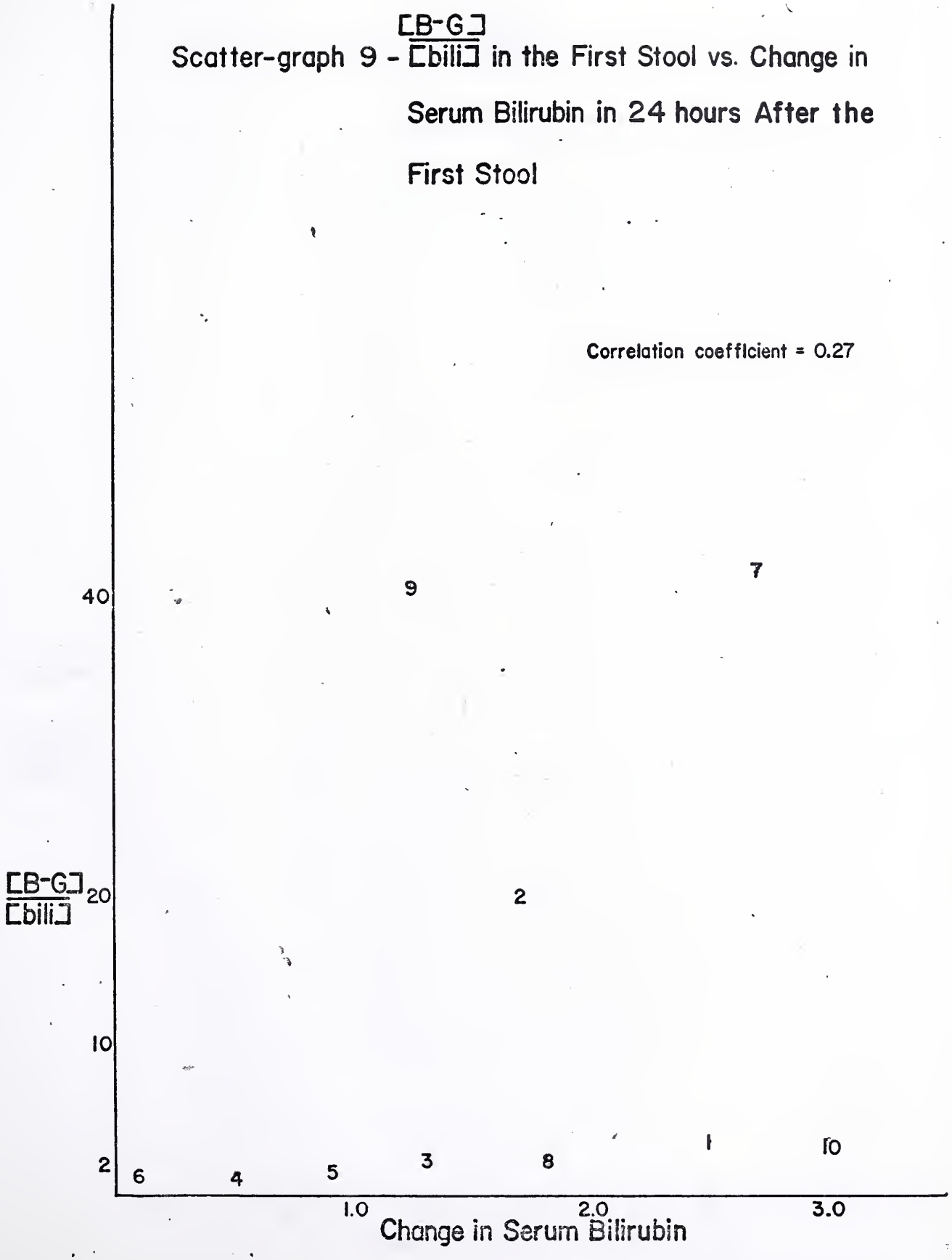


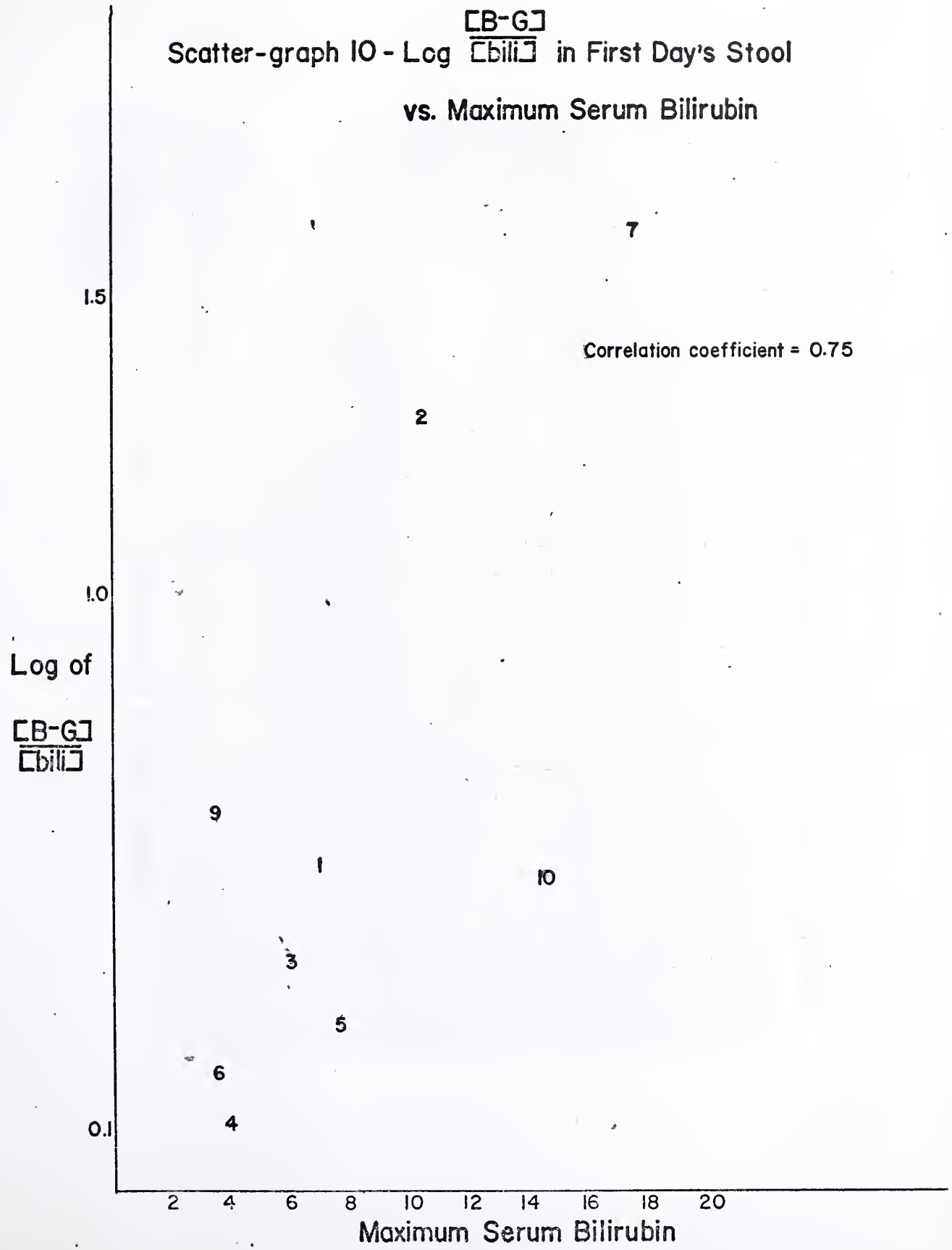
Scatter-graph 8 - $\frac{[B\text{-}G]}{[bili]}$ in First Stool vs.

