A STUDY OF SERUM BILIRUBIN LEVELS IN RELATION TO KERNIKTERUS AND PREMATURITY

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(RECEIVED FOR PUBLICATION NOVEMBER 30, 1955)

The staining of the basal ganglia in various conditions not associated with iso-immunization has aroused much interest in recent years. With the introduction of replacement transfusion in the treatment of haemolytic disease, kernikterus due to this disease has become a comparative rarity.

Kernikterus has been described associated with acholuric family jaundice, idiopathic familial hyperbilirubinaemia, and in 1950 two groups of workers, Aidin, Corner and Tovey in Great Britain and Zuelzer and Mudgett in America, independently drew attention to the fact that kernikterus was sometimes associated with prematurity only.

The clinical picture of the premature infant who becomes jaundiced on the third or fourth day of life and by the sixth day exhibits profound jaundice, disinclination to feed, head retraction, changes in muscle tone and cyanosis, and in most cases dies on the sixth or seventh day have been described by Aidin *et al.* (1950), and more recently by Crosse, Meyer and Gerrard (1955).

The post-mortem appearances of kernikterus have aroused much speculation as to the metabolism of the breakdown products of haemoglobin and the pathogenesis of the disease. It has been suggested that bilirubin is the sole cause of the damage to the basal nuclei (Pickles, 1949; Küster and Krings, 1950) whilst other workers have claimed that it is due to some abnormal pigment such as mesobilifuchsin which arises from an underlying metabolic disorder (Schmorl, 1904; Bevis, 1953). The suggestions have been made too that the primary lesion may be asphyxial with a secondary bilirubin invasion (Govan and Scott, 1953; Aidin *et al.*, 1950) or the combination of hyperbilirubinaemia and hypoglycaemia (Gerrard, 1952).

Claireaux, Cole and Lathe (1953) produce convincing evidence that whatever else has occurred the pigment in the brains of these infants at necropsy is bilirubin. Whether the bilirubin is toxic or not, it does appear to be the common factor of all these theories.

Day (1954) has given an indication of the possible mode of action of bilirubin by showing experimentally that oxygen uptake of brain tissue from decapitated rats was depressed by 25% when saturated with bilirubin in concentrations of 20 mg. %.

Waters and Britton (1955) have recently produced staining of the brain in newborn rats by intraperitoneal injection of bilirubin, thus confirming the work of Frohlich and Mirsky (1942). An interesting observation of these latter workers has been their inability to produce staining in animals of over 10 days old.

There is circumstantial evidence which supports the view that bilirubin crosses from the circulation into the cerebral tissue with comparative ease in the newborn (Dereymaeker, 1949; Dine, 1954). It has been shown by Mollison and Cutbush (1951) that kernikterus seldom occurs in infants affected by haemolytic disease if the serum bilirubin levels do not rise above 18 to 20 mg. %.

It appears that bilirubin is likely to be the causative factor of kernikterus, whatever the mode of production of the hyperbilirubinaemia.

A search of the literature revealed very little information on the normal serum bilirubin levels in the full-term and premature infant. Davidson, Merritt and Weech (1941), doing daily bilirubin estimations on 94 newborn infants (no mention was made of maturity or weight), produced a normal curve rising to 4·8 mg. on the second day and then falling. They found that there was a very wide deviation of normal, but they correlated their bilirubin levels in the first 10 days of life with those of cord bilirubin levels and stated that the higher the cord bilirubin level the higher the serum level would rise on succeeding days. Other workers have been unable to confirm this. Davidson found also that where the levels rose high they were reached on the fourth day and were about 12·5 mg. per 100 ml.

Obrinsky, Allen and Anderson (1954) concluded from their daily bilirubin estimations on 27 pre-

mature and 12 full-term babies that there was a slightly higher degree of hyperbilirubinaemia in premature babies, but there was considerable overlapping of individual members. They agreed that the maximal level was reached on the fourth day.

More recently, Billing, Cole and Lathe (1954) estimated the plasma bilirubin levels in 49 babies whose weights varied from $2\frac{1}{2}$ to $10\frac{1}{2}$ lb. They divided these into 1 lb. weight groups and showed that the smallest babies reached the highest levels (up to 14 mg. per 100 ml.) on the sixth day, whereas the larger ones rose only to 2·5 mg. and usually on the second day.

