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Bilirubin-binding Cerebral Lipid

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In the pathomechanism of kernicterus several factors may play a role.

(i) Prolonged bilirubinaemia due to increased haemolysis [22]; or an immaturity [8], inhibition [1, 2, 23, 31] or deficiency [10, 13, 51] of the glucuronyl-transferase enzyme system.

(ii) An immature blood-brain or blood-CSF barrier. Several clinical and experimental [3, 9, 14, 20, 32, 37] observations have been explained in accordance with this fact. Alternatively, on the basis of experimental data DIAMOND and SCHMID [16] emphasized the importance of damage suffered at delivery and deny the role of the blood-brain barrier's immaturity.

(iii) Blood and tissues accumulate bilirubin [7, 38, 41, 55]. The bulk of serum bilirubin is bound to albumin [33, 39, 42, 52], but binding to erythrocytes has also been reported [53]. In the treatment of neonatal jaundice albumin alone or in conjunction with exchange transfusion has been recommended [40, 45] on the basis of the affinity of bilirubin to albumin. Care must be taken when prescribing drugs, since several compounds compete for albumin linkage [20, 26, 39].

(iv) Bilirubin inhibits respiration and uncouples oxidative phosphorylation [18, 54]. According to recent investigations, bilirubin inhibits the function of several enzyme systems [4]. BLANC and JOHNSON [6] assume that bilirubin itself is the toxic agent and is capable of entering into, and interfering with the metabolism of ganglion cells. This view was challenged by HAYMAKER et al. [20] who state that only necrotic cells can take up bilirubin and intracellular bilirubin is not the cause, but a mere indication of cell necrosis. It is not known why some parts of the brain (e.g. the corpora striata, the thalamus and hippocampus) tend to, while other parts of the brain do not, become stained in the presence of high levels of plasma bilirubin [5]. COWGER et al. [12] demonstrated that the chemical structure and lipid solubility of bilirubin are responsible for its toxic effect, while other bile pigments having a more polar character are less toxic.

CLAIREAUX et al. [11] identified the pigment recovered from the

stained brain tissue as bilirubin of the so-called indirect type and described a bilirubin-retaining property of a brain lipid fraction, but failed to determine the chemical nature of the latter.

This problem has now been investigated by us by means of thin-layer chromatography. The method has the advantage of identifying the bilirubin-containing lipid spots by means of specific reagents, and of making it possible to analyse small quantities, allowing to study rat brains and brains of kernicteric newborn infants.

Methods

(*i*) *Experiments in vitro*. Newborn and adult Wistar strain rats were decapitated and slices of their brains incubated with bilirubin solution.

(*a*) At the beginning of our experiments incubation of brain slices was performed during 24 hours at 4°C in rat serum containing 500 mg/100 ml of bilirubin, then the slices were washed twice with physiological saline and homogenized (see *iv*).

(*b*) In part of the experiments the incubation time was shortened. 5 g brain was homogenized with 6 ml bilirubin solution consisting of a) solvent: 2.5 ml Krebs-Ringer solution (15) containing 25 mg bovine albumin and b) solvent 500 mg per cent bilirubin dissolved in *N* Na_2HPO_4 (for complete dissolution adding a few drops of 2*N* KOH is necessary), just prior uniting and using the solutions 10 mg ATP dissolved in 1 ml H_2O was added to solution b). Incubation time was 10 minutes at 37°C, then 20 minutes at 4°C. As a control, a brain sample incubated without bilirubin was used.

(*ii*) *Experiments in vivo* were performed on 5 Wistar rats under ether anaesthesia; bilirubin dissolved in heparinized blood was introduced intracarotically. The bilirubin solution was prepared by dissolving bilirubin in a small volume of 0.1 *N* Na_2CO_2 and added to heparinized rat blood plasma to a final concentration of 150 mg per 100 ml. Thereafter the pH was adjusted to 7.8 and the plasma was united with the erythrocytes just prior to infusion. 12 ml/kg of bilirubin-containing blood was infused during 15 minutes and the same amount of blood was withdrawn. After 45 minutes the rats were decapitated, the brains isolated, sliced, and 1 g of brain was homogenized in a cooled Potter homogenizer.