Maximum Serum Bilirubin

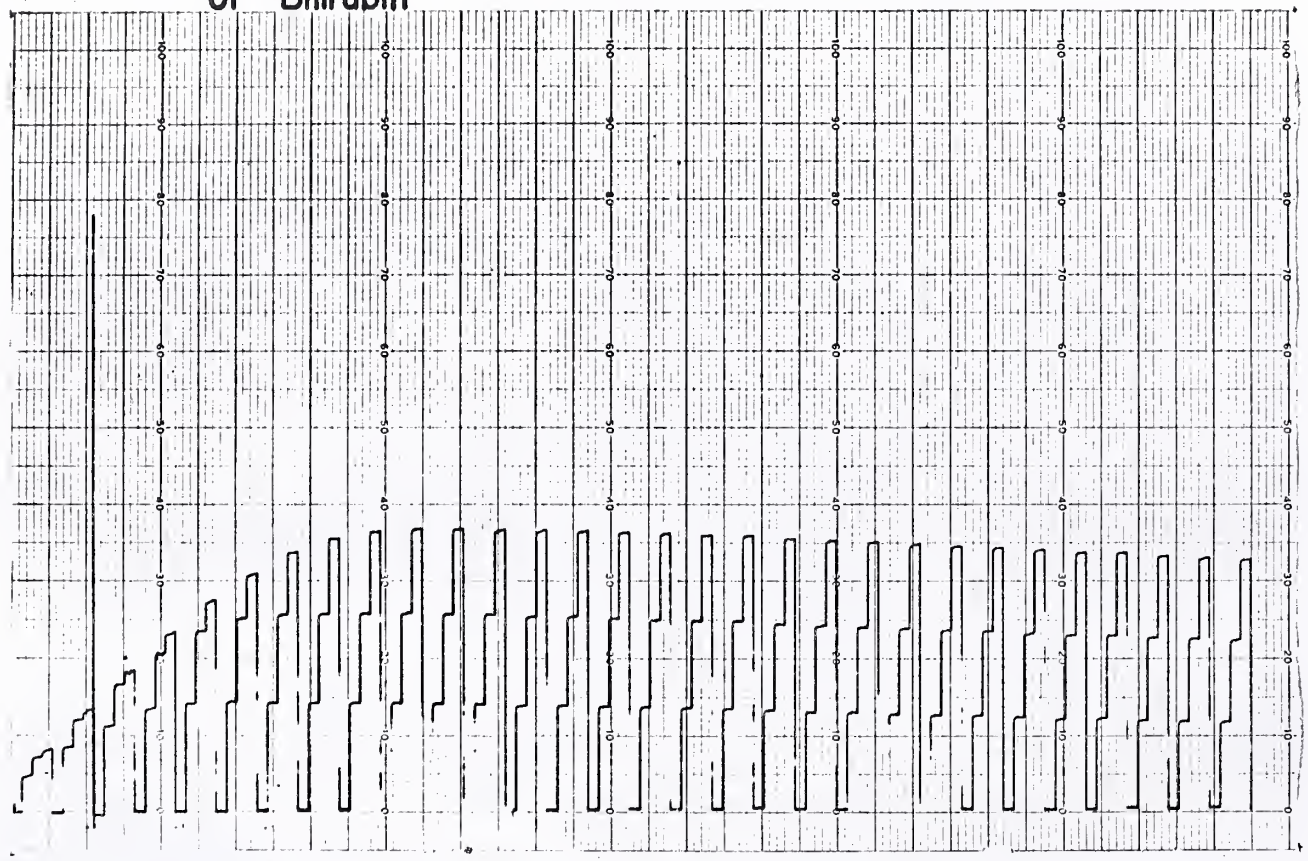


Scatter-graph 9 - $\frac{[B\text{-}G]}{[bili]}$ in the First Stool vs. Change in Serum Bilirubin in 24 hours After the First Stool