McLean, Lucey and Harris (1955) have studied the serum bilirubin levels on 60 premature infants and the relationship of these levels to the degree of kernikterus in those who did not survive. They report the levels reached in the four babies with kernikterus in the absence of iso-immunization as being 22·8 mg., 13·2 mg., 15·2 mg. and 12·6 mg. per 100 ml. respectively. They make the interesting observation that there is a remarkable drop in the serum bilirubin levels immediately preceding death. This has been noted too by Hsia (1954) in the Seventh M. & R. Pediatric Research Conference Report on Haemolytic Disease.

The present investigation was undertaken with four objects in view: (1) To establish the range of serum bilirubin levels in 46 premature babies who did not develop kernikterus and 47 babies over $5\frac{1}{2}$ lb.; (2) to compare these levels with those of babies who developed kernikterus; (3) to ascertain whether there was any correlation between the bilirubin levels on the second day and the appearance of signs of kernikterus on the sixth day; and (4) to ascertain whether there was any correlation between the serum and the C.S.F. levels of bilirubin and the latter's relationship to kernikterus if any.

Material

The investigation passed through various phases.

Phase 1. Forty-eight premature babies, 46 of whom did not develop kernikterus and two who did, and 47 babies over $5\frac{1}{2}$ lb. were admitted to Sorrento Premature Baby Unit or to one of the lying-in wards at Sorrento Maternity Hospital during that phase. Blood samples were collected from these babies on the second, fourth and sixth days.

Phase 2. Because it was noted that the two affected babies in Phase 1 had high serum bilirubin levels on the second day, specimens were taken on the second day only from the next 51 babies admitted to Sorrento Premature Baby Unit. Four of these developed kernikterus.

Parallel Investigation

A further series was running concurrently in another unit. This consisted of 46 babies of whom five were affected. Serum and C.S.F. estimations of bilirubin were done on various days during the first week of life. It was found fairly early in this series that unless clinical jaundice was marked there was no detectable bilirubin in the C.S.F. so the latter part of this phase was restricted to babies showing marked jaundice and is, therefore, largely selected. All babies in Phases 1 and 2 who developed signs of kernikterus were submitted to lumbar puncture and the bilirubin level estimated in the spinal fluid: these babies were added to this series. Eleven babies in all developed kernikterus and were included in this investigation.

Iso-immunization was excluded serologically in all cases developing kernikterus.

Methods

Venous samples of blood were collected by syringe from the femoral vein in all cases and allowed to clot and retract for two to four hours in dry centrifuge tubes at room temperature. The quantity of bilirubin in the serum was estimated by van den Bergh's reaction using a slight modification of the technique of Malloy and Evelyn (1937) in which total bilirubin is determined by treatment with diazotized sulphanilic acid in aqueous methanol solution, without precipitation of the proteins. A measure of the direct bilirubin is obtained by carrying out a parallel determination in which water is used in place of methanol. Because of the difficulty of obtaining pure bilirubin an artificial standard was used, consisting of a solution of methyl red at *p*H 4·63 (King and Coxon, 1950).

Diazo Reagents. A .. 0·1% sulphanilic acid in 0·1 hydrochloric acid
B .. 0·5% sodium nitrate solution
was prepared at monthly intervals and stored in the refrigerator

Immediately before use 0·5 of B was mixed with 10 ml. of A.

Phosphate Buffer. 0·15% anhydrous sodium phosphate (Na_2HPO_4).

Standard Solution. Analar methyl red, 290 mg., is dissolved in 100 ml. of pure glacial acetic acid. (This stock solution keeps indefinitely if stored in a well-stoppered bottle in a cool dark place.)

Serum or plasma, 0·5 ml., was added to 9·5 ml. of phosphate buffer and three tubes were set up as follows:

(1) 'Blank', containing 1 ml. diazo reagent A + 4 ml. methanol
(2) 'Direct', containing 1 ml. mixed diazo reagent + 4 ml. water
(3) 'Total', containing 1 ml. mixed diazo reagent + 4 ml. methanol.