(*iii*) The brains from two kernicteric infants and of one with oesophageal malformation and in the first case the bone marrow too, were examined.

Case 1 was a 1050 g premature born from a mother with pregnancy toxaemia. On the first day there were episodes of apnoea and spasms mainly localized to the face and arms. There was no Rh incompatibility. Jaundice on the second day was slight, on the third day, distinct. On the fourth day plasma bilirubin was 24 mg per 100 ml and an exchange transfusion was carried out. Next day the plasma bilirubin level was 17.5 mg per 100 ml, subsequently it decreased to near normal. Neurological symptoms and respiratory disturbances also ceased only to reappear a day later. Then the muscles were rigid, the apnoeic attacks and spasms occurred more and more frequently until respiratory paralysis had ensued. Artificial respiration was instituted but the baby died on the fourteenth day.

Case 2 was a premature born with 1550 g. Soon after delivery severe respiratory disturbance and pulmonary haemorrhage were apparent. The haemorrhage improved after the administration of protamine sulphate and calcium but intermittent positive pressure breathing had to be instituted. Then pneumonia and grave jaundice appeared and at a plasma bilirubin level of 15.3 mg per 100 ml an exchange transfusion was performed. On

the sixth day of life the condition deteriorated, spasms appeared, and on the eighth day the baby died with respiratory paralysis and cardiac failure.

Case 3. A premature newborn weighing 1800 g with oesophageal atresia was subjected to surgery on the first day of life. Respiration was maintained artificially but obstruction of the tracheal tube by thick mucus was difficult to overcome. Jaundice was clinically insignificant. Owing to increasing scleroedema and cardiac failure death ensued on the eigth day.

In all the three cases necropsy was done 4 hours after death. The brain and bone marrow were examined immediately. The coloured nuclear parts of the brain (thalamus, pons) and, as a control, non-coloured parts of the grey matter were subjected to extraction.

(*iv*) *Extraction procedures*

a) Sliced rat brains and samples of the newborn infants' brains or bone marrow (1 g each) were homogenized and extracted in a cooled Potter homogenizer with 17 parts of Folch reagent during 5 minutes, centrifuged and the lower part was again homogenized with 10 ml inversed Folch reagent (chloroform : methanol, 1 : 2) completed with 0.5 ml distilled water. After centrifugation the supernatants were united, evaporated *in vacuo*, and the concentrated extracts were put directly on thin-layer plates.

b) The incubated brain samples were washed with saline to remove the incubation mixture and then homogenized and centrifuged 3 times with 17 ml of Folch reagent for 5 minutes. An Erlenmeyer flask was filled with the united upper layers and put into a ten times larger glass beaker filled with distilled water just to cover the Erlenmeyer flask (double beaker-method of FOLCH *et al.* [19]). On the next day the 2—3 cm thick yellow layer on top of the watery phase was pipetted off, 2% butanol was added, and the solution was saturated with ammonium sulphate. The yellow colour entered the butanol phase and the watery phase became colourless. The butanol phase was evaporated to dryness *in vacuo* and dissolved in Folch reagent prior to chromatography.

c) 1 g brain was homogenized with 17 ml of Folch reagent, centrifuged, and after adding 5% (referred to the organic phase, 0.5 ml) water, homogenized for 5 minutes. After centrifugation the two upper layers were united. In part of the experiments the extracts so obtained were used without purification. For more accurate work we united the two upper layers and completed the solvent with chloroform to obtain a chloroform : methanol ratio of 2 : 1. The extract so obtained was evaporated to one half volume and shaken with 1/5 volume of 0.88% KCl solution in a glass-stoppered tube. After centrifugation the upper layer was drawn off and the lower layer shaken with 1/5 volume of 0.88% KCl solution saturated with Folch reagent. After repeated centrifugation the upper layer was united with the original upper layer, while the lower layer was shaken with 1/5 volume of water saturated with Folch reagent. All the upper layers were united and concentrated to 1/5 volume *in vacuo* and dialyzed at 4°C overnight, then evaporated to dryness. The residue of both the upper and the lower layer was dissolved in Folch reagent and subjected to thin-layer chromatography [47].