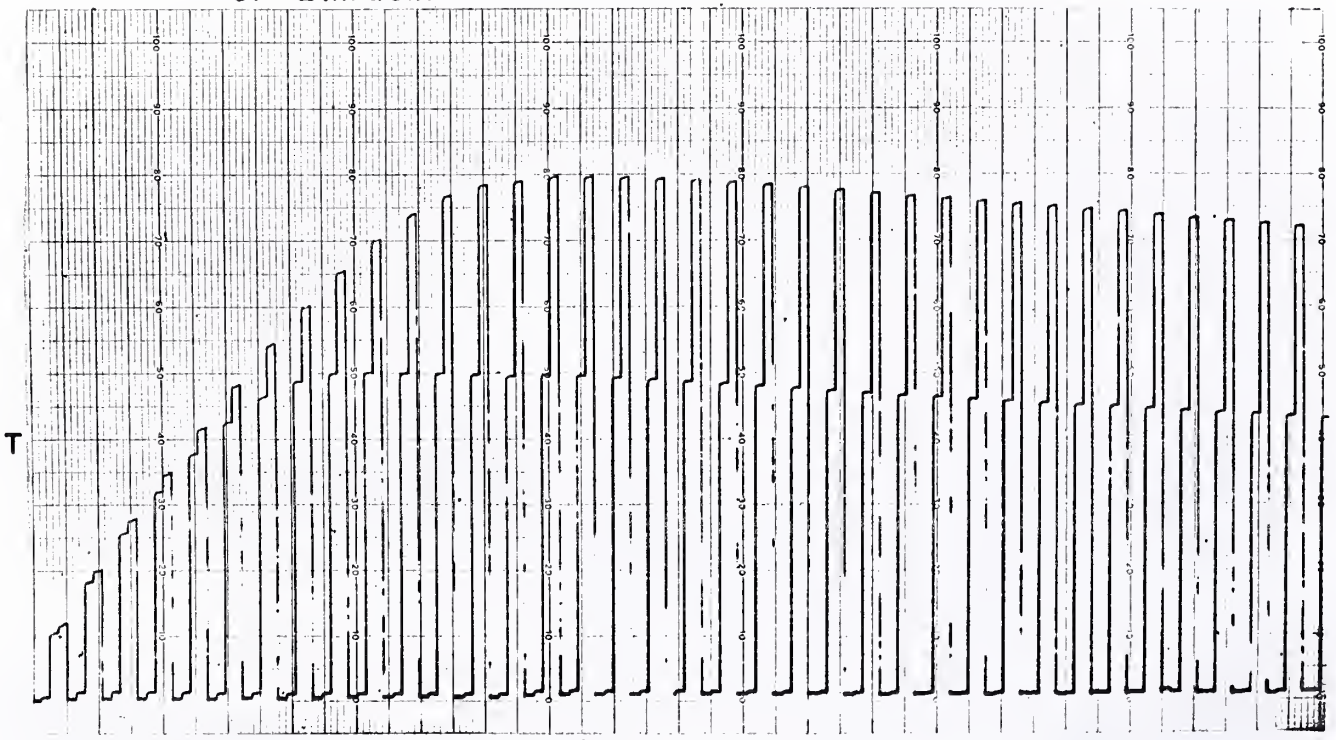

Scatter-graph 10 - Log [B-G]/[bili] in First Day's Stool
vs. Maximum Serum Bilirubin
Correlation coefficient = 0.75
Log of [B-G]/[bili]
1.5
1.0
0.1
7
2
9
1
10
3
5
6
4
2
4
6
8
10
12
14
16
18
20
Maximum Serum Bilirubin