To each tube 3 ml. of the diluted serum or plasma was added and mixed well. After 30 minutes the colours were measured against the blank, using Ilford filter No. 624. The colour of about 10 ml. of the methyl red standard solution was measured using water as a blank.

$$\frac{\text{Reading of tube 3}}{\text{Reading of standard}} \times 0{\cdot}4 \times 8 \times 20 = \text{mg. total bilirubin per 100 ml. [serum or plasma}$$

The 'direct' bilirubin was calculated similarly, the equation being:

$$\frac{\text{Reading of tube 2}}{\text{Reading of Standard}} \times 0{\cdot}4 \times \frac{8}{3} \times 20 = \text{mg. direct bilirubin per 100 ml. [serum or plasma}$$

The quantity of indirectly reacting bilirubin was obtained by subtraction of the directly reacting figure from the total.

Estimations of Bilirubin in C.S.F. Cerebrospinal fluid, 1 ml., was added to 2 ml. buffer and to this was added 1 ml. of mixed diazo reagent and 4 ml. methanol as for serum dilutions. No blank was done and total bilirubin colour was measured against water. The calculation was:

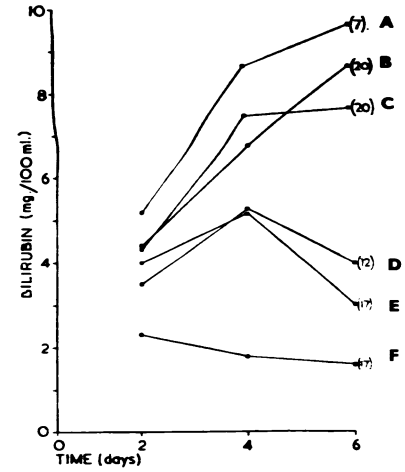
$$\frac{\text{Test}}{\text{Standard}} \cdot 0{\cdot}4 \times 8 \text{ mg. bilirubin per 100 ml. C.S.F.}$$

In no case was there more than 3·2 mg. of directly reacting pigment and this only in some of the mature babies showing very small quantities of bilirubin. The quantities of directly reacting pigment were usually less than 2 mg. and as these substances are not considered toxic the levels were disregarded. The total bilirubin in the C.S.F. was estimated as the figures were so low that differentiation was difficult and without significance.

Results

Fig. 1 shows the average serum bilirubin levels in the 93 babies in Phase 1 who did not develop kernikterus. They are split into 500 g.-birth-weight groups and the averages calculated and plotted. This figure shows a definite difference in the trend of the curves. The bilirubin levels of babies weighing less than 2,000 g. at birth are still tending to rise on the sixth day, whilst those of the babies in the weight group 2,000-2,500 g. are levelling out and those of the larger babies are falling by the sixth day. The babies in the highest weight group, i.e., over 3,500 g., reach their highest bilirubin levels on the second day and these have dropped by the fourth. Fig. 2 shows the weight group 2,500-3,000 g. The lowest curve is the average of the 12 babies in that weight group. The top curve is the statistically calculated maximum limits and the middle curve shows the maximum readings we obtained from the babies in that weight group.

Maximal statistical normals were calculated for all weight groups and are plotted in Fig. 3. The curves are similar to those in Fig. 2 except for the curve shown for the smallest babies, up to 1,500 g. The readings obtained from these babies showed very little spread and gave a smaller statistical standard deviation. This is the reason why the maximal statistical normal for this weight group



A—up to 1,500 g. (3 lb. 4 oz.).
B—1,500-2,000 g. (3 lb. 4 oz.-4 lb. 6 oz.).
C—2,000-2,500 g. (4 lb. 6 oz.-5 lb. 8 oz.).
D—2,500-3,000 g. (5 lb. 8 oz.-6 lb. 10 oz.).
E—3,000-3,500 g. (6 lb. 10 oz.-7 lb. 10 oz.).
F—over 3,500 g. (7 lb. 10 oz.).