Serum and erythrocyte lipids were obtained by the modified [28] method of REED *et al.* [43].

(*v*) *Thin-layer chromatography*

Lipid fractions of the extracts were separated by ascending chromatography on 250 μ activated Kieselgel G-(Merck) layers in the following systems.

1) For the separation of lipids of different polarity: a) chloroform-methanol-water (65 : 25 : 4); or b) chloroform-methanol-water-ammonium hydroxide (65 : 27 : 4 : 0.1) (lower phase);

2) for the separation of different gangliosides. n-butanol-pyridine-water (3 : 2 : 1);

3) for the separation of apolar lipid fractions from polar ones, the modified [28] double solvent system of SACHS and WOLFMAN [44] were used.

For visualization and identification of the fractions, the following sprays were applied. As general lipid-dyes, bromothymolblue [24, 46] or oxytetracycline [29]; for visualizing gangliosides, BIAL's orcinol reaction [21]; for the demonstration of phospholipids, the molybdenum blue reagent of ZINZADZE [17;] for the indentification of bilirubin, the diazo reagent of JENDRASSIK and GRÓF [25]. Reference substances: lecithin and cephalins prepared from brain [34, 35]; cerebral ganglioside [49] consisting mostly of the monosialoganglioside GM_1 [50]; crystalline bilirubin (Reanal, Budapest).

All bilirubin-containing materials were protected from light. The chemicals were of analytical grade, the solvents were redistilled.

RESULTS

(i) For the comparison of their lipid fractions, serum and erythrocyte [27, 28] extracts were chromatographed simultaneously with brain extracts. In Fig. 1 the following

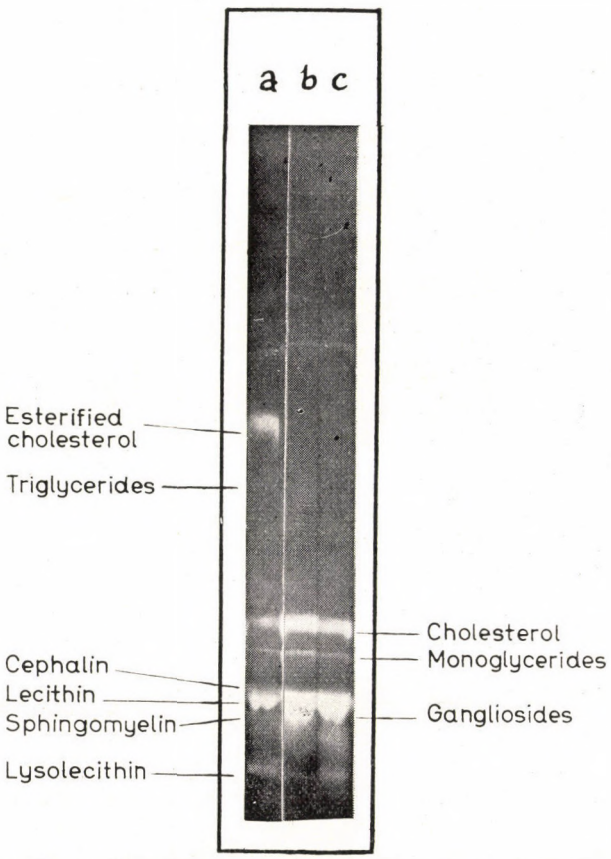

FIG. 1. Thin-layer chromatogram of brain lipid extracts (*c*), compared to lipid fractions of serum (*a*) and erythrocytes (*b*). Extraction with chloroform-methanol (2 : 1) SACHS and WOLFMAN's [44] modified [28] solvent system; oxytetracycline spray [29]

brain lipids are to be seen: next to the start line is lysolecithin, then follow gangliosides, free fatty acids, sphingomyelin, lecithin, cephalin, monoglycerides, cholesterol, triglycerides and esterified cholesterol fractions.