## CHART I - Variation in Color Development with Different Amounts of Bilirubin

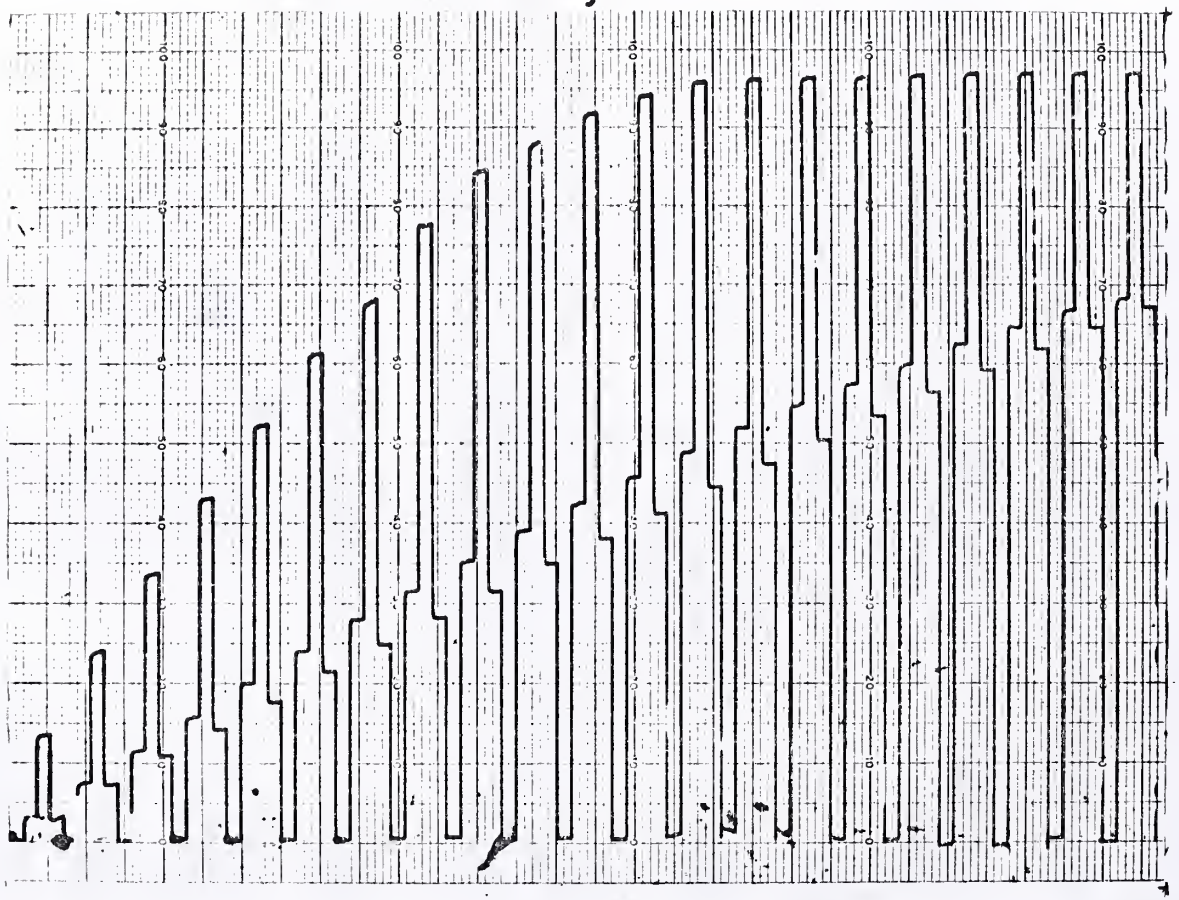



no. 1 - Blank
no. 2 - 30 mcg. bili.
no. 3 - 50 mcg. bili.
no. 4 - 70 mcg. bili.

## CHART 2 - Variation in Color Development with Different Amounts of Bilirubin



## CHART 3 - Variation in Color Development with Different Reagents in the Bilirubin Assay




no. 1 - Blank
no. 2 - 3.5 ml. reagent 1 (0.4 ml. hydrogen peroxide)
no. 3 - 5.0 ml. reagent 1
no. 4 - 3.5 ml. reagent 2 (0.8 ml. hydrogen peroxide)

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