FIG. 1.—Average bilirubin levels in 93 babies in Phase I.

gives the lowest curve of the three groups of premature babies, although the average readings for this group were the highest curve. This is of importance in that there may well be a lower serum bilirubin

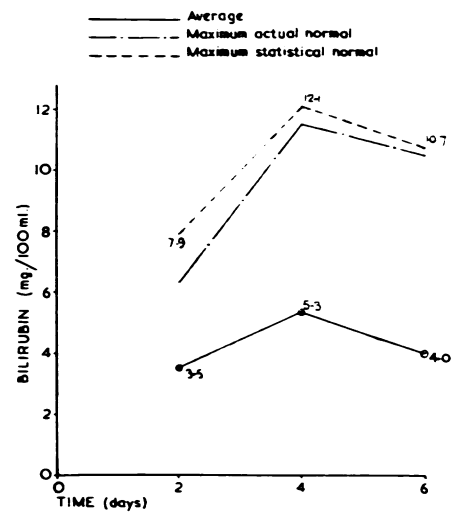
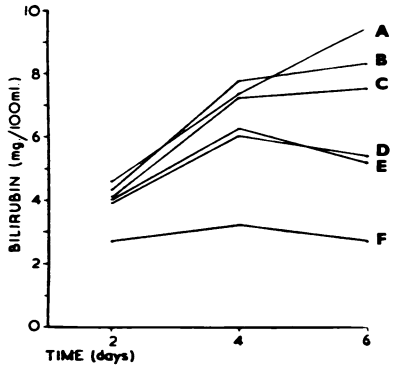


FIG. 2.—Maximum statistical normals and maximum actual normals in the weight group 2,500-3,000 g. (5 lb. 8 oz.-6 lb. 9 oz.).

level at which staining of the basal ganglia takes place in the smallest babies and would seem to add some statistical evidence for that clinical observation.



A—1,500-2,000 g. (3 lb. 4 oz.-4 lb. 6 oz.).
B—2,000-2,500 g. (4 lb. 6 oz.-5 lb. 8 oz.).
C—up to 1,500 g. (3 lb. 4 oz.).
D—3,000-3,500 g. (6 lb. 10 oz.-7 lb. 12 oz.).
E—2,500-3,000 g. (5 lb. 8 oz.-6 lb. 10 oz.).
F—over 3,500 g. (7 lb. 12 oz.).

FIG. 3.—Maximal statistical normals (all weight groups).

Fig. 4 shows the actual maximal levels of bilirubin obtained for all weight groups in Phase 1 of the investigation, against which are plotted bilirubin readings of the 12 cases of kernikterus on the days that the specimens were taken. It suggests that kernikterus is very likely to occur in any baby whose serum bilirubin level rises above 18 mg. per 100 ml. This is in agreement with the conclusions of many workers observing babies affected by haemolytic disease.

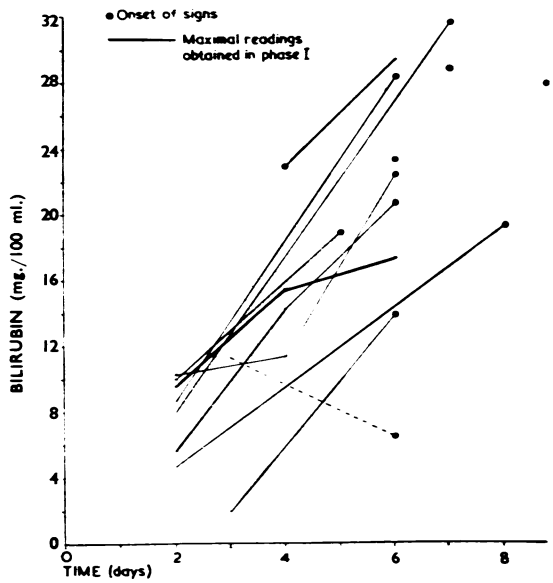


FIG. 4.—Serum bilirubin levels in babies with kernikterus.

Two infants who died of kernikterus with levels below 18 mg. per 100 ml. weighed 2 lb. and 2 lb. 8 oz. respectively. They both collapsed suddenly on the sixth day, the collapse being the first sign of the condition apart from the jaundice which had not been thought to be severe. Following the collapse both babies were moribund until they died about six hours later. The samples were taken shortly before death in both cases when the bilirubin level may have been dropping rapidly. One baby had a high level of bilirubin (11·3 mg. per 100 ml.) on his third day and the inference is that the level had been very much higher in the period between the collection of the two specimens.