Chromatography of the lipid extracts prepared according to FOLCH [19] or SUZUKI [47] in an n-butanol : pyridine : water (3 : 2 : 1) solvent system revealed at least two spots characteristic of gangliosides and giving a positive reaction with Bial's reagent. In the extracts of brains incubated with bilirubin there were 1 to 3 chromatographic spots of bilirubin (yellow and diazo positive) the site of which coincided with that of the violet, BIAL positive, ganglioside spots. — If extracted with the double beaker method of FOLCH, the chromatogram of the watery phase revealed several yellow and diazo positive spots coinciding with the gangliosides termed by SVENNERHOLM [50] GT_1 (trisialoganglioside), GD_{1a} (disialoganglioside), GD_{1b} (disialoganglioside), GM_1 (monosialoganglioside), which could be made visible by Bial's reagent. The bilirubin-containing spots were ninhydrin-positive. Since cephalin is also ninhydrin positive, for differentiation between phospholipids the solvent system chloroform : methanol : water (65 : 25 : 4) and Zinzadze's molybdenum blue phospholipid reagent were used. The sites of the bilirubin-containing lipid spots and those of the Zinzadze positive spots differed considerably. Phospholipids giving a Zinzadze reaction were found on the upper part of the chromatogram, while the strongest bilirubin containing spot was near to the start line (Fig. 2).

(ii) Examination of bilirubin in the rat's brain after introduction by intracarotic infusion. These brains differed from those of the control rats by their intensive yellow colour. Lipids obtained by extraction procedure a) gave 3 diazo positive spots on the chromatogram in the chloroform : methanol : water system. These spots coincided with the gangliosides GT_1, GD_{1a}, GD_{1b}, GM_1 which could be made visible by Bial's reagent, while sites of the phospholipid spots differed from them. The yellow colour of crystalline bilirubin remained in these systems on the start line (Fig. 3).

(iii) Brain extracts of Case 1 and 2, and that of the spinal cord of Case 1 yielded only one diazo-positive spot; its site was close to the start line in the chloroform : methanol : water system. In the n-butanol : pyridine : water system the diazo positive spot was near the front. In both solvents this spot was Bial positive (crystalline bilirubin, as mentioned above, remained at the start line). Sites of the phospholipids differed from those of the bilirubin containing spots; this was particularly obvious on chromatography in the chloroform : methanol : water system (Figs 4, 5).

In contrast to the two kernicteric cases, on the chromatogram of brain lipids of Case 3 the site of the intensive yellow-coloured spot coincided with that of crystalline bilirubin in both solvent systems.