Fig. 5 shows the serum bilirubin levels in the 41

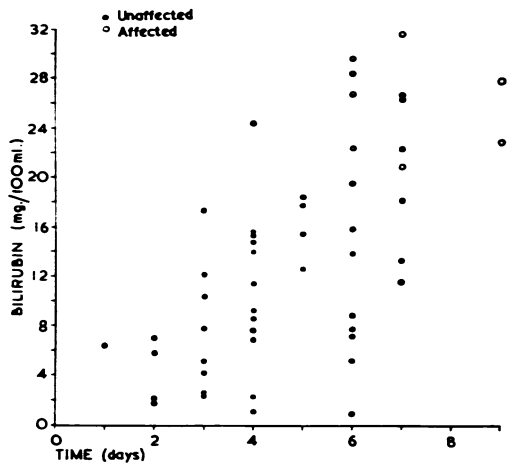


FIG. 5.—Serum bilirubin levels in unaffected and affected babies.

premature babies submitted to lumbar puncture in the first week of life who did not develop kernikterus and the 11 babies who did. The figure is interesting in that it shows samples taken from four babies who had serum bilirubin levels of over 18 mg. per 100 ml. and did not develop signs of kernikterus. At this time it was of interest to find out how high the serum bilirubin levels could rise without signs of kernikterus developing.

Fig. 6 shows the C.S.F. levels of bilirubin in the same 52 babies. There is on the whole a higher level of bilirubin in the C.S.F. of infants developing kernikterus than in the unaffected ones, but there is no correlation between the C.S.F. level of bilirubin and the appearance of signs of kernikterus.

Fig. 7 includes all babies on whom a second day bilirubin reading was obtained. The number of cases is plotted against the bilirubin levels and the levels of babies developing kernikterus is shown. It is interesting that four of the 16 babies who had

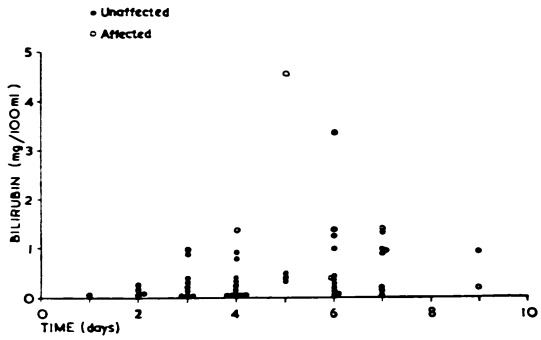


FIG. 6.—Bilirubin levels in C.S.F. in unaffected and affected babies.

second day levels of over 8 mg. per 100 ml. developed kernikterus. It seems to indicate that early biochemical prognosis of the infant likely to develop kernikterus may be possible. There were, however, two babies who developed kernikterus who had levels well below 8 mg. on the second day.

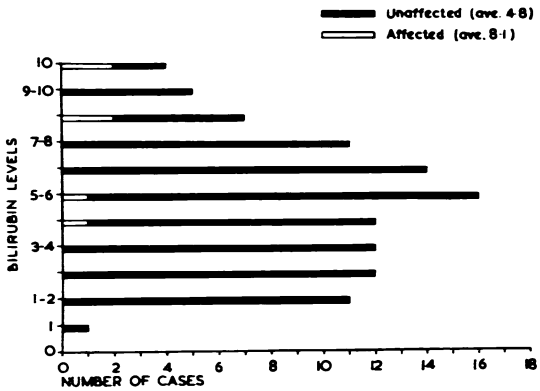


FIG. 7.—Bilirubin levels on the second day in Phases I and II (103 cases, 6 affected).

Discussion

Neonatal mortality has shown little significant change in the last 10 years when it is compared with the fall in infant mortality. Prematurity accounts for the great majority of the neonatal deaths and the proportion of spasticity amongst prematurely born babies is five times that amongst full-term babies (Asher and Schonell, 1950). The greatest mortality amongst premature babies is in the first 48 hours but, having survived this period, the premature baby is still exposed to the hazards of infection, aspiration and late effects of cerebral haemorrhage, which account for the relatively small death rate in the subsequent four weeks. There is, however, a constant increase in the mortality rate amongst premature babies towards the end of the first week of life. The majority of babies dying in this period die of kernikterus and those babies who develop kernikterus and survive are probably the major portion of the spastic, mentally defective and deaf children who are born prematurely. At Sorrento Premature Baby Unit the incidence of kernikterus in 1954 was 3·6% of all admissions and there was a 25% survival rate in those who developed the condition.

The present investigation was undertaken in the hope that it would be possible to foretell biochemically which babies would develop signs of the condition and at what levels of serum bilirubin the cerebral tissue would be damaged. There is little doubt that an infant with a serum bilirubin level higher than 18 mg. per 100 ml. runs a grave risk of developing kernikterus. Of some 350 specimens tested in the course of this investigation only 20 have been over 18 mg. %, and of these 12 babies have had kernikterus, i.e., 60% of babies reaching serum bilirubin levels of over 18 mg. per 100 ml. have developed the condition. Only the two moribund babies already discussed developed kernikterus with observed levels of less than 18 mg. %.

The treatment of hyperbilirubinaemia seems to be replacement transfusion with the sole object of reducing the bilirubin level below dangerous levels and keeping it down until liver function is sufficiently mature to convert bilirubin to the directly-reacting pigments which can be excreted. The technical difficulties of replacement transfusion at the age of a week are many but not insuperable. Replacement transfusion through the umbilical cord of a baby 5 or 6 days old is fraught with many more hazards than replacement transfusion at a few hours old. Replacement transfusion through the femoral vein using the saphenous vein as the portal of entry is technically a highly skilled procedure in a very small baby and outside the scope of many of the smaller units caring for premature babies. Therefore it would be preferable if the baby liable to develop kernikterus could be picked out early for transfer or immediate treatment using the umbilical cord.

Fig. 8 shows that the hyperbilirubinaemia was present on the second day in four of the six babies who developed kernikterus later. Replacement transfusion of the 16 babies with levels of over 8 mg. bilirubin per 100 ml. on the second day might

have prevented those four cases but not the two who had levels below 8 mg. per 100 ml.

It is not yet known exactly how the conversion of the bilirubin to the directly-reacting pigments takes place in the liver, but presumably the enzyme system responsible is synthesized in the immature liver in response to some stimulus which may well be the level of bilirubin itself. The less mature the liver the higher the bilirubin must rise before it is sufficient to stimulate production of the enzyme. If this hypothesis be true it should be possible to isolate the enzyme or some substance containing the enzyme which may then prevent the hyperbilirubinaemia.

The work of Day (1954) suggests a third possibility in the treatment of hyperbilirubinaemia. He showed that the bilirubin may be oxidized *in vitro* by cytochrome C and methylene blue, and thereby reversed the effect on the oxygen uptake of rat cerebral tissue. A similar compound with less toxic effects may be synthesized and injected into a jaundiced premature baby.

Replacement transfusion is, however, a recognized form of treatment of hyperbilirubinaemia in haemolytic disease and seems to offer the only method at present available by which toxic levels of bilirubin may be prevented and thus reduce the neonatal mortality and morbidity rate.

Summary

The present study shows the curves of serum bilirubin levels on 93 babies of all weight groups and indicates that babies under 2,000 g. (4 lb. 6 oz.) have levels still rising on the sixth day, babies of 2,000 to 2,500 levelling out and levels of babies over 2,500 g. are falling by the sixth day.

Premature babies developing kernikterus have generally higher serum levels of bilirubin and the majority have levels of over 18 mg. % at the onset of signs. Kernikterus is very likely to occur where serum levels of bilirubin rise above 18 mg. %.

There appears to be no critical level of bilirubin in the C.S.F. at which staining of basal nuclei takes place and there is no correlation between the bilirubin levels in the serum and that in the C.S.F.

It is suggested that all jaundiced premature babies should have serial bilirubin estimations, the frequency of those estimations depending on the level of bilirubin and the rate of rise of that level.

It is suggested further that replacement transfusion may be effective in the prevention of kernikterus in babies whose serum bilirubin levels are above 18 mg. % or whose rate of rise is rapid.

I am grateful to Dr. V. Mary Crosse for permission to study her patients and for her advice and encouragement during the preparation of this paper. I wish to thank Dr. A. H. Henley and the laboratory staff at Little Bromwich Hospital for their assistance, and also Miss Lane, Sister Murray, Sister Rowlly, Sister McGlynn and their staffs for their patience and invaluable help during the course of this study.

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