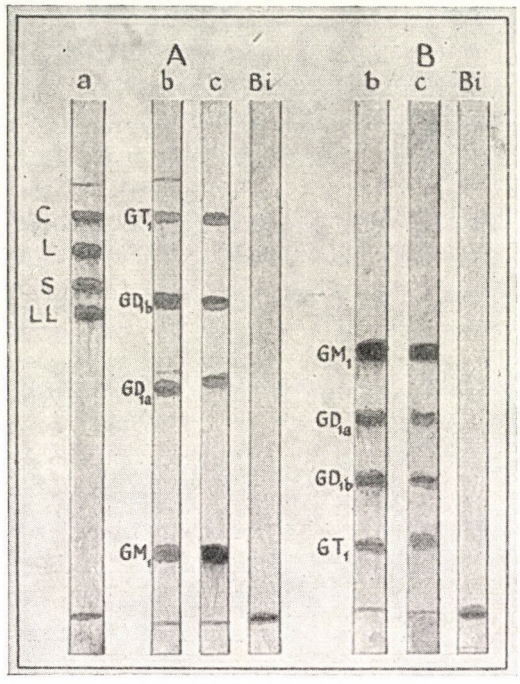

FIG. 2. Scheme showing the lipid fractions of rat brain incubated with 250 mg per 100 ml of bilirubin solution. Extraction with chloroform : methanol (2 : 1). Solvents: A: Chloroform:methanol : water (65 : 25 : 4). B: n-butanol : pyridine : water (3 : 2 : 1). *a:* molybdenum-blue reaction of ZINZADZE; *b:* BIAL reaction; *c* and Bi: diazo reaction. C: cephalin; L: lecithin; S: sphingomyelin; Ll: lysolecithin; GM_1, GD_{1a}, GD_{1b}, GT_1 gangliosides according to SVENNERHOLM [50]. Bi: crystalline bilirubin in chloroform

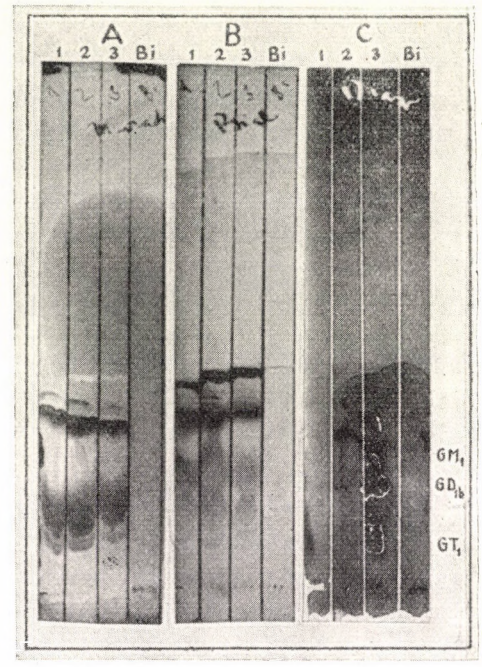

FIG. 3. Thin-layer chromatogram of brain-lipid extracts of rats treated with 1.5 mg bilirubin by intracarotic infusion. (3 ml heparinized blood with 150 mg per 100 ml plasma bilirubin level.) Extraction procedure according to SUZUKI [47]. Solvents A, B, C: chloroform : methanol : water (65 : 25 : 4). A: molybdenum-blue reaction of ZINZADZE, B: BIAL reaction, C: diazo reaction. Bi: crystalline bilirubin in chloroform

DISCUSSION

In both the *in vitro* and the *in vivo* experiments, we used adult Wistar rats. Since our aim was to throw light on the bilirubin-lipid linkage, we introduced bilirubin intracarotically into the organism. So most of the bilirubin bypassed the liver and entered directly the brain. KÜSTER [30] produced kernicterus in adult rabbits similarly, by intracarotic infusion.

We found that the purity and exact ratio of the solvents were essential for the stability of the minimal amount of lipid-linked bilirubin and for the stability of tri- and disialogangliosides which are easily transformed into monosialogangliosides. The yellow colour is characteristic of bilirubin only immediately after chromatography; after some time the sialic acid-containing ganglioside spots became yellow and even brown. The diazo reaction carried out on the still wet

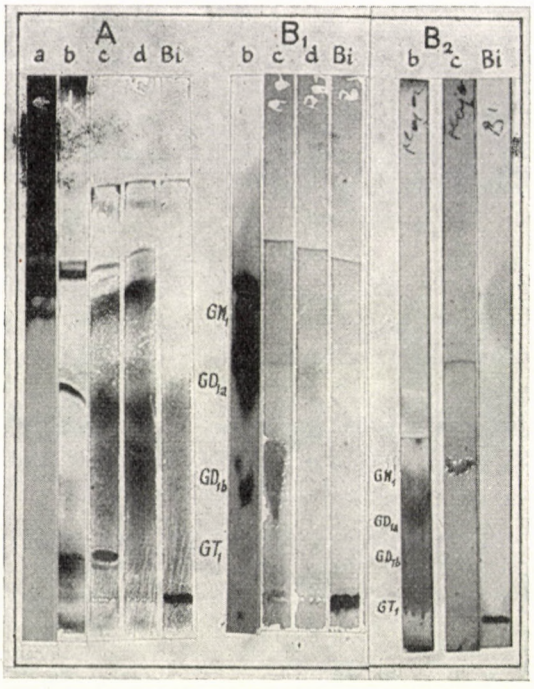

FIG. 4. Thin-layer chromatogram of brain lipids of Case 1 and Case 2 (*a b, c*) and control brain (*d*). Bi: Bilirubin standard. Extraction according to SUZUKI [47]. Solvents, A: chloroform : methanol : water (65 : 25 : 4); B: (B_1, Case 1; B_2, Case 2) n-butanol : pyridine: water (3 : 2 : 1). *a:* molybdenum-blue reaction of ZINZADZE; *b:* BIAL reaction; *c, d* and Bi: diazo reaction

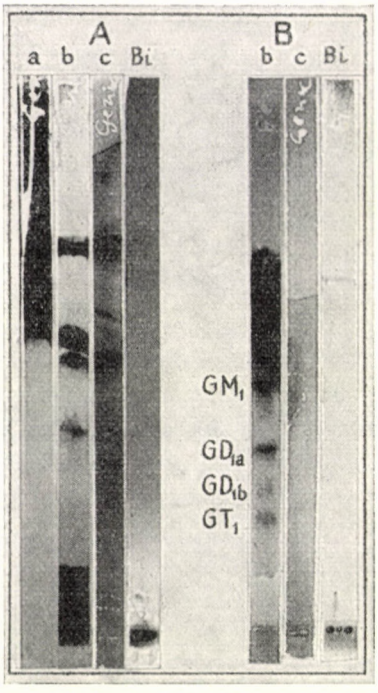

FIG. 5. Thin-layer chromatogram of lipids of the bone marrow (Case 1). Extraction according to SUZUKI [47]. Solvents: A: chloroform : methanol : water (65 : 25 : 4); B: n-butanol : pyridine : water (3 : 2 : 1). *a, b, c:* extract of bone marrow, Bi: bilirubin reference. *a:* molybdenum-blue reaction of ZINZADZE, *b:* BIAL reaction, *c* and Bi: diazo reaction. GM_1, GD_{1a}, GD_{1b} GT_1 gangliosides according to SVENNERHOLM [50].

thin-layer slides yielded the most satisfactory information. It has to be remembered that lipid-bound bilirubin gives an immediate diazo reaction whilst unbound bilirubin reacts only after some delay.

In respect of the origin and effects of the bilirubin-lipid linkages, the role of three factors must be emphasized.

1. Albumin concentration of the bilirubin solution. In experiments both *in vitro* and *in vivo*, and also in the case of kernicteric newborns, bilirubin entered the brain from an albumin-containing medium. In the experiments *in vitro* the albumin content was only 0.45 per 100 ml. Bilirubin was present at a concentration higher than the binding capacity of albumin. In the experiments *in vivo* the employed heparinized blood contained as much as 150 mg per 100 ml of bilirubin and even when it had be-

come diluted in the rat's circulation, the level was 30 mg per 100 ml, still exceeding the bilirubin-binding capacity of albumin. In the kernicteric newborn infants (Cases 1 and 2) the plasma bilirubin level was at the limit of the human albumin's bilirubin-binding capacity; this is why an exchange transfusion was done. In Case 3 the plasma bilirubin level was not determined.

Thus, in all the experiments as well as the kernicteric infants bilirubin was carried to the brain by albumin-containing media. In the experiments the amount of albumin was much less, in the kernicteric infants just sufficient for the binding of bilirubin.

2. In all the brains, whatever the origin of their bilirubin content, and in spite of the different extraction procedures and different chromatographic systems, the result unequivocally pointed to the role of gangliosides in binding the bilirubin. To this referred the sites of the bilirubin-containing spots giving a violet colour with Bial's reagent on the thin-layer chromatogram. When partitioned, the larger part of gangliosides was found in the aqueous phase, for example with the method of SUZUKI and in Folch's "double beaker" system. The yellow colour appearing in the watery phase speaks in favour of the gangliosides which are known to be soluble in water and organic solvents. From the above-mentioned data the conclusion might be drawn that the gangliosides have a definite role in binding the bilirubin entering the brain. As ERNSTER [18] has pointed out, some lipid having a great affinity to bilirubin must have a role in the origin of kernicterus. According to him "may be the brain cells concentrate bilirubin transferring it from albumin to cellular proteins, possibly to the very lipoprotein structures on which bilirubin then exerts its damaging effect". In this regard we have to mention the two possibilities raised by MENKEN et al. [36], by which bilirubin exerts its toxic effect on the brain: either the concentration of bilirubin is higher in the nervous system than in the liver or the spleen, or the neurons are more susceptible to its toxic effect.

According to our experiments, there is no controversy between the two assumptions. Owing to the concentrating effect of gangliosides the bilirubin concentration may be extremely high (higher than in the liver or the spleen) at certain sites of the neurons, and as a consequence the neurons have a peculiar sensitivity to bilirubin.

3. The gangliosides are taking up the bilirubin and presumably concentrate it. This, however, does not yet answer the question where and how bilirubin exerts its toxic effect. Since in the organism, too, there is a partition between watery and lipid phase, i.e. between the cytoplasm and the lipoprotein organelles, it may be assumed that those bilirubin molecules are bound by the gangliosides which molecules have contacted the ganglion cells, and these transfer them to the lipoprotein structures where the bilirubin exerts its toxic

effect. The insufficient amount of albumin for binding bilirubin and the undeveloped or damaged blood-brain barrier makes the contact of bilirubin with the ganglion cells possible. The insufficiency of the blood-brain barrier did not play a role in our experiments *in vitro* since in the experiments *in vivo* bilirubin was introduced into normal adult rats having a healthy blood-brain barrier, but the toxic action of part of the unbound bilirubin was not inhibited. In the immature infants, the underdeveloped blood-brain barrier, an injury at birth, furthermore an incomplete bilirubin-albumin binding may have played a role in the development of kernicterus.

Thin-layer chromatography proved useful not only for studying the bilirubin-lipid linkage, but it was found to represent a more sensitive indicator than any histochemical method. By its means it was possible to extract the amount of lipid-bound bilirubin necessary for chromatography and for identifying in the previously elaborated solvent systems. In this connection the experiment of DIAMOND and SCHMID [16] has to be mentioned, who could demonstrate deposition of isotope material in the brain of animals which received ^{14}C labelled bilirubin bound to albumin without any sign of damage to the central nervous system. Since our kernicteric infants showed significant nervous symptoms and since their plasma bilirubin concentration just reached the critical level, thin-layer chromatography seems to allow to demonstrate the critical cerebral bilirubin level causing damage in the nervous system.

Since regional and developmental differences in brain gangliosides have recently been described [48] we must raise the question whether this fact was one of the causes why certain brain areas become icteric while others remain unaffected.

In the brain of our Case 3 no lipid-linked bilirubin could be demonstrated; in this infant both the clinical findings and the fact that it died on the third day of life, have rendered the presence of a kernicterus improbable.

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SUMMARY

Rat brains incubated with bilirubin, rat brains made kernicteric *in vivo*, and brain lipid extracts from two kernicteric newborn infants have been studied for lipid bound bilirubin. According to its partitional distribution, R_f value and thin-layer chromatography, bilirubin is bound to the cerebral gangliosides.

It is assumed that cerebral gangliosides have the property of binding, retaining and concentrating bilirubin, thus giving the possibility for bilirubin to exert its toxic effect.